

# Female-biased nectar production in the protandrous, hermaphroditic shrub *Salvia hierosolymitana* (Lamiaceae)

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**Abstract.** Flowering progresses upward along vertical inflorescences in the protandrous dichogamous shrub *Salvia hierosolymitana* (Boiss.). Flowers' gender nectar production rates and their vertical distribution were recorded in two populations (northern and central Israel) over 3 years. Female-phase flowers produced significantly more nectar than male-phase flowers and were more abundant at the inflorescences' base. Thus, nectar availability gradually decreases along inflorescences. Female-biased nectar production can benefit plants by increasing pollinator visits to female-phase flowers, enhancing pollination success when pollen is scarce. In congruence with this hypothesis, the following observations suggest that pollen in *S. hierosolymitana* may be in short supply: (1) freshly dehisced anthers contained 40% of sterile pollen; (2) pollen counts on female-phase stigmas were low (mean  $\pm$  s.e.  $11.6 \pm 1.56$ ); and (3) counts of germinated pollen tubes at the pistils' base were even lower ( $5.02 \pm 0.54$ ). The nectar gradient along the inflorescence may also be adaptively beneficial in other aspects. Foraging insects that follow this gradient are expected to move from female-phase flowers near the inflorescences' base, to male-phase flowers closer to the top. Thus, reducing the risk of geitonogamy and promoting outcrossing while moving from male-phase flowers of one individual to female-phase flowers of another.

## Introduction

Nectar, which is produced by plants as a food reward for pollinators, varies in quantity and composition both within and among species (e.g. Nicolson and Thornburg 2007; Keasar *et al.* 2008). Part of the variation in nectar production rates is heritable, and thus can be shaped by natural selection (Leiss *et al.* 2004; Kaczorowski *et al.* 2008). In dichogamous hermaphrodite plants, male and female functions are separated in time, creating the potential for different selection pressures on the two sex phases (Carlson 2007). Indeed, nectar production rates in hermaphrodite plants are often sexually dimorphic. Male-biased nectar production (higher in male-phase than in female-phase flowers) occurs in some species of dichogamous hermaphrodites (Bertsch 1987; Carlson 2007), while female-biased production has been reported from others (Carlson and Harms 2006).

One possible adaptive explanation for gender-biased nectar production involves the limiting factors of reproductive success in the two sex phases. In many plant species, male reproductive success is limited by opportunities for pollen export, while female fitness is limited by resources for seed and fruit maturation (Stanton *et al.* 1986; Andersson 1994; for a general review). Thus, male-phase flowers are expected to gain more

from attracting insect visits than female-phase flowers, selecting for increased nectar production in the male phase (Charnov 1982; de Jong *et al.* 2008; in the context of sex allocation, Bell 1985). In other species female fruit set is limited by pollen receipt rather than by resources. This can provide a selective pressure for female-phase flowers to attract more pollinators through enhanced nectar production (Burd 1994; Wilson *et al.* 1994; Ashman *et al.* 2004; Knight *et al.* 2005).

A second complementary hypothesis has mainly been applied to explain female-biased nectar production. According to this hypothesis, this bias plays a role in enhancing outcrossing in plants with vertical inflorescences, by influencing the movement patterns of insect foragers. Female-phase flowers in protandrous, acropetally flowering plants are located at lower positions along the inflorescences compared with male-phase flowers. Increased nectar production during the female phase, combined with the high abundance of female-phase flowers at the inflorescence base, generates a pattern of decreasing nectar productivity from the base of the inflorescences towards their tops (Pyke 1979). Foraging bees on vertical inflorescences tend to arrive at the inflorescence base, climb upward, and leave the inflorescence before reaching the top flowers (Pyke 1979; Waddington and Heinrich 1979; Best and Bierzychudek 1982; Orth and Waddington 1997). By

moving upward along the inflorescence, foragers are expected to encounter flowers with progressively lower volumes of nectar. Low nectar volumes in the upper, male flowers may induce foragers to leave for lower flowers on another plant, thereby promoting cross-pollination.

In the present study, we characterised nectar production patterns in the male and female sexual phases of the dichogamous hermaphroditic shrub *Salvia hierosolymitana* (Boiss.) (Lamiaceae). After establishing that female-phase flowers produce nectar at a higher rate than male-phase flowers, we used a descriptive approach to identify possible selective benefits of this female-biased production. We assessed the viability of pollen in the population, as well as the number of pollen grains on stigmas and the number of pollen tubes at the base of styles of female-phase flowers. This was done because low pollen quality and/or quantity may select for increased nectar production by female-phase flowers to enhance pollen import. We also tested whether the floral arrangement along the inflorescences generates decreasing gradients of nectar production and female-phase flower abundance, and whether insect foragers move from the bottom of the inflorescences upwards. Such gradients, if they exist, may provide a selective benefit to female-biased nectar production by promoting cross-fertilisation.

## Materials and methods

### Plant material

The study was conducted during the flowering seasons of 2003–07 in natural populations of *S. hierosolymitana* (Boiss.) at the following locations: the Mount Meron nature reserve (northern Israel, altitude 800 m); the Hebrew University's botanical gardens at the Mount Scopus and Givat-Ram campuses in Jerusalem (central Israel, altitude 750 m); Nahal-Refaim, Nes-Harim and Even-Sapir sites at the outskirts of Jerusalem (altitude 700 m). Plant density in the studied populations ranged from 100 to 200 shrubs per 1 km<sup>2</sup>.

*S. hierosolymitana* is an herbaceous perennial native to the eastern Mediterranean (Clebsch and Barner 2003). In Israel, it blooms during March–April and is pollinated mostly by solitary large bees of the genus *Anthophora*. The flowers are arranged in whorls, which are situated at intervals of 2–4 cm along vertical inflorescences, and flowering is indeterminate. There are six flower buds in a whorl, 7–10 whorls develop per inflorescence and there are 1–5 inflorescence stems per plant. Blooming progresses from the inflorescence base upwards, so that 2–4 whorls bloom simultaneously. Buds within a whorl open gradually (Fig. 1a, *S. hierosolymitana* in full bloom). The time from anthesis to flower abscission is  $48.6 \pm 2.5$  h (s.d.) under controlled conditions (Conviro chamber, growth conditions 16/8 h light/dark and 24/20°C, respectively). The exact durations of the male and female phases within this period are very variable, and are strongly affected by temperature and other environmental factors (Yehoram Leshem, unpubl. data). It is not known to what extent the plants are self-compatible. As in most members of the Lamiaceae family, *Salvia* flowers have a superior ovary of two fused carpels that form four distinct locules each with one basal ovule (Heywood 1978). The fruits consist of four one-seeded indehiscent achene-like nut-lets. The mean ( $\pm$ s.d.)

number of seeds per fruit in our study populations was  $3.2 \pm 0.87$ , ( $n = 400$  fruits, data not shown).

### Determination of floral sex phases

The flowers of *S. hierosolymitana* are protandrous–hermaphroditic. During the male phase, the stigma faces upwards and its lobes are clustered together. During the female phase, the stigma bends down and its lobes bifurcate (Fig. 1b–g). When the stigma is completely bent and bifurcated, most of the pollen is already discharged, suggesting little overlap between the female and the male phases. Thus, the position of the stigma is useful as a marker of the flower's sex phase. To assess the reliability of this marker, we determined stigma receptivity before and after bifurcation. Angiosperm stigmas are known to exhibit high levels of reactive oxygen species (ROS) activity when they are mature and most receptive to pollen (McInnis *et al.* 2006). Flowers were sampled in the field, placed in cooled containers and brought to the laboratory within 60–120 min for determination of ROS activity. We used DAB stain (3,3'-diaminobenzidine, Sigma-Aldrich, Rehovot, Israel), which polymerises on contact with H<sub>2</sub>O<sub>2</sub> and produces a reddish-brown precipitate, as an indicator. Staining was performed as described by Thordal-Christensen *et al.* (1997). Pictures were taken with a Coolpix 4500 camera (Nikon, Japan) attached to a binocular stereoscope (Olympus, Center Valley, PA, USA). This procedure confirmed that staining increases dramatically as the aperture of the stigma lobes widen (Fig. 1e–g). Thus, stigma bifurcation served as a good indicator for the determination of flower sex phase during field work.

### Nectar production measurements

Nectar was collected from flowers at the male and female phases, using 5- $\mu$ L micropipettes (BLAUBRAND intraMARK, Wertheim, Germany). Plants were covered with nets (20 mesh) at sunset before measurement, and remained covered until measured. Samples were taken at three time points of each day: after sunrise, (which served as t<sub>0</sub> during the study), 90–120 min later (t<sub>1</sub>), and 90–120 min after t<sub>1</sub> (t<sub>2</sub>). This sampling period overlaps the morning foraging period of *Anthophora* (Stone *et al.* 1999). The plants were netted between sampling periods. Nectar sugar concentration (w/w %) was measured with a hand-held refractometer (Bellingham and Stanley, Tunbridge Wells, UK). In each sampling period, 20 flowers of each gender phase were sampled from a few inflorescences on 4–6 plants. Individual flowers, rather than plants, were treated as the sampling unit, since within-plant variability in nectar production and composition often exceeds between-plant variability (Marden 1984; Herrera *et al.* 2006; Keasar *et al.* 2008). Sampling was destructive; therefore, nectar samples for t<sub>0</sub>, t<sub>1</sub> and t<sub>2</sub> were taken from different flowers. Nectar production rate per flower was calculated as the difference between the recorded nectar volume at time t<sub>x</sub> and the mean volume at time t<sub>x-1</sub>, divided by the time elapsed between these measurements.

### The vertical distribution of male- and female-phase flowers

The inflorescences of 15 plants were divided into three vertical segments of equal lengths. The plants were sampled randomly



**Fig. 1.** Male and female phases in *Salvia hierosolymitana* (Boiss.) flowers, stigma receptivity, pollen viability and pollen tube growth during these phases. Vertical inflorescence at full bloom (a), anthesis (b), male phase (c), female phase (d). Representative pictures of 3,3'-diaminobenzidine stain of the stigma before anthesis (e) and during male (f) and female (g) phases. Note the increase in dark-brown precipitate, which indicates increase in reactive oxygen species activity. Distance between horizontal ruler bars presented in b–d represent 1 mm. Scale bar for e–g is the same. Representative pictures of MTT (methylthiazolyldiphenyl-tetrazolium bromide) staining of pollen grains during anthesis (h), 2 days after anthesis (i) and pollen grains sampled during anthesis and boiled at 80°C for 1 h before MTT treatment (j). Scale bar for h–j is the same. Representative pictures of Aniline blue staining of pollen tubes in pistil base during male (k) and female (l) phases. White arrows in (k) indicate xylem location in the pistil while white arrows in (l) indicate callose deposits of pollen tubes. Scale bar for k, l is the same.

from the Mount Meron population during its peak bloom, on a single day in 2003. The total number of open flowers and the proportions of flowers in male and female phases in each segment were recorded. These data, combined with per-flower nectar production rates, allowed estimation of nectar production by all male- and female-phase flowers in each segment.

#### Pollen grain measurements

**Viability assessment:** Viability of pollen grains during the two flower gender phases was determined by

methylthiazolyldiphenyl-tetrazolium bromide (MTT, Sigma) staining as previously described (Khatun and Flowers 1995; Rodriguez-Riano and Dafni 2000). Two anthers of one flower from each of four plants (totalling eight anthers) were sampled at the Even-Sapir and Givat-Ram sites during anthesis and 2 days later (during the female phase), and were brought to the laboratory in cooled containers. Pollen grains were treated with 0.9% MTT for 15 min, and were then inspected under a light microscope. The number of viable grains (identified by deep-red staining) was determined in a sample of ~200 pollen grains from each anther.

A control for the staining procedure was performed with fresh pollen grains that were collected at anthesis, and boiled at 80°C for 1 h before MTT treatment. We expected no staining of pollen grains in this control, since boiling reduces viability to zero.

**Pollen counts:** Counts of pollen grains on stigmas during the male and female flower sex phases were performed in five natural populations in and around Jerusalem during the 2007 season. Twenty male-phase and 20 female-phase flowers from several plants were sampled in each population. Pollen grains were observed and counted under a stereomicroscope (Olympus). Pollen tubes were also checked at the pistil base during the male and female flowering phases through staining of stigmas with Aniline blue. Briefly, 20 flowers from each gender phase were sampled at each of the studied populations. Twenty recently fallen flowers, which had completed their female phase, were collected in each of four of the populations as well. Flowers were brought to the laboratory for analysis in a cool container, and were used within 60–120 min of picking. Staining was performed as described previously by Pagnussat *et al.* (2005). Pollen tubes located at the pistils' base were counted under an epifluorescence microscope (Olympus IX70; Olympus Optical Co. Ltd, Tokyo, Japan) using the UV fluorescence filter. Representative images were taken with a Coolpix 4500 camera (Nikon, Japan).

**Assessment of spontaneous self-fertilisation:** To test for the possibility of pollen transfer in *S. hierosolymitana* in the absence of insect vectors, 6–8 inflorescences were covered with nets in the Givat-Ram and Mount Scopus populations before anthesis. The number of pollen grains was determined on female-stage stigmas 2 days later. Since handling of the nets under field conditions may cause some artificial pollen spread, the same protocol was also performed on flowers from plants grown under controlled room conditions inaccessible to insect visitors.

#### Observations of pollinator behaviour

The foraging behaviour of *Anthophora plumipes*, the primary pollinator of *S. hierosolymitana*, was studied at the Mount Meron site during the 2003 season. We recorded 27 foraging bouts (sequences of visits on a single inflorescence by one individual) on 13 inflorescences from 13 shrubs. To identify landing and departure locations, the inflorescences were divided into three vertical segments of equal lengths. Each observation included recording of the bee's landing and departure positions, and its movement directions: upward, downward or sideward flights.

#### Data analysis

Nectar production rates and concentrations in male-phase and female-phase flowers within each location and year combination were compared using *t*-tests. ANCOVA was used to compare nectar production rates between the sex phases (the fixed factor) in all samples combined, while controlling for sampling hour (the covariate). One-sample *t*-tests were employed to determine whether the proportion of female-phase flowers deviated from 0.5 in the bottom, middle and top segments of the inflorescences. The effects of the position in the inflorescence (bottom, middle or top) on the proportions of female-phase flowers, and on the number of open flowers, were assessed using ANOVA followed by Tukey's post-hoc tests. Proportions were arcsine-transformed before analysis. Statistical analyses were performed using JMP (SAS Institute, Cary, NC, USA) and SPSS version 14.0.

## Results

### Nectar production

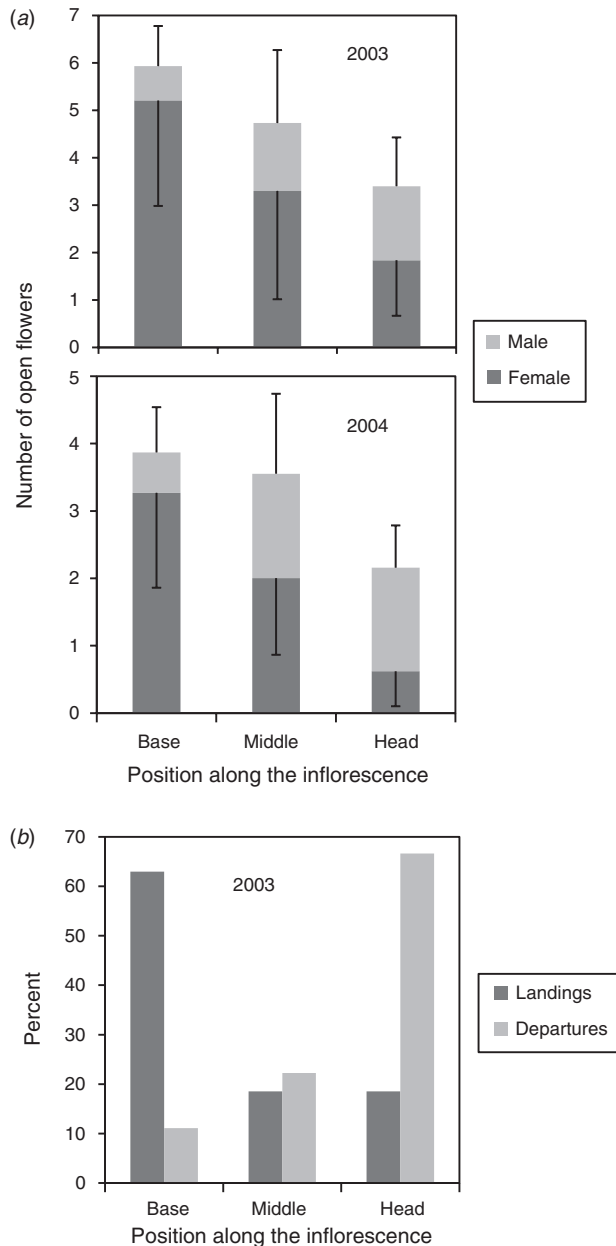
Nectar production during the flowers' female stage was higher than during the male stage at all seasons and locations. In Mount Meron, during 2003 and 2004, the female-phase production rates were 1.5–3.1 times higher than in the male phase. The same pattern was observed during 2004 and 2005 in Mount Scopus where female-phase production rates were 2.9–3.3 times higher than male-phase production rates (Table 1). Production rates were significantly influenced by the hour of sampling, but the effect of floral sex phase remained highly significant after controlling for the effect of sampling hour (ANCOVA, effect of sampling hour:  $F_{1,175} = 16.57$ ,  $P < 0.001$ , effect of floral sex phase:  $F_{1,175} = 242.86$ ,  $P < 0.001$ ). Nectar sugar concentrations did not differ between the sex phases, and ranged from 28 to 32% (Table 1).

At full bloom, most flowers along the inflorescence base were at their female phase, but this percentage significantly dropped towards the inflorescence top (Fig. 2a). The proportion of female-phase flowers significantly differed from 0.5 in the lowest portion of the sampled inflorescences in both flowering seasons ( $t_{14} = 9.70$ ,  $P < 0.001$  in 2003,  $t_{18} = 7.02$ ,  $P < 0.001$  in 2004). In the middle and top segments, this difference was significant in only one of the years (middle segment:  $t_{14} = 2.46$ ,  $P = 0.037$  in 2003,  $t_{18} = 1.08$ ,  $P = 0.29$  in 2004; top segment:  $t_{14} = 0.46$ ,  $P = 0.65$  in 2003,  $t_{18} = -4.48$ ,  $P < 0.001$  in 2004). The distribution of male-phase flowers complemented this pattern, as they were more abundant at the top part of the inflorescences than at their

**Table 1. Differential nectar production and sugar concentrations in *Salvia hierosolymitana* flowers during male and female phases**

Data for each date are based on two sampling periods of 90–120 min within the range of hours indicated in the table. Different letters within the rows indicate statistically significant differences at  $P < 0.01$  after performing Student's *t*-test. In each sampling period, 20 flowers from each gender phase were sampled from 4 to 6 different plants  $\pm$  s.e. Means  $\pm$  s.e. are reported

Date	Location	Hour	Nectar production ( $\mu\text{L/h}$ )		Sugar concentration (%)	
			Male	Female	Male	Female
1.iv.05	Mount Scopus	7:00–11:00	0.31 $\pm$ 0.08a	0.89 $\pm$ 0.15b	29.9 $\pm$ 1.49a	28.3 $\pm$ 0.69a
29.iii.04	Mount Scopus	6:30–9:30	0.32 $\pm$ 0.07a	1.07 $\pm$ 0.17b	31.1 $\pm$ 1.38a	32.3 $\pm$ 0.98a
14.iv.04	Mount Meron	6:00–10:00	0.25 $\pm$ 0.07a	0.77 $\pm$ 0.12b	30.4 $\pm$ 1.91a	31.03 $\pm$ 1.13a
31.iv.03	Mount Meron	6:00–10:00	0.28 $\pm$ 0.08a	0.43 $\pm$ 0.13b	28.1 $\pm$ 1.03a	29.2 $\pm$ 1.18a



**Fig. 2.** Number and gender distribution of open flowers (a), and bee landing and departures (b) along inflorescences at peak bloom, at Mount Meron. Data were collected in 2003 and 2004 for (a), and in 2003 for (b). Error bars (pointing downward for female-phase flowers and upwards for male-phase flowers) are 1 s.d.

bottom. The inflorescence base had about twice the number of flowers compared with the inflorescence head in both seasons (Fig. 2a). We multiplied the mean number of male- and female-phase flowers in each segment by their mean nectar production, and summed the results for the two sex phases, to estimate nectar production per segment. This calculation indicates the existence of a nectar production gradient along the inflorescence: Nectar production at the inflorescence base was 2-fold higher than its head in 2003, and 2.9-fold higher in 2004.

### Pollen measurements

MTT staining of pollen grains sampled during the two sex phases detected a remarkable decrease in viability, from  $59.5\% \pm 2.5$  (s.e.) during anthesis to  $4\% \pm 0.7$ , 2 days later. Pollen grains that were collected at anthesis and boiled for 1 h at  $80^\circ\text{C}$  before MTT treatment exhibited no staining at all (Fig. 1 h–j). The number of pollen grains recorded on the stigmas of female-phase flowers was relatively low in all sampled populations. However, it was still much higher (~10-fold) than the number of pollen grains on the stigmas of male-phase flowers (Table 2). The number of pollen tubes at the pistils' base during the female phase was very low in all populations, on average barely exceeding the number of ovules per flower. This result was confirmed by sampling fallen flowers, which completed their female phase attached to the plant. The average number of pollen tubes in these flowers was similar to the numbers observed in the sampled female-phase pistils.

Most of the stigmas from flowers that were netted in the field before anthesis to exclude insect visits were pollen-free [94% ( $n=36/38$ ) in Givat-Ram and 85% ( $n=17/20$ ) in Mount Scopus]. The frequencies of pollen-free stigmas in room-grown plants were very high as well: 95% ( $n=20/21$ ) and 92% ( $n=24/26$ ) for plants originating from Givat-Ram and Mount Scopus, respectively. Thus, we conclude that spontaneous self-fertilisation can occur in *S. hierosolymitana* but at a very low rate, and that the majority of the pollen grains recorded on stigmas of non-covered plants were due to pollinator activity.

### Pollinator foraging behaviour

Most of the bees' foraging bouts (64%) started at the inflorescence base, 18% of the bouts started at the middle segment of the inflorescence, and the remaining 18% bouts started at the top segment. The bees moved upwards in all cases. The departure position in most bouts (67%) was the inflorescence head, while in some cases bees left the inflorescence at its base (11% of the bouts) or at its middle segment (22% of the bouts), without reaching the top flowers of the inflorescence (Fig. 2b).

### Discussion

Our observations indicate that the female phase in *S. hierosolymitana* flowers produces 2–3-fold more nectar than the male phase (Table 1). As far as we know, this is the first report of gender differences in nectar production for this species. This gender-biased production pattern, combined with a higher abundance of female-phase flowers at the base of the inflorescences than near their top, contribute to the formation of nectar production gradient along the inflorescences.

Increased nectar production was hypothesised to reflect selection for pollinator attraction by female-phase flowers, when their fitness is limited by fertilisation opportunities, rather than by resources. Pollen availability is a major factor affecting reproductive success in many plants (Ashman *et al.* 2004; Wesselingh 2007). We did not conduct manipulative experiments to directly test for pollen limitation in *S. hierosolymitana*. Nevertheless, the following indirect evidence suggests that viable pollen may be indeed in short supply for female-phase flowers in this species: Laboratory pollen measurements located only 12 grains, on average, on stigmas during their female phase. Only 5–6 pollen tubes per

**Table 2.** Number of pollen grains on stigma and germinating pollen tubes in style base during flowers' male and female phases at various natural populations of *Salvia hierosolymitana*For representative images of germinating pollen tubes in pistils see Fig. 1*k, l*.  $n=20$  flowers from each population  $\pm$  s.e.; n.d. – not detected

Location	No. of pollen grains on stigma		No. of germinating pollen tubes in style base		
	Male phase	Female phase	Attached flowers		Fallen flowers
			Male phase	Female phase	
Mount Scopus	1.3 $\pm$ 0.60	11.2 $\pm$ 1.54	0.36 $\pm$ 0.16	6.4 $\pm$ 0.44	6.5 $\pm$ 0.71
Givat-Ram	1.4 $\pm$ 0.53	13.7 $\pm$ 2.10	0.18 $\pm$ 0.13	5.5 $\pm$ 0.66	5.2 $\pm$ 0.60
Evan-Sapir	1.1 $\pm$ 0.29	10.6 $\pm$ 1.65	0.17 $\pm$ 0.12	4.4 $\pm$ 0.62	3.4 $\pm$ 0.37
Nes-Harim	1.5 $\pm$ 0.41	10.1 $\pm$ 1.14	0.21 $\pm$ 0.16	4.6 $\pm$ 0.52	4.7 $\pm$ 0.44
Nahal-Refaim	1.1 $\pm$ 0.54	12.6 $\pm$ 1.37	0.10 $\pm$ 0.09	4.2 $\pm$ 0.46	n.d.
Average	1.3 $\pm$ 0.47	11.6 $\pm$ 1.56	0.20 $\pm$ 0.13	5.02 $\pm$ 0.54	4.95 $\pm$ 0.55

flower were found at the pistil base, both in female-phase pistils and in pistils from fallen flowers that abscised naturally from plants (Table 2). Moreover, MTT staining indicated that 40% of the produced pollen is sterile, and that its viability decreases dramatically from 60 to 4% by the end of the female phase (Fig. 1*h–j*). This partial viability reduces the probability of germination even if the pollen is successfully transferred to the stigma. This may partially explain the failure of 60% of pollen grains to germinate in our sample of female-phase flowers (Table 2). *S. hierosolymitana* flowers have 4 ovules to be fertilised (as most members in the Lamiaceae family, Heywood 1978), and only 3.2 of them, on average, develop into seeds. A possible interpretation for this observation is that pollen quantity and/or quality may limit fertilisation in the insect-pollinated *S. hierosolymitana*. The high abundance of sterile pollen grains in the anthers during the flowers' male stage suggests that *S. hierosolymitana* may exhibit pollen heteromorphism, i.e. produces both fertile and sterile morphs (Till-Bottraud *et al.* 2005).

If pollen limits female fitness, then selection for pollinator attraction is expected to operate on female-phase flowers. The observed female bias in nectar production may reflect this selection, as female-phase flowers seem to invest more resources in food rewards for pollinators than male-phase flowers. The higher pollen loads on female-phase stigmas as compared with male-phase flowers suggest that female-phase flowers received more effective pollinator visits. This could result from a higher frequency of visits to the high-rewarding female-phase flowers, and/or increased pollen transfer efficiency via longer visit durations in the higher-rewarding flowers (Conner *et al.* 1995). Additional possible explanations for the higher pollen loads in female-phase flowers is that these flowers are older than male flowers, and have therefore provided more time for pollen accumulation on the stigmas; that the morphology of female-phase stigmas (facing downwards with bifurcated lobes) enhances the ability of pollen to accumulate; or that female-phase stigmas are more sticky, thereby facilitating the adherence of pollen grains.

Female-biased nectar production was further proposed to promote cross-pollination by affecting the foraging patterns of insect pollinators. This hypothesis is also compatible with our observations of *S. hierosolymitana*: the increased nectar production during the female phase, combined with the high abundance of female-phase flowers at the inflorescence base,

lead to decreasing nectar productivity from the base of the inflorescences towards their tops. This nectar production gradient may reinforce the tendency of insect foragers to move from bottom to top. Indeed, all bees observed in the present study foraged upwards on *S. hierosolymitana* inflorescences. When shifting between plants, insect visitors are predicted to move from male-phase flowers at the top of one inflorescence to female-phase flowers at the bottom of the next. This movement pattern could benefit the plants by increasing outcrossing, and reducing geitonogamy. However, spontaneous selfing (as observed in our study) and low geitonogamous pollen transfer may be one of the reasons for the low pollen loads observed on female-phase stigmas. This suggests that the reproductive strategy of *S. hierosolymitana* may involve mainly cross-fertilisation, even at the risk of not receiving sufficient pollen.

At the proximate level, the higher nectar production of female-phase flowers may result from their location near the base of the inflorescences, close to the photosynthetic tissues that form a source of assimilates. This hypothesis assumes that nectar production rates are limited by carbon availability, so that flowers situated near carbon sources have a competitive advantage over distal flowers. In support of this assumption, the central flowers of the dichasian inflorescences of *Petrocoptis grandiflora* produce more nectar than first- or second-order lateral flowers (Gutián and Navarro 1996). However, drastic defoliation (reduction of carbon sources) did not reduce nectar production rates in *Polemonium fliosissimum* (Zimmerman and Pyke 1988) and in *Alstroemeria aurea* (Aizen and Raffaele 1996). Thus, nectar production rates do not seem to be universally carbon-limited. To separate the effects of flower gender and vertical location on nectar production in *S. hierosolymitana*, the existence of a vertical gradient in nectar production within flowers of a given gender, or differences in production between male and female flowers at the same position, should be assessed. Such tests were beyond the scope of the present study.

Our classification of the flowers' sexual phase was based on stigma morphology and ROS activity. These markers correlate well with stigma receptivity, which was much higher during the female phase (43.5% mean pollen germination rate) than during the male phase (15.8% pollen germination, derived from Table 2). This finding validates our use of the morphological marker for identification of the flowers' sex phase. It also suggests that pollen deposition on male-phase stigmas might be wasteful, since the germination prospects of such pollen are low. Under

conditions of pollen limitation, it may be adaptive for the plants to reduce such wastage by producing attractive female-phase flowers.

Pollen removal rates from anthers by visiting pollinators provide a further explanation for reduced male investment in nectar production. Thomson and Thomson (1989) reported cases where a single visit of a bee in *Erythronium grandiflorum* flowers removed 85% of the anthers' pollen. Thus, if only a few visits are needed for complete pollen release from the anthers, no further benefit arises from producing extra nectar for male function. The pollen removal efficiency of bee visitors was not studied in this research, and further work is needed to clarify these essential features in *S. hierosolymitana*.

Competition among pollen grains that arrive in larger numbers than needed to fertilise all ovules, and choice of the fittest ones by the maternal tissue, can be interpreted as sexual selection (Charnov 1979; Delph and Havens 1998; Skogsmyr and Lankinen 2002). In this study the observed ratio of floral ovules/germinating pollen tubes was almost 1, a finding that suggests little competition among pollen tubes and/or female choice. Thus, this report may contribute to the ongoing debate about whether sexual selection actually occurs in plants (Charlesworth et al. 1987; Lyons et al. 1989; Grant 1995; Winsor et al. 2000).

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