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The endangered *Iris atropurpurea* (Iridaceae) in Israel: honey-bees, night-sheltering male bees and female solitary bees as pollinators

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- *Background and Aims* The coastal plain of Israel hosts the last few remaining populations of the endemic *Iris atropurpurea* (Iridaceae), a Red List species of high conservation priority. The flowers offer no nectar reward. Here the role of night-sheltering male solitary bees, honey-bees and female solitary bees as pollinators of *I. atropurpurea* is documented.
- *Methods* Breeding system, floral longevity, stigma receptivity, visitation rates, pollen loads, pollen deposition and removal and fruit- and seed-set were investigated.
- *Key Results* The main wild pollinators of this plant are male eucerine bees, and to a lesser extent, but with the potential to transfer pollen, female solitary bees. Honey-bees were found to be frequent diurnal visitors; they removed large quantities of pollen and were as effective as male sheltering bees at pollinating this species. The low density of pollen carried by male solitary bees was attributed to grooming activities, pollen displacement when bees aggregated together in flowers and pollen depletion by honey-bees. In the population free of honey-bee hives, male bees carried significantly more pollen grains on their bodies. Results from pollen analysis and pollen deposited on stigmas suggest that inadequate pollination may be an important factor limiting fruit-set. In the presence of honey-bees, eucerine bees were low removal–low deposition pollinators, whereas honey-bees were high removal–low deposition pollinators, because they removed large amounts into corbiculae and deposited relatively little onto receptive stigmas.
- *Conclusions* Even though overall, both bee taxa were equally effective pollinators, we suggest that honey-bees have the potential to reduce the amount of pollen available for plant reproduction, and to reduce the amount of resources available to solitary bee communities. The results of this study have potential implications for the conservation of this highly endangered plant species if hives are permitted inside reserves, where the bulk of *Oncocyclus* iris species are protected.

Key words: Endangered, *Iris atropurpurea*, pollination, pollinator effectiveness, *Apis mellifera*, night-sheltering, eucerine bees, solitary bees, pollen removal, pollen deposition, stigma receptivity, pollen viability.

INTRODUCTION

The conservation of biodiversity has become one of the major goals of present environmental policies, given the current pace of global species loss (Loreau *et al.*, 2001). Recent assessments estimate that one in five of the world's plant species is facing extinction (Kew/Natural History Museum/IUCN, 2010). Plant–pollinator interactions play a significant role in maintaining the functional integrity of most terrestrial ecosystems (Ollerton *et al.*, 2011*b*). There is mounting evidence of large scale global declines of wild-bee populations and parallel diversity declines of bees and insect–pollinated plants across Europe (Biesmeijer *et al.*, 2006). Landscape modification, habitat fragmentation and the associated decline in suitable forage plants have been pinpointed as major global threats to bee diversity and pollination services (Biesmeijer *et al.*, 2006, Tylianakis *et al.*, 2007).

Assessing the effectiveness of different flower visitors for pollination and subsequent seed-set is central to addressing questions about the ecology and evolution of plant reproduction. Pollinator effectiveness is a function of multiple interacting traits of both flower and flower visitors, influenced by animal behaviour, morphology and relative sizes. Quantity (visitation rates) and quality components (pollen removal and deposition) of pollination service have been widely used in the literature to rank individual pollinators and to determine overall pollinator importance (*sensu* Waser and Price, 1983; Herrera, 1987, 1988; Olsen, 1997; Mayfield *et al.*, 2001; Ivey *et al.*, 2003; Sahli and Conner, 2007; Ne'eman *et al.*, 2009; Watts *et al.*, 2011).

Honey-bees (*Apis mellifera*; Apidae) vary widely in their effectiveness as pollinators of native plants (Paton, 1995; Butz Huryn, 1997; Frietas and Paxton, 1998; Celebreeze and Paton, 2004) and are often inferior pollinators compared with other native flower visitors (Schaffer *et al.*, 1983; Taylor and Whelan, 1988; Westerkamp, 1991; Paton, 1993; Vaughton, 1996; Gross and Mackay, 1998; Hansen *et al.*, 2002), and in some cases are responsible for a reduction in seed-set (Paton, 1993; Vaughton, 1996; Whelan *et al.*, 2009). However, in some systems they have comparable pollination effectiveness with other bees (Cresswell *et al.*, 1995; Fishbein and Venable, 1996; Thomson and Goodell, 2001; Ivey *et al.*, 2003; Watts *et al.*, 2011). In natural plant communities, honey-bees are less important as pollinators than they are in agricultural systems, because in most regions honey-bees are not a native species (Ollerton *et al.*, 2011*a*). Although honey-bees are native to the Mediterranean region of Israel, beekeepers maintain very high bee densities (90 000 beehives scattered in 6300 locations, with an average area of 1·5 km^2 for any beehive location) (Shavit *et al.*, 2009). Consequently, their establishment and spread, particularly into nature reserves, has the potential to reduce the pool of resources available to other native bee communities, and negatively impact on plant fitness, including rare and endangered species (Butz Huryn, 1997; Gross and Mackay, 1998; Hansen *et al.*, 2002; Goulson, 2003; Celebreeze and Paton, 2004; Dupont *et al.*, 2004).

Primary floral resources utilized by bees include pollen, nectar, oil, resin and gums, although flowers functioning as a shelter or sleeping place could also be considered a possible reward offered by a particular plant (Faegri and van der Pijl, 1979; Simpson and Neff, 1983). Flowers have been utilized as night shelters by a variety of solitary male bee species (Dafni *et al.*, 1981; Gaglianone, 2000; Sapir *et al.*, 2005; Monty *et al.*, 2006). In many species of aculeate Hymenoptera, males form sleeping aggregations on exposed plants in the late afternoon or early evening and disperse early the following morning (Alcock, 1988, and references therein).

Iris atropurpurea is a Red List species endemic to the coastal plain of Israel (Shmida and Pollak, 2007). In 2000, the total area containing *I. atropurpurea* populations within nature reserves was estimated to be 1·44 km^2 (Cohen and Avishai, 2000). Some populations are threatened by extinction, and about one-third have already disappeared (Cohen and Avishai, 2000; Sapir *et al.*, 2003). Previous studies have reported that the main visitors to *Oncocyclus* irises are male eucerine (Apidae; tribe Eucerini) (Ivri and Eisikowitch, 1988; Sapir and Shmida, 2002; Sapir *et al.*, 2005; Monty *et al.*, 2006), but until now there has been no attempt to quantify the pollination services of these bees directly.

Studying the reproductive biology of *Iris atropurpurea* is important given its rarity and conservation status. In this paper, we present data on observations and experiments in which the pollination effectiveness of honey-bees was compared with that of male solitary bees on both male and female fitness components. We augmented data from field surveys with the analysis of pollen loads from female solitary bees. Specifically, three questions were addressed. (1) What are the main flower visitors of *I. atropurpurea*? (2) Which bee species are the most effective pollinators in terms of visitation rate, pollen deposition and pollen removal? (3) Are honey-bees as effective as solitary bees as pollinators of *I. atropurpurea*?

MATERIALS AND METHODS

Study species

Iris atropurpurea Baker [*Iris* section *Oncocyclus* (Siemssen), Iridaceae] is a rhizomatous clonal geophyte characterized by a large solitary flower on the stem (Avishai and Zohary, 1980; Sapir and Shmida, 2002). The floral morphology of all *Oncocyclus* irises is similar; the inner vertical tepals (standards) and outer horizontal tepals (falls) form three functionally separate bilabiate units (meranthium), each resembling a single gullet flower or tunnel (Goldblatt and Bernhardt, 1999). The roof of each tunnel is formed by an expanded petaloid style, and the base is shaped by the outer falls which functions as a landing platform for insect visitors. Each flattened anther is pressed against the upper surface of the tunnel. The stigma lies above the apex of the anther at the entrance of each tunnel (Fig. 1A). *Iris atropurpurea* flowers have no nectaries and thus offer no nectar reward (Avishai, 1977). Thus, *I. atropurpurea* flowers are phenotypically specialized (*sensu* Ollerton *et al.*, 2007). Flowers colour varies greatly among, and even within, populations from deep purple, to brown or nearly black. The fruits are a capsule (mean length = 68·9 ± 18·1 mm, mean width = 21·8 ± 4·6 mm, $n = 51$) which is divided into three parts and collectively contain many seeds (mean = 21·5 ± 18·2, range 1–93, $n = 225$).

Observations and experiments were conducted at Yaqum, Israel (coastal plain; dry Mediterranean climate, 32·25°N, 34·85 °E, 20 m a.s.l.) between February and March 2009–2011. Additional observations were made at Netanya (32·20 °N, 34·51 °E, 35 m a.s.l.) during 2011. Although by law, all *Iris* species in Israel are protected (Cohen and Avishai, 2000), both populations studied are not located in official nature reserves. These populations are isolated fragments threatened by urban development and growth of agricultural areas. The *Iris atropurpurea* population at Yaqum (area 156 650 m^2) is surrounded by avocado groves, *Eucalyptus* trees, other fruit groves and agriculture fields. At Yaqum, honey-bee hives are abundant throughout the site and are used for avocado pollination. The Netanya population is the largest in Israel (370 579 m^2) and is threatened by new housing developments. Although no commercial bee hives are located in Netanya, honey-bees were frequently observed.

Iris atropurpurea plants are clonal with growth via underground rhizomes which creates dense patches of genotypically identical plants (ramets). Separation of clones is clear, based on the gap between patches. In this study, the assumption was that ramets within a patch are closely related plants, hence are self-incompatible if crossed (Sapir *et al.*, 2005). Clones were randomly selected in different patches across entire populations. To determine visitation rates, individual flowers were monitored for diurnal visitors during 10-min observations periods spaced evenly throughout the day (0800–1600 h), with a total of 193 periods. Flowers were observed from distances of 2–4 m. For each visit we recorded the species of flower visitor, behavioural aspects such as stigma contact, pollen collection, and number of flowers visited per foraging bout and the number of tunnels entered. Those insects which could not be identified in the field were collected for identification in the laboratory.

Pollen viability and stigma receptivity

Pollen viability and stigma receptivity were tested at Yaqum in 2011 using MTT (thiazolyl blue tetrazolium bromide; M-2128, Sigma-Aldrich Inc.; Rodriguez-Riaño and Dafni,

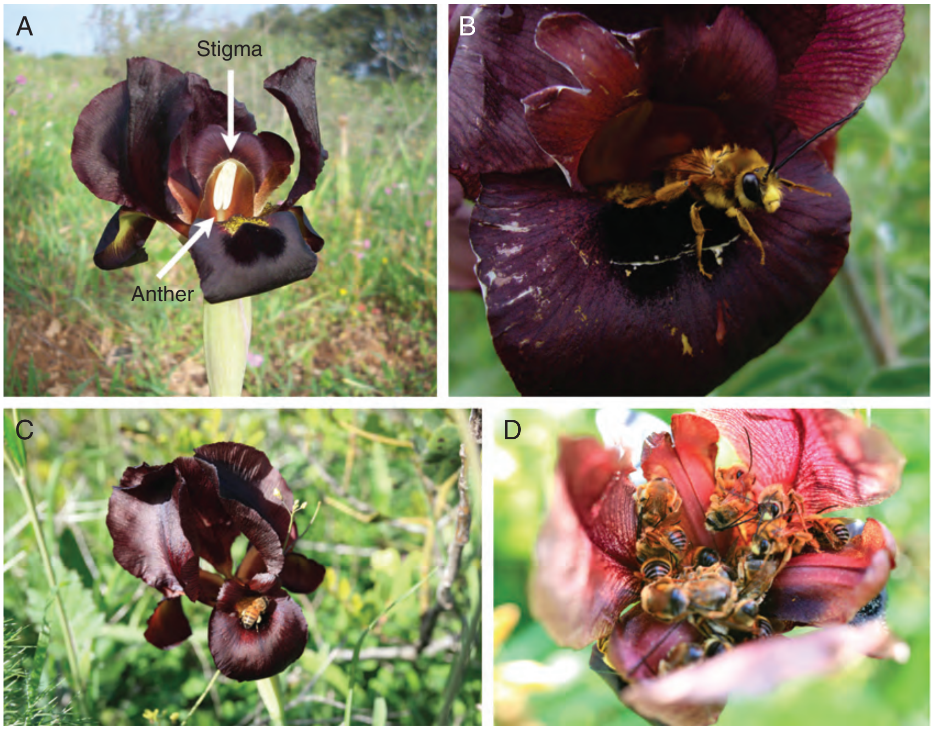

FIG. 1. (A) Flower structure of *Iris atropurpurea*: the inner vertical standards and outer horizontal falls form three functionally separate bilabiate units (meranthium), each resembling a single gullet flower or tunnel. (B) Male of *Synhlonia spectabilis* leaving iris flower after sunrise, showing the thorax lacking pollen. (C) Diurnal flower visitor *Apis mellifera* showing corbiculae loaded with iris pollen. (D) Single species aggregates of male *S. spectabilis* with >30 individuals sheltering in and on top of the three tunnels. Images: (A) Elisa Mancuso, (B) Stella Watts, (C, D) David Firmage.

2000). Fresh pollen grains from five flowers were collected in the field immediately after anthesis and brought back to the laboratory. Flowers of five developmental stages were tested for both pollen viability and stigma receptivity: (1) recently open buds (0 −1 d); (2) newly open flowers (1–2 d); (3) mature flowers (3 d); (4) old flowers with open tunnel but wilting standards (4 d); (5) wilting standards and falls, tunnel almost closed (5 d). Pollen grains were removed from anthers, mounted on a microscope slide and stained with MTT. One hundred pollen grains were counted and the proportion of stained grains was calculated ($n = 25$ each with five replications per pollen sample). We used MTT to test the receptivity of all three stigmas from five plants for each flower developmental stage (100 papillae per sample were counted; $n = 25$ each with five replications per sample).

Breeding system

Although *Oncocyclus* irises are reported to be completely self-incompatible (Avishai and Zohary, 1980; Sapir *et al.*, 2005), self-pollination has been found in populations of *Iris atropurpurea* at Shafdan and in populations of *I. haynei* in Gilboa (Y. Sapir, unpubl. res.). Therefore, we examined self-compatibility and autogamous pollination in populations of *I. atropurpurea* by bagging late-stage buds with fine mesh bags. Flowers were treated as follows: (*a*) open (unbagged) control – no treatment ($n = 38$); (*b*) emasculated treatment – flowers were carefully emasculated to remove anthers and left unbagged throughout the duration of the observation period ($n = 26$); (*c*) autogamy – bagged unmanipulated flowers were left covered with exclusion bags until the end of the observation period ($n = 26$); (*d*) self-compatibility – bagged flowers were carefully emasculated and hand-pollinated with pollen from other flowers on the same individual ($n = 30$); (*e*) xenogamy – bagged flowers were carefully emasculated and hand-pollinated with pollen from flowers from donor plants located at a distance of at least 10 m ($n = 22$). After the autogamy and xenogamy experiments flowers were re-covered. In addition, fruits were collected to represent open-pollination treatments. Natural fruit-set was calculated as the percentage of flowers that produced fruits per clone, and seed-set was recorded as the number of seeds per capsule.

The effect of pollen load on stigma on the number of seeds per capsule was tested in Netanya in 2009. Twenty flowers, each from a different clone, were bagged in late-bud stage and left overnight. The following morning, bags were removed and different numbers of pollen grains were deposited on stigmas using anthers from flowers >20 m away. The number of pollen grains deposited was assigned to any of three groups: few (1–10), intermediate number (10–100) and many (> 100). Pollen was deposited by brushing the anther against the receptive side of the stigma (one stigma per flower). Immediately after deposition, actual number of pollen grains deposited was counted using a ×20 magnifying glass. Flowers were re-covered and left until they completely wilted (approx. 6 d later). Five weeks later, fruits were collected and all seeds were counted.

Solitary-bee surveys

During three consecutive seasons (2009–2011 in Yaqum and 2011 in Netanya) the flowers of *Iris atropurpurea* were surveyed for sheltering male bees. We focused on night-sheltering visits because prior observations showed that bees rarely sheltered in flowers during the day time. Flowers were monitored throughout entire flowering seasons from late January to late March. The tunnels of each flower were sampled either 1 h before sunset or 1 h before sunrise. Each observer examined all open flowers in a designated area of the population and each session lasted up to 60 min. In total, 72 h of observations were carried out over 73 mornings/nights. When bees were found, flowers were tagged and we recorded the number of individuals per species per tunnel and the total number of flowers surveyed. Each year a sample of bees was collected from flowers and deposited individually into labelled vials for later identification and pollen analyses. Voucher specimens and pollen slides are deposited in the collection of the Laboratory of Pollination Ecology, Institute of Evolution, University of Haifa, Israel.

Pollen deposition by solitary bees

Pollen deposition by solitary bees was examined in Netanya and Yaqum during the peak of bee aggregation between 17 and 22 March 2011. A total of 27 stigmas (one stigma per flower) at Netanya and 25 stigmas at Yaqum were collected from flowers hosting male eucerine bees. Levels of natural pollination were also examined by randomly selecting 30 flowers and collecting stigmas (one stigma per flower) at Yaqum, and 26 flowers at Netanya. During the sampling period, no visits by honey-bees were observed to iris flowers at Netanya, whilst at Yaqum honey-bees had switched to avocado flowers, and so visits to iris flowers were rare.

Fruit-set by solitary bees

We initially attempted to estimate fruit-set following single visits to virgin flowers by male solitary bees. However, their unpredictable behaviour when searching for a suitable night shelter and their rapid flight, made it impossible to follow the bees. In 2010, we bagged 60 late-stage buds in four different areas of the Yaqum population. All the other open flowers surrounding the target flowers (varying flower ages) were bagged to prevent visitation, so that only the experimental flowers remained open. Despite our efforts, no visits by male bees were recorded. Therefore, the effectiveness of solitary bees at facilitating fruit and seed production could only be evaluated by tagging and monitoring flowers which hosted solitary male bees.

Prior diurnal and early evening observations showed that honey-bees were frequent visitors to *Iris atropurpurea* throughout and across all seasons in Yaqum. Honey-bee visitation was also noted in the Netanya population in 2009 and 2010, but less frequently in 2011 when bees preferred bushes of *Retama raetam* (Fabaceae). Thus, due to the strong overlap between honey-bee and solitary-bee visitation, it was not possible to identify which taxa facilitated fruit-set. Consequently, results are referred to as solitary bees plus (+) *Apis*. It should also be noted that female solitary bees were occasional diurnal visitors.

Pollen deposition by honey-bees

To evaluate the role of honey-bees as pollinators, pollen deposition was investigated using single visits to 37 virgin flowers. The flowers were treated as follows: (*a*) control flowers left bagged throughout the duration of the experiment ($n = 39$ stigmas), and (*b*) virgin flowers exposed to pollination ($n = 56$ stigmas). Every flower was bagged in late-bud stage with a fine-mesh bag and left for 24 h prior to collection. Bags in the pollination treatment were removed the following day at 0800 h to allow single visits by honey-bees, and flowers were watched until all target flowers were visited. Immediately after single visits, flowers were re-covered. On completion of the experiment, control and visited stigmas were collected using fine forceps and stored in separate Eppendorf tubes. Levels of natural pollen deposition were also examined by randomly selecting flowers over three seasons and collecting stigmas (one stigma per flower).

Pollen removal by honey-bees

Pollen removal by honey-bees was investigated using multiple visits to 30 virgin flowers (one anther per flower; treatment anthers, $n = 45$; control anthers, $n = 45$) on 2 March 2011. Flowers were bagged in late-bud stage and left overnight. Treatment bags were removed from flowers the following morning at 1000 h to allow visitation by honey-bees. Target flowers were watched until 1230 h and control bags were left on for the duration of the experiment. Total observation time was 100 min. When observations were concluded, visited and control anthers were removed with fine forceps and stored in separate Eppendorf tubes. To calculate the number of pollen grains per anther, each anther was placed into fresh vials containing a mixture of aniline blue and distilled water, crushed and placed in a sonic bath for 10 min. To count pollen grains, 1 μL of solution was pipetted into a haemocytometer and placed on a slide. A total of ten slides per anther were counted under a microscope. The number of pollen grains per flower was calculated by multiplying by the number of anthers per flower (see Dafni, 1992).

Fruit- and seed-set by honey-bees

Fruit-set and seed production by honey-bees was evaluated using 133 late-stage buds. The flowers were treated as follows: (*a*) open pollinated (unbagged control – no treatment ($n = 28$); (*b*) bagged control – flowers left covered with exclusion bags until the end of the observation period ($n = 49$); (*c*) treatment flowers – experimental virgin flowers ($n = 56$). The experiment was conducted 4–7 March 2009. Flowers were randomly selected and tagged over a 4-d period and assigned to treatments and bagged controls, and left overnight. Each morning the treatment bags were removed at 0800 h to only allow visitation by honey-bees and at 1500 h flowers were re-covered. The time period was based on prior honey-bee visitation field data, allowing for flowers to be re-covered before male bees started searching for night shelters. The flowers were subsequently monitored and, when the standards and falls had completely wilted the bags were removed. Fruits from experimental flowers were collected and the number of seeds per fruit counted.

Pollen loads

In 2008, 2009, 2010 and 2011, a random sample of 227 male bees found sheltering in flowers was captured and killed using ethanol. We counted all the pollen grains deposited over the entire body and pooled data from the three body areas (thorax, occuli and frons) most likely to contact the outer face of the stigma. Iris pollen grains are easy to recognize due to their relatively large size (100 μm), and so all pollen grains were counted using a binocular microscope. We also analysed pollen from the scopas of 75 female bees (18 species) caught on other co-flowering plant species over three seasons. To obtain pollen samples, small cubes of fuchsin-stained gelatin were rubbed over scopas and melted on glass slides. All iris pollen grains were counted. Given that bees may pick up iris pollen previously deposited by other flower visitors, we considered species with ≥5 pollen grains per slide as verification of iris visitation.

Data analysis

Statistical analyses were performed using SPSS version 17·0 for Windows (2008, SPSS Inc., Chicago, IL, USA). Data were assessed for expectations of normality and homogeneity of variances with particular statistical tests. Data used in non-linear regressions were square-root transformed to improve normality. Pollen load and pollen deposition data were not normally distributed; therefore Mann–Whitney *U*-tests were used with a Bonferroni correction ($\alpha = 0{\cdot}05$ divided by the number of comparisons), yielding a critical value of 0·0034 (15 comparisons) and 0·0025 (20 comparisons) against which all *P*-values were tested. All means in the text are presented ± s.d., and medians are indicated as required.

RESULTS

Pollen viability and stigma receptivity

Maximum pollen viability for *Iris atropurpurea* was 97·8 ± 2·3 % and 97·7 ± 2·1 % (mean ± s.d.) for recently open buds and young flowers (1–2 d), respectively (Fig. 2A). There was significant variation in percentage of pollen viability among pollen age groups (ANOVA; $F = 33{\cdot}43$, d.f. = 5, $P < 0{\cdot}0001$). Pollen viability decreased gradually with flower age. After day 5, viability was still high for old flowers with wilting standards, and falls and tunnel almost closed.

Maximal stigmatic receptivity occurred in buds and young flowers (1–2 d), with an average of 97·7 ± 2·3 % and 98·4 ± 1·5 % papillae stained, respectively (Fig. 2B). Stigmatic papillae remained firm for the first 3 d. A rapid change occurred on day 3 in stigma appearance, and its papillae appeared to wilt. After days 4 and 5, stigma receptivity decreased rapidly, and on day 5 less than half of papillae remained stained (Fig. 2B), indicating that even in old flowers with wilting standards and falls, and when tunnels were almost closed (4–5 d), the stigmas remain receptive. Thus, the end of female function is after flowers have wilted, similar to the male function.

Experimental pollen load in 2009 in Netanya was between 16 to approx. 800 grains per stigma. The artificial pollination resulted in fruit production in 12 out of 20 flowers pollinated. Probability of producing a fruit was not associated with the amount of pollen deposited (General Linear Model with binomial distribution of the errors: $F_{1,21} = 1{\cdot}16$, $P = 0{\cdot}294$). Linear regression with Poisson distribution of the errors revealed a significant linear slope of 0·085 ($t = 3{\cdot}69$, $P = 0{\cdot}001$), i.e. for a production of one seed, 11·8 pollen grains are needed.

Breeding system

Autogamy treatments produced no fruits indicating self-incompatibility (Table 1). Within-clone crosses (geitonogamy treatments) in the Netanya population resulted in 10 % fruit-

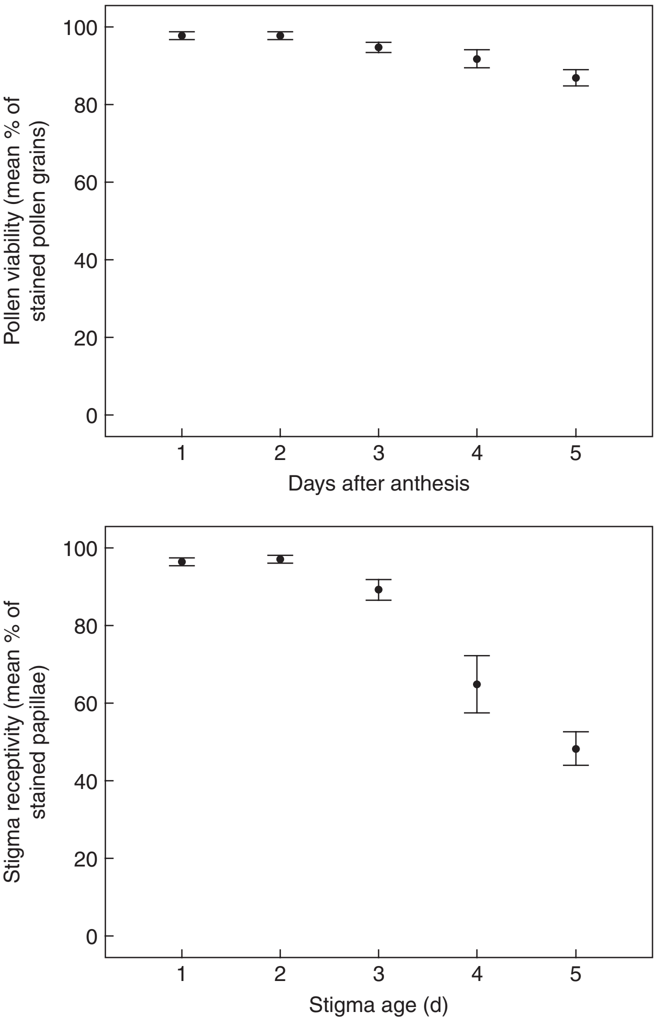

FIG. 2. (A) Pollen viability (%, as tested with MTT staining) after anthesis from flowers of five developmental stages (given as days after anthesis); $n =$ 25, with five replications per pollen sample, and 100 pollen grains per sample. (B) Stigma receptivity (%, as tested with MTT staining) in relation to stigma age; 100 papillae per sample were counted.

TABLE 1. *Natural and experimental fruit and seeds set in flowers of* I. atropurpurea *in Yaqum 2009 and 2011 and in Netanya 2011*

Year	Treatment*	No. of flowers	No. of fruits	No. of seeds per capsule (mean ± s.d.)	Fruit-set (%)
			Yaqum		
2009	1. Open pollination	1562	152	13·7 ± 12·9	9·7
2011	1. Open pollination	793	89	14·4 ± 0·60	11·2
2009	3. Autogamy	22	0	0	0
			Netanya		
2011	1. Open pollination	38	13	23·5 ± 15·8	34·2
	2. Emasculated	26	5	23·3 ± 11·3	19·2
	3. Autogamy	26	0	0	0
	4. Self-compatibility	30	3	2·0 ± 0·0	10·0
	5. Xenogamy	22	20	31·1 ± 21·0	90·9

*, No treatment; 2, anthers were emasculated and left unbagged; 3, bagged unmanipulated; 4, bagged flowers emasculated and hand-pollinated from other flowers of the same clone; 5, bagged flowers emasculated and hand-pollinated with pollen from flowers of other individuals from different clones in the same population.

set, although the mean number of seeds sired per capsule and seed dry weight was low. Natural fruit- and seed-set measured in Netanya at the end of the season yielded a lower value than fruit-set following the experimental treatments (22·0 %; $n =$ 2233 flowers in 143 clones). Supplementary hand cross-pollination treatments revealed higher fruit-set and seeds per fruit compared with control flowers (Table 1). Natural reproductive parameters in Netanya were all higher than in Yaqum: number of flowers per clone (General Linear Model with Poisson distribution, $P = 0{\cdot}005$), fruit-set (Mann–Whitney *U*: $P < 0{\cdot}001$), seed-set (General Linear Model with Poisson distribution: $P < 0{\cdot}0001$) and seed weight (Mann–Whitney *U*: $P < 0{\cdot}0001$) (Fig. 3).

Diurnal observations of flower visitors

During the spring of 2009, 2010 and 2011, observations at Yaqum showed that *Apis mellifera* was the most frequent diurnal visitor to *Iris atropurpurea*. Bees from nearby hives (in some cases ≤50 m) typically landed on the fall and crawled inside the flower tunnel, rotating their bodies upside down to collect pollen. They also burrowed into flowers which were not fully open to harvest pollen. Honey-bees were rarely seen to move long distances between clones; instead they moved short distances between flowers, (usually ≤1 m). After gathering pollen they exited tunnels backwards, then hovered in mid-air for a few seconds before either re-entering the same tunnel or moving on to the next one. This behaviour was repeated until corbiculae were loaded with pollen (Fig. 1C), when bees returned to hives.

Other diurnal visitors were relatively rare and included female eucerine bees of the subgenus *Eucera* (*Synhalonia spectabilis*; Apidae), mining bees (*Anthophora plumipes*; Apidae) and mason bees (*Chalicadoma sicula*; Megachilidae). In total, only 30 visits were recorded during this study.

The daily pattern of visitation by honey-bees was bimodal with a peak during the middle of the day, followed by a second peak in the mid-afternoon (Fig. 4). Honey-bee visitation rate differed across years ($\chi^2 = 12{\cdot}46$; d.f. 2; $P < 0{\cdot}005$); on average, honey-bees visited more iris tunnels per minute in 2011 than in 2010 and 2009 (Mann–Whitney *U*: 2011 vs. 2010: $Z = -3{\cdot}10$, $P < 0{\cdot}005$; 2011 vs. 2009: $Z = -3{\cdot}06$, $P < 0{\cdot}005$) (Table 2). However, there was no significant difference between visitation rates by honey-bees during 2009 and 2010 ($Z = -9{\cdot}57$, $P > 0{\cdot}05$).

Night-sheltering visitation

During three consecutive seasons, the tunnels of 13 717 flowers of *Iris atropurpurea* were sampled for sheltering bees (Table 3). The total number of bees found in flowers was highest in 2009, when 31 individuals were recorded in a single flower (Fig. 1D). The number of bees found in flowers across years ranged from 184 to 525 individuals (Table 3).

Non-linear regressions between the number of bees found sheltering in individual flowers versus change over time (*d* number) were highly significant in all three seasons (Yaqum 2009: $r^2 = 0{\cdot}30$; $F = 25{\cdot}38$; $P < 0{\cdot}001$; 2010: $r^2 = 0{\cdot}27$; $F = 25{\cdot}19$; $P < 0{\cdot}001$; 2011: $r^2 = 0{\cdot}30$; $F = 51{\cdot}31$; $P < 0{\cdot}0001$; Netanya 2011: $r^2 = 0{\cdot}40$; $F = 167{\cdot}31$; $P < 0{\cdot}0001$) (Fig. 5). In 2011, significantly fewer bees were observed in tunnels in the Netanya population in comparison with the Yaqum population (Mann–Whitney *U*: $Z = -5{\cdot}08$, $P < 0{\cdot}0001$; $Z = -4{\cdot}48$, $P < 0{\cdot}0001$). In 2009, the peak of aggregation of male bees occurred between 11 and 21 March at the end of the flowering season, when there were significantly fewer open flowers available in the population ($t = -3{\cdot}4$; d.f. $= 5$; $P = 0{\cdot}017$). In 2009, more bees were found in flowers than in subsequent years (Table 3). Across all seasons, the number of male bees found in iris flowers gradually increased with day number and peaked approx. 40 d after commencement of flowering (Fig. 5). Bees were found mostly in monospecific aggregations in all populations across years, and some flowers hosted male bees in the same tunnels over consecutive nights (Table 3).

Across all seasons, the assemblages of insects found in flowers were dominated by medium-sized male bees (mean = 12·1 mm ± 1·3; range = 9·2–14·2 mm, $n = 35$) belonging to the Eucerini tribe. We collected a total of 227 individuals representing ten species, including three previously undescribed species (Table 4). In 2009 and 2010, early into each season, a few *Chalicadoma sicula* males were also

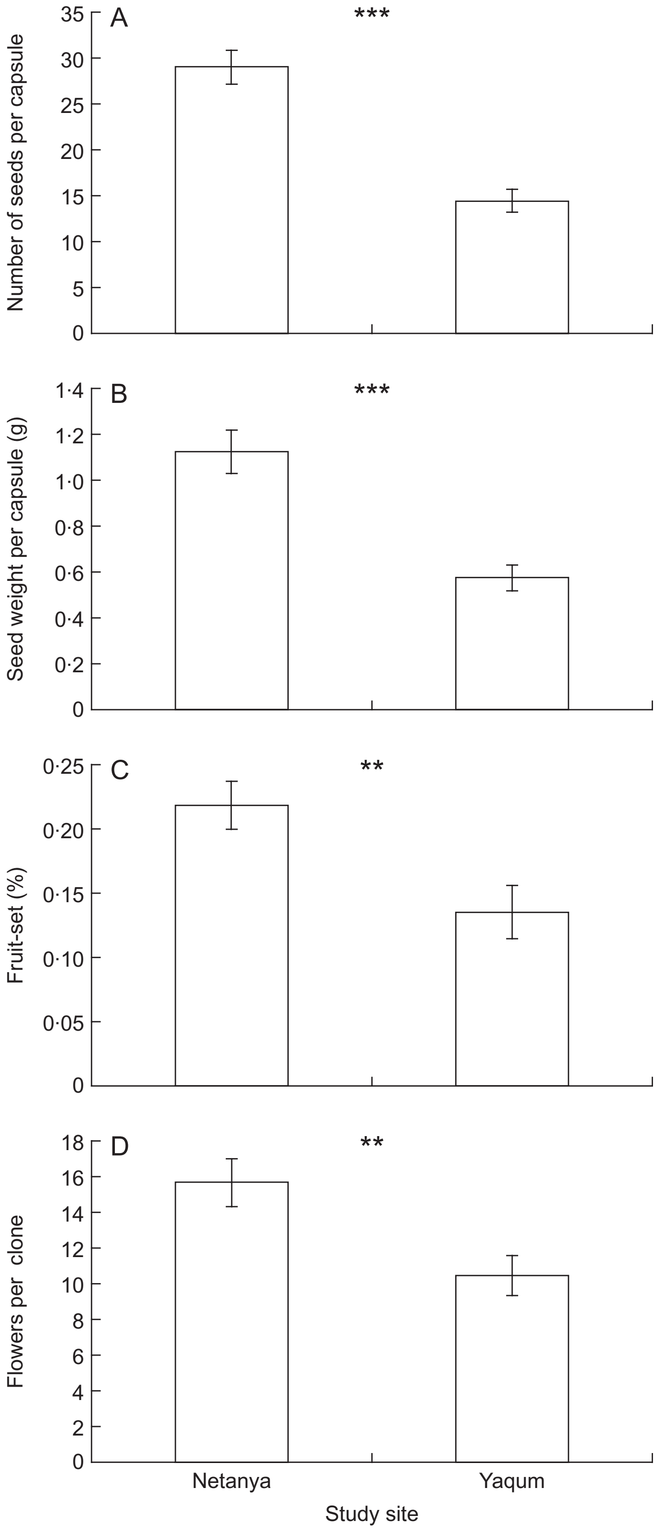

FIG. 3. Reproductive traits in populations of *Iris atropurpurea* at Yaqum and Netanya: (A) number of seeds per capsule; (B) seed weight per capsule; (C) fruit-set; (D) number of flowers per clone. Bars are mean values (± s.e.). Asterisks denote significant differences: ** $P < 0·01$; *** $P < 0·001$.

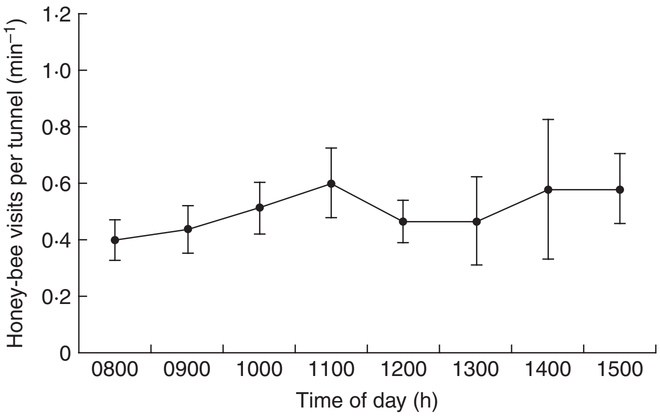

FIG. 4. Diurnal patterns of honey-bee visitation at Yaqum. Data were pooled across three seasons and calculated as visits per minute. Error bars are standard error of means.

TABLE 2. *Visitation rates to* Iris atropurpurea *by* Apis mellifera

Year	Mean (median) visits (± s.d.) per minute per tunnel	Mean (median) visits (± s.d.) per minute per flower
2009	0·41 (0·3) ± 0·31[a]	0·28 (0·2) ± 0·29[a]
2010	0·45 (0·3) ± 2·85[a]	0·29 (0·2) ± 0·27[a]
2011	0·75 (0·6) ± 0·58[b]	0·49 (0·5) ± 0·34[b]

Mann–Whitney *U*-tests, $P > 0·05$. Medians followed by the same letters do not differ significantly.

observed in flowers at Yaqum, but these individual carried very little pollen on the thorax (range 0–6 grains). The paucity of data for these males precluded statistical comparison. Overall, *Synhalonia spectabilis* was by far the commonest flower visitor (Table 4). Other hymenoptera visitors also found sheltering in tunnels of iris flowers included wasps (*Vespula germanica*; Vespidae) and the bumble bee (*Bombus terrestris*; Apidae) (only one record). Iris flowers were also visited by a taxonomically wide range of insects belonging to the orders Araneae, Coleoptera, Heteroptera and Homoptera, of which chafer beetles (*Oxythyrea* spp.; Coleoptera) were the second most abundant insects in 2009 (21·6 %). However, none of these insects carried iris pollen, so they were not considered to be pollinators.

Pollen loads

There were significant differences among some taxa for the number of iris pollen grains deposited on their bodies (thorax, occuli and frons; $\chi^2 = 19·56$, d.f. $= 5$, $P < 0·005$, entire body; $\chi^2 = 20·13$, d.f. $= 5$, $P < 0·0001$ (Fig. 6). Overall, the density of pollen grains carried by male solitary bees was low; 21·4 % of all bees found in flowers bore no iris pollen (range = 0 − 1525), 46·4 % carried ≤ 5 pollen grains and only *Synhalonia mediterranea* carried on average >200 pollen grains. Although *S. mediterranea* carried more pollen grains on the body than that of all other taxa, a Bonferroni adjustment for the 15 comparisons rendered this finding insignificant (Fig. 6). Sheltering bees in the Netanya population carried significantly more iris pollen grains on their bodies than bees in the Yaqum population (data pooled from 2008 and 2011;

TABLE 3. *The number and percentage of flowers hosting night-sheltering male bees in the populations of* I. atropurpurea *at Yaqum (2009, 2010 and 2011) and Netanya (2011 only)*

Year	Total no. of flowers checked	No. of sampling mornings/ nights	Mean (range) no. of flowers checked each morning/night	Mean % of flowers hosting bees	Total no. of bees	Mean no. of bees per flower	Maximum no. of bees in a single flower	% of flowers hosting bees in the same flower tunnels over consecutive nights
2009	3868	19	223·1 (22–486)	22·5 ± 37·1	525	2·9 ± 4·2	31	–
2010	3915	21	217·1 (12–419)	6·0 ± 9·0	217	1·0 ± 1·0	8	17 (2–4 nights)
2011	2598	18	133·5 (9–414)	18·7 ± 19·4	506	2·0 ± 1·8	12	19 (2–3 nights)
2011	3336	15	219·8 (6–613)	4·0 ± 4·1	184	1·5 ± 1·5	11	16 (2–3 nights)

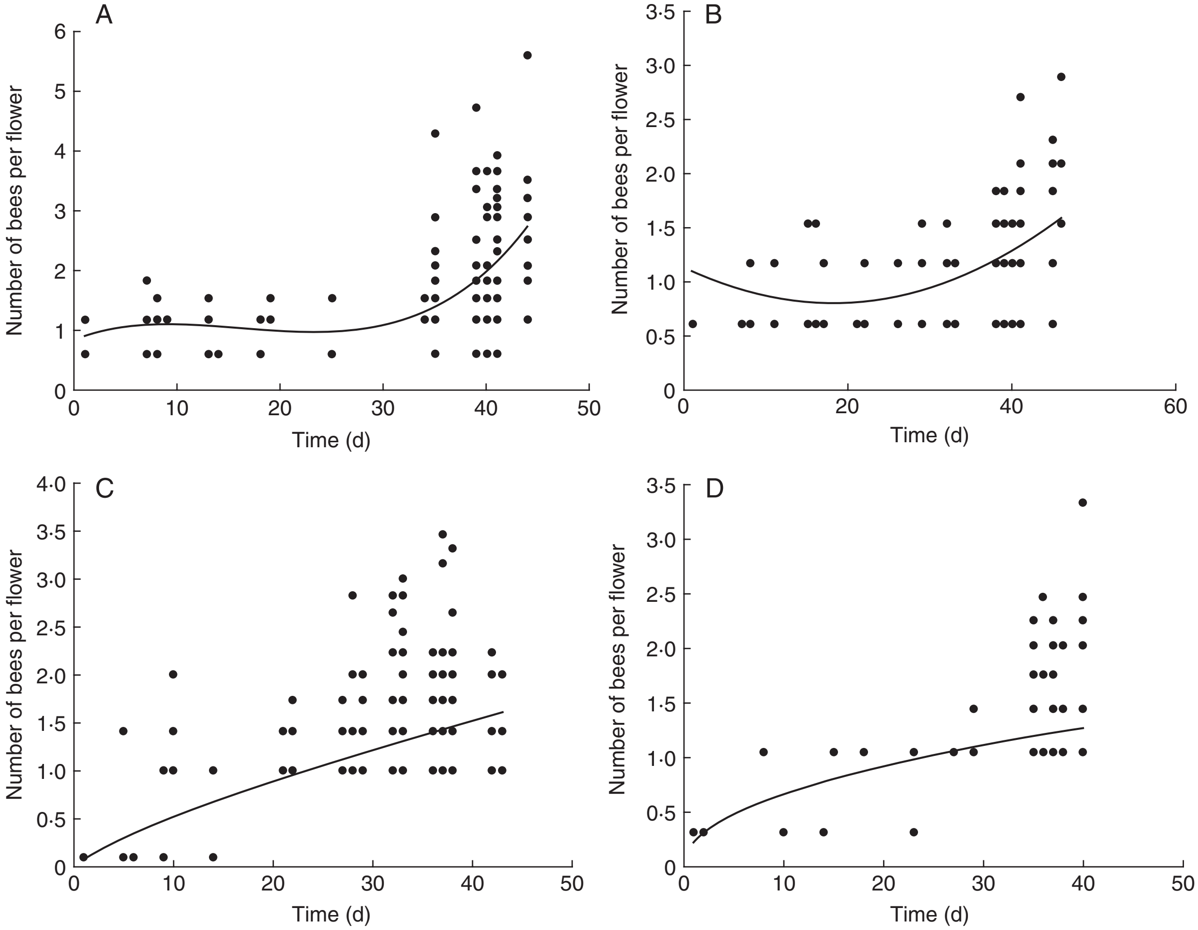

FIG. 5. Non-linear regressions showing the relationship between the number of male bees per flower sheltering in tunnels of *I. atropurpurea* flowers as a function of days from the start of the observations, starting (A) 6 February, 2009 (Yaqum); (B) 21 January, 2010 (Yaqum); (C) 14 February, 2011 (Yaqum); (D) 6 February, 2011 (Netanya; square-root transformed). (A, B) Lines represent polynomial best-fit models: (A) $y = 0{\cdot}0001x^3 - 0{\cdot}0049x^2 + 0{\cdot}0644 + 0{\cdot}8491$; $r^2 = 0{\cdot}302$; $F = 25{\cdot}38$; $P < 0{\cdot}001$; (B) $y = 0{\cdot}00003x^3 - 0{\cdot}0017x^2 + 0{\cdot}0273x + 0{\cdot}07314$; $r^2 = 0{\cdot}277$; $F = 25{\cdot}19$; $P < 0{\cdot}001$. (C, D) Lines represent exponential and power best-fit models, respectively: (C) $y = 0{\cdot}0902x^{0{\cdot}7665}$; $r^2 = 0{\cdot}30$; $F = 51{\cdot}31$; $P < 0{\cdot}0001$; (D) $y = 0{\cdot}7101e^{0{\cdot}0177x}$; $r^2 = 0{\cdot}40$; $F = 167{\cdot}31$; $P < 0{\cdot}0001$.

Mann–Whitney *U*: $Z = -5{\cdot}60$, $P < 0{\cdot}001$). The pollen data revealed that 25 % of female bees, representing seven species carried ≥5 iris pollen grains per scopa sample. The number of iris pollen grains per sample averaged 7·58 ± 16·11 (range = 0–77). The species which carried the most iris pollen grains were *Anthophora plumipes* (mean = 12·6 ± 22·4, $n = 12$ bees), *Eucera cypria* (mean = 14·3 ± 15·3, $n = 9$ bees) and *E. nigrilabris* (mean = 11·7 ± 25·9, $n = 8$ bees).

Pollen deposition by honey-bees

The mean number of pollen grains deposited on iris stigmas after single visits by *Apis mellifera* did not differ significantly from stigmas of open-pollinated flowers across years (ANOVA: $F = 1{\cdot}56$, d.f. = 3, $P = 0{\cdot}20$; Table 5). The average number of pollen grains found on virgin stigmas of control flowers (bagged flowers) was significantly different from stigmas visited by honey-bees ($t = -5{\cdot}5$, d.f. = 1, $P < 0{\cdot}001$). Note that due to

TABLE 4. *List of eucerine male bee species sampled from* I. atropurpurea *flowers at Yaqum and Netanya 2008, Yaqum 2009 and 2010, at Yaqum and Netanya in 2011*

	2008		2009		2010		2011		
Eucerini species	*n*	%	*n*	%	*n*	%	*n*	%	2008–2011 (%)
Eucera bidentata	1	3	1	1	9	18	2	4	6
Eucera sp. 1*	–	–	7	9	1	2	1	2	4
Eucera graeca	–	–	–	–	1	2	–	–	1
Eucera sp. 2†	12	23	6	8	–	–	3	6	9
Eucera sp. 3*	–	–	7	9	1	2	2	4	4
Eucera nigrilabris	2	4	3	4	–	–	–	–	2
Eucera parnassia	–	–	–	–	1	2	–	–	1
Synhalonia mediterranea	1	3	15	20	1	2	–	–	7
Synhalonia rufa	–	–	1	1	–	–	–	–	1
Synhalonia spectabilis	37	67	36	48	35	72	41	84	65
Total	53	100	76	100	49	100	49	100	227

*S. Risch (Leverkusen, Germany, unpubl. res); †B. Tkålcu (Prague, Czech Republic, unpubl. res).

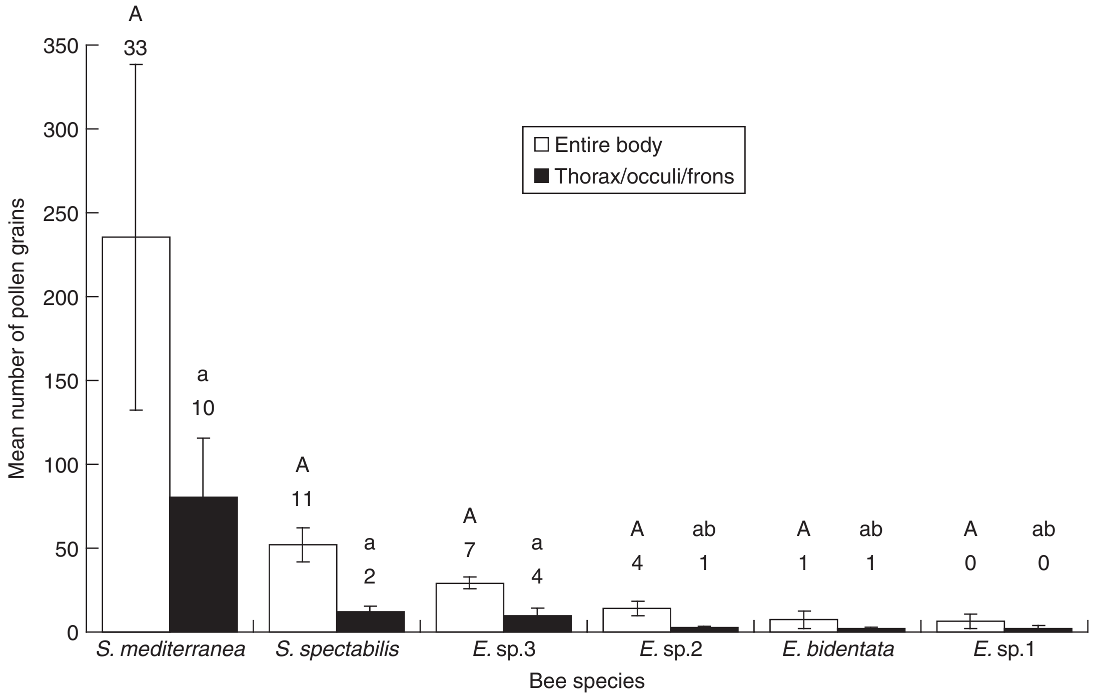

FIG. 6. Mean number of *Iris atropurpurea* pollen grains found on the bodies of the most abundant night-sheltering bee species. Data are pooled from populations at Yaqum and Netanya 2008; Yaqum 2009, 2010, 2011 and Netanya 2011. Error bars are standard error of means. Numbers at the top of each bar indicate medians. Different letters indicate significant differences (Mann–Whitney *U*-tests with Bonferroni correction, $\alpha = 0{\cdot}0034$). Upper- and lower-case letters are used to distinguish between different parts of the body: A = entire body, a = thorax, occuli and frons.

the morphology of *I. atropurpurea*, removing stigmas from flowers can result in contamination of the stigma from pollen on the anther but, putatively, this would not create bias among treatments, as all stigmas were removed using the same method.

Pollen removal by honey-bees

Significant differences were found in the median number of pollen grains per anther between visited and bagged flowers (Mann–Whitney *U*: $Z = -22{\cdot}35$, $P = 0{\cdot}0001$). The mean (median) number of pollen grains remaining per anther after multiple visits was 11 842 (5000) versus 43 250 (39 000) for virgin anthers. Honey-bees removed 75 % of pollen on virgin anthers during 100 min. The mean number of pollen grains per flower was calculated as 129 750 for virgin flowers, whereas the mean number of pollen grains remaining after multiple visits was 35 526.

Pollen deposition

The median number of pollen grains deposited on iris stigmas varied significantly among treatment groups ($\chi^2 = 39{\cdot}86$, d.f. = 6, $P < 0{\cdot}0001$). Open-pollinated and sheltering-bee treatments deposited more pollen grains on iris stigmas in Netanya than in Yaqum. However, after a Bonferroni adjustment for the 20 comparisons, only bagged controls from Yaqum (four sample medians) were significantly different from the other six treatments ($P < 0{\cdot}0024$; Fig. 7). There were no significant differences in the median number of pollen grains deposited per stigma between open pollination in Yaqum and Netanya (Mann–Whitney U: $Z = -1{\cdot}27$, $P = 0{\cdot}20$). Similarly, open pollination and *Apis mellifera*, and sheltering bees and *A. mellifera* treatments were statistically indistinguishable (Fig. 7).

TABLE 5. *Pollen deposition by* Apis mellifera *after a single visit in 2009 season, and pollen deposition on stigmas of naturally pollinated flowers in* Iris atropurpurea *in Yaqum population during three seasons*

Treatment	No. of flowers	Mean no. of pollen grains deposited on stigmas (± s.d.)
Open pollination 2009	49	23·1 ± 25·0^{a}
Open pollination 2010	21	27·3 ± 19·0^{a}
Open pollination 2011	30	36·8 ± 42·8^{a}
Apis single visit	56	27·3 ± 21·3^{a}
Apis single visit control	39	9·6 ± 13·1^{b}

Means with the same letter do not differ significantly (*post-hoc* Tamhane test; $P > 0{\cdot}05$)

Fruit- and seed-set by honey-bees

Fruit- and seed-set experiments following multiple visits by *Apis mellifera* indicated that the mean number of seeds per capsule between honey-bee and open-pollinated control flowers (no treatment) did not differ significantly ($t = -0{\cdot}44$, $P = 0{\cdot}77$). Bagged control treatments did not result in fruit-set. Naturally pollinated flowers yielded a slightly lower percentage fruit-set than those pollinated by honey-bees in 2009 (Table 6).

Fruit-set by sheltering bees

Fruit-set from flowers hosting sheltering bees varied between years and between sites. The mean number of fruits from each treatment varied significantly among groups (ANOVA: $F = 7{\cdot}48$, d.f. $= 2$, $P < 0{\cdot}0001$). *Post-hoc* tests of multiple comparisons showed that two sample means were highly significantly different from one another (Table 6). Low fruit-set was observed at Yaqum in all three seasons, despite recording the highest proportion of flowers hosting bees in 2009 and in 2011 (Table 3). The mean number of seeds per capsule was significantly higher in the Netanya population than at Yaqum, in 2009 and 2011, whereas sheltering-bee treatments were statistically indistinguishable.

DISCUSSION

The main wild pollinators of *Iris atropurpurea* are medium-sized male eucerine bees. Although a wide diversity of other insect taxa regularly sheltered in the flower tunnels, these

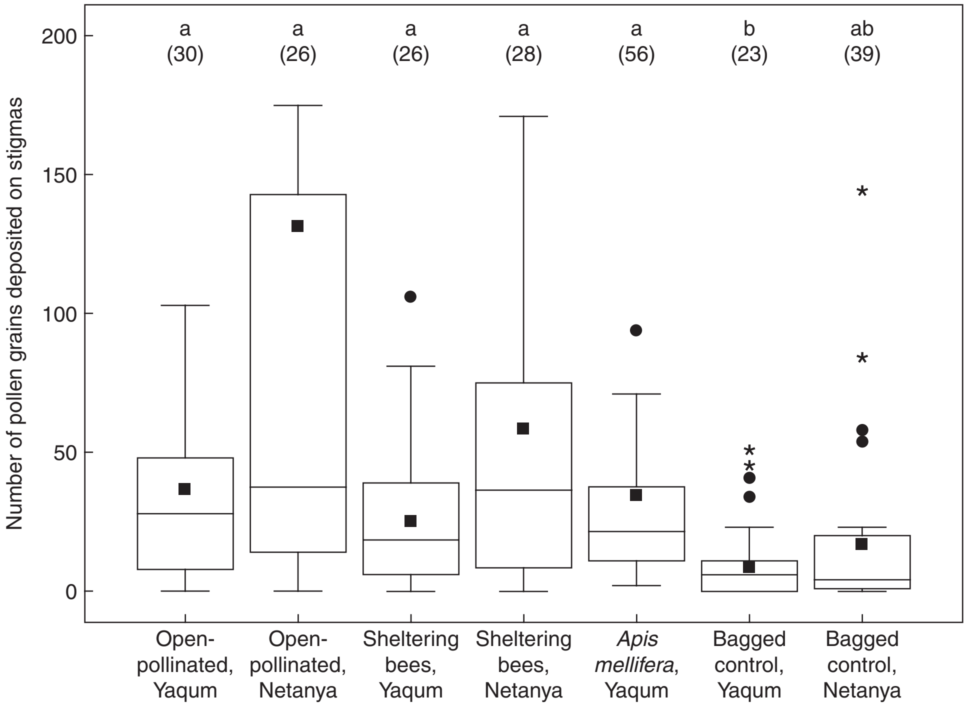

FIG. 7. Number of pollen grains deposited on iris stigmas following single visits by *Apis mellifera* at Yaqum, and number of pollen grains deposited on stigmas following multiple visits by sheltering bees in Netanya and Yaqum when bees were aggregated. Box plots show the median (horizontal line) and ranges from the 25th and 75th percentiles, the black square is the mean, and the tips of the whiskers indicate the fifth and 95th percentiles. The circles and stars represent outliers. Sample sizes are given in parenthesis. Different letters above of the error bars indicate significant differences (Mann − Whitney U-tests with Bonferroni correction, $\alpha = 0{\cdot}0025$).

visitors were not considered as pollinators because they did not carry pollen. Honey-bees were found to be as effective as night-sheltering bees at pollinating this species. Indirect evidence of visitation to iris flowers from pollen loads revealed that one-quarter of female solitary bees carried iris pollen, and thus were considered as potential pollinators. Nevertheless, we acknowledge that direct measures of effectiveness components and more field observations and experiments are needed to clarify the role of female solitary bees in the pollination of *I. atropurpurea*.

Supplementary cross-pollination substantially increased fruit-set (Table 1), indicating that *Iris atropurpurea* is pollen limited, in agreement with other *Oncocyclus* iris studies (Sapir *et al.*, 2005; Segal *et al.*, 2006; Shimrat, 2008), However, no mate-limitation (*sensu* Campbell and Husband, 2007) exists among crosses within populations, crosses among near populations, and crosses among far population (Shimrat, 2008; Sapir and Mazzucco, 2012). Reproductive parameters were all significantly higher in the Netanya population (Fig. 3) which is not surprising given this site was used as a refuge for plants transferred from disturbed habitats (Y. Malihi, Israel Nature and Parks Authority, Israel, pers. comm.), resulting in a genetic mixture derived from various genetic sources, while Yaqum is smaller and more local, and putatively less genetically variable.

Night-sheltering surveys of *Iris atropurpurea* populations showed that the majority of bees belonged to the *Eucerini* tribe, supporting earlier works by Sapir and Shmida (2002) and Sapir *et al.* (2005), although this is the first detailed study in which bee pollinators have been identified to species level. We observed not only temporal variation in bee assemblages, but variation in their abundance between years. Despite such variation, however, *Synhalonia spectabilis* was consistently the most frequent visitor across all seasons (Table 4).

Honey-bees were frequent diurnal visitors across all seasons (Table 2 and Fig. 4); they were the first to visit recently opened flowers and they removed large amounts of pollen, in agreement with other studies (e.g. Paton, 1993; Vaughton, 1996; Whelan *et al.*, 2009). During multiple visits, honey-bees removed approx. 90 000 times more pollen from anthers than they deposited on stigmas. The majority of pollen was stored in the corbiculae making it inaccessible for transfer onto stigmas. Furthermore, honey-bees removed pollen that could otherwise be transferred to stigmas by more effective pollinators. By late afternoon when male eucerine bees started searching for flowers to shelter in, we observed many anthers stripped of pollen, possibly influencing amounts subsequently transferred onto the bodies of sheltering bees the following evening. Indeed, repeated pollen depletion by honey-bees may explain why male bees in the Yaqum population carried significantly fewer pollen grains than bees in the Netanya population.

Pollination by male eucerine bees was facilitated at dusk by the movement in and out of flowers, when bees visited a sequence of flowers in search of a suitable shelter, as observed in a previous study (Sapir *et al.*, 2005). Notably, we found that the average number of pollen grains carried on the bodies of most sheltering bees was low (Fig. 6). Low densities of iris pollen found on the bodies of sheltering bees may also be attributed to the extent to which pollen is groomed when leaving flower tunnels (Rademaker *et al.*, 1997; S. Watts, unpubl. obs.), pollen displacement when bees aggregated together in flowers and pollen depletion by honey-bees. In the Netanya population (although not significant), twice the amount of pollen was deposited on stigmas for sheltering bees and open-pollination treatments compared with those in the Yaqum population (Fig. 7).

Some studies have found that honey-bees alter pollination levels by reducing the amount of pollen available to native pollinators (Paton, 1993), reduce seed-set by depleting pollen (Vaughton, 1996; Celebrezee and Paton, 2004; Whelan *et al.*, 2009) or, where active pollen-collecting bees remove more pollen and deposit less, they may reduce total pollen transfer (Wilson and Thompson, 1991). Here we suggest that honey-bees can have a potentially negative effect on the pollination of *Iris atropurpurea* for two reasons: (1) honey-bees reduce the amount of pollen available for plant reproduction via the male fitness component; (2) honey-bees can potentially reduce the amount of resources available to solitary-bee populations for brood provision.

It was found that honey-bees and sheltering bees deposited equivalent amounts of pollen on stigmas (Fig. 7), but a possible caveat important to highlight here is pollen deposition by sheltering bees was based on unrestricted visits when bees were aggregated, compared with single visits by honey-bees. Therefore, if pollen deposition increased as a flower received successive honey-bee visits (e.g. Young and Stanton, 1990), for this component, honey-bees should be more effective pollinators. Given that honey-bees deposited similar amounts of pollen as naturally pollinated flowers (Table 5), we conclude that both taxa were equally effective at depositing pollen on the stigmas of *I. atropurpurea*, and for the female component of fitness were functionally equivalent (see Zamora, 2000). Our findings are in accordance with other studies which reported similar pollen deposition efficiencies between honey-bees, solitary bees or bumble bees (e.g. Cresswell *et al.*, 1995; Freitas and Paxton, 1998; Thomson and Goodell, 2001; Watts *et al.*, 2011).

Similarities were also found between honey-bees and eucerine bees across successively visited flowers with respect to female fitness components. Seed production following multiple visits by honey-bees was not only statistically indistinguishable from that of open-pollinated treatments, it was also comparable to that of fruit-set from flowers tagged as hosting male bees, and to naturally pollinated flowers (Tables 1 and 6). However, even though both taxa could be considered functionally equivalent in terms of their contribution to fruit-set, we were unable to identify a single most effective pollinator, so any fruit-set was also likely to be by female solitary bees/and or honey-bees.

Sapir *et al.* (2005) proposed that fruit-set might be limited by the number or the activity of night-sheltering male solitary bees. However, our data suggested that inadequate pollination may be an important factor limiting fruit-set rather than bee abundance alone, given that eucerine bees were frequently found in flowers, and *S. spectabilis* was one of the most abundant bee species in the plant community across all seasons (S. Watts, unpubl. res.). In fact, even though we found a higher proportion of flowers hosting bees, and more bees sheltering in flowers in the Yaqum population, fruit- and seed-set

TABLE 6. *Number of fruits set in flowers of* Iris atropurpurea *following multiple visits by* Apis mellifera *compared with naturally pollinated flowers and amount of fruit-set for each treatment in flowers tagged as hosting night-sheltering bees*

Population	Year	Treatment	No. of flowers	No. of fruits	Mean no. of seeds (± s.d.)	Fruit-set (%)
Yaqum	2009	Open pollination	1562	152	13·7 ± 12·9*	9·7
		Apis multiple visits	47	6	13·0 ± 9·9*	12·8
		Bagged control	22	0	0	0
Yaqum	2009	Sheltering bees + *Apis*	102	14	9·7 ± 6·2^{a}	13·7
	2010	Sheltering bees + *Apis*	119	4	–	3·3
	2011	Sheltering bees + *Apis*	187	15	11·6 ± 13·8^{a}	8·0
Netanya	2011	Sheltering bees + *Apis*	102	30	23·0 ± 13·4^{b}	29·4

* Indicates no significant difference (*t*-test, $P > 0·05$). Means followed by the same letter do not differ significantly (*post-hoc* Tamhane test. $P > 0·05$).

were still significantly lower than in the Netanya population (Table 3 and Fig. 3).

In conclusion, in the presence of honey-bees, male eucerine bees were low removal–low deposition pollinators, whereas honey-bees were high removal–low deposition pollinators. Even though overall, both bee taxa were equally effective pollinators, we suggest that honey-bees not only have the potential to reduce the amount of pollen available for plant reproduction, they also have the potential to reduce the amount of resources available to solitary bee communities. The results of this study have potential implications for the conservation of this highly endangered plant species if hives are permitted inside reserves, where the bulk of *Oncocyclus* iris species are protected (Shavit *et al.*, 2009).

In the terminology of Fenster *et al.* (2004), Ollerton *et al.* (2007), Vázquez and Aizen (2006) and Watts *et al.* (2011), *Iris atropurpurea* has high apparent generalization but low realized generalization and can be considered to be a moderate ecological generalist (a number of species of medium-sized bees provide pollination services) but a functional specialist as most pollinators belong to a single functional group (Fenster *et al.*, 2004, although see discussion by Ollerton *et al.*, 2007).

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