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FEMALE-BIASED NECTAR PRODUCTION IN THE PROTANDROUS, HERMAPHRODITIC SHRUB SALVIA HIEROSOLYMITANA: POSSIBLE REASONS AND CONSEQUENCES

by

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Discussion Paper #494

December 2008

מרכז לחקר הרציונליות

CENTER FOR THE STUDY OF RATIONALITY

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Female-biased nectar production in the protandrous, hermaphroditic shrub Salvia hierosolymitana: possible reasons and consequences

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ABSTRACT

Sexual selection theory states that male reproductive success is commonly limited by opportunities for fertilization, while female reproductive success is more often resource-limited. This creates higher selective pressure on males to attract mating partners as compared with females. Similar reasoning, when applied to plant reproduction, predicts higher investment in pollinator-attracting traits, such as nectar production, in male flowers than in female flowers. Contrary to this prediction, nectar production by female-phase flowers in the protandrous hermaphrodite shrub Salvia hierosolymita (Boiss.) (Lamiaceae) was significantly higher than in male-phase flowers in two populations over three years. Female-biased nectar production may reflect selection for pollinator attraction by female-phase flowers, possibly due to pollen limitation. In support of this interpretation, (a) the number of pollen grains on female-phase stigmas was substantially higher than on male-phase stigmas, suggesting that the female phase received more insect visits; (b) the number of germinating pollen grains in female-phase styles only slightly exceeded the number of ovules per flower, therefore pollen availability may restrict female fitness. Proportions of femalephase flowers decreased from the base of the inflorescences towards their top. This creates a vertical gradient of nectar production, which may help reduce geitonogamous pollination by effecting pollinator behavior.

<u>Key words</u>: female choice, nectar production, pollination, pollen limitation, *Salvia hierosolymita*, sexual selection theory

INTRODUCTION

The evolution of sexually dimorphic traits is of fundamental importance for many organisms (Hedrick and Temeles, 1989). Sexual selection theory maintains that the asymmetry in reproductive roles between males and females selects for different traits in the two sexes. According to this theory, the reproductive success of males is commonly limited by the number of fertilizations they can obtain. Therefore, traits that make males more attractive to females (secondary sexual traits) are expected to be selected. Females, on the other hand, invest more resources in gamete production and other aspects of parental care; hence their reproductive success is often resource-limited. Selection should therefore increase the ability of females to choose suitable mates, rather than maximizing their mating opportunities (Andersson, 1994).

In insect-pollinated plants, reproduction is mediated by pollinator vectors. Plants are selected to attract pollinating insects, rather than conspecifics of the opposite sex. The validity of applying sexual selection theory to plant reproductive systems is therefore not self-explanatory. While still debated, sexual selection arguments have been increasingly used in recent years to explain reproductive phenomena in plants (Skogsmyr and Lankinen, 2002, Delph and Ashman, 2006). The male component of plant fitness is expected to be limited by the efficiency of pollen export by pollinators, while the female component of fitness should be limited by resources for seed production (Stanton et al., 1986). Higher investment in traits that attract pollinators is expected in male flowers (or hermaphroditic flowers in their male phase) than in female (or female-phase) flowers (Bell, 1985). It has further been argued that such traits can be considered secondary sexual characters (Carlson, 2007).

In dichogamous hermaphrodite plants, male and female functions are separated in time, creating a potential for different selection pressures on the two sex phases (Carlson, 2007). Nectar is produced by flowers as a food reward for nectarivores, and can therefore be considered a pollinator-attracting character. Nectar production rates in hermaphrodite plants are often, indeed, sexually

dimorphic. As predicted by sexual selection theory, nectar production rates are higher in male-phase than in female-phase flowers in several species. In other species, on the other hand, flowers produce more nectar when they are in their female phase than during their male phase (Carlson and Harms, 2006). A possible explanation for female-biased nectar production is that the assumption of resources as a limiting factor for female reproductive success does not hold for all plants. Female success may be pollen-limited, rather than resource limited, in some species, leading to selection for female traits that attract pollinators (Burd 1994; Wilson et al., 1994; Ashman et al., 2004; Knight et al., 2005). Two testable predictions arise from this interpretation. First, the female phase in plants with female-biased nectar production should receive more insect visits, and hence a higher import of pollen, than the male phase. Second, such female-phase flowers should exert rather little choice of pollen grains.

In the present study we adopted a descriptive approach to test these predictions. We first documented female-biased nectar production in the Mediterranean hermaphrodite shrub *Salvia hierosolymitana* (Boiss.) (Lamiaceae). We then compared pollen import to male-phase vs. female-phase flowers, by counting pollen grains on stigmas in both sexual phases. Finally, we estimated the potential for female choice of mates in this species by counting germinating pollen grains in female-phase pistils.

MATERIALS AND METHODS

<u>Plant Material</u> - The research was conducted during the flowering seasons of 2003-2007 in several populations of *Salvia hierosolymitana* at the following locations: 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). *S. hierosolymitana* blooms in

Israel during March-April and is pollinated mostly by solitary large bees of the genus *Anthophora*. Plant density in the studied populations ranged 100-200 shrubs per 1 km². The flowers are arranged in whorls, which are situated at intervals of 2-4 cm along vertical inflorescences. Blooming progresses from the inflorescence base upwards, but not all buds in a whorl open simultaneously (Fig. 1A, *S. hierosolymitana* in full bloom). The time from anthesis to flower abscission is 48.6 (± 2.5) h under controlled conditions (unpublished data). 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 VH, 1978). The fruits consist of four one-seeded indehiscent achene-like nut-lets. The mean (±SD) number of seeds per flower in our study populations was 3.2± 0.87, (n=400 flowers, data not shown).

Determination of floral sex-phases

S. hierosolymitana's flowers 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). 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 it bifurcates. 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 minutes for determination of ROS activity. We used DAB stain (3,3'-diaminobenzidine, SIGMA), which polymerizes on contact with H_2O_2 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, Japan). This procedure confirmed that staining increases dramatically as the aperture of the stigma lobes' widens (Fig. 1 E-G). Thus, stigma bifurcation served during field work as a good indicator for the determination of flower sex phase.

<u>The vertical distribution of male- and female-phase flowers</u>: The inflorescences of 15 plants were divided into three vertical segments of equal lengths. The total number of open flowers and the proportions of flowers in male/female phases were determined in each segment.

Nectar production measurements: Nectar was collected from flowers at the male/female phases, using 5μ L micropipettes (BLAUBRAND intraMARK, Germany). Plants were covered by nets (20 mesh) at sunset prior to measurement after ascertaining that flowers contained no nectar (due to pollinators' dusk activity), and remained covered until measurements. Samples were taken at three time points of each day: after sunrise, (which served as t0 during the study), 90-120 minutes later (t1), and 90-120 minutes after t1 (t2). The plants were netted between sampling periods. Nectar sugar concentration (w/w%) was measured with a hand-held refractometer (Bellingham and Stanley, UK). In each sampling period, 20 flowers of each gender phase were sampled from 4-6 plants. Sampling was destructive, therefore each flower was sampled only once. Nectar production rate per flower was calculated as the difference between the recorded nectar volume at time t_x and the mean volume at time t_{x-1} , divided by the time elapsed between these measurements.

<u>Pollen grains and tubes measurements</u>: During the 2007 season, the number of pollen grains on stigmas was assessed during the male and female flower sex phases. Pollen measurements were conducted in five natural populations in and around Jerusalem. Pollen grains were observed and counted under a binocular - stereoscope (OLYMPUS, Japan).

<u>Germination of pollen tubes</u> was assessed at the pistil base during the male and female flowering phases through staining of stigmas with Aniline blue (Pagnussat et al., 2005). In brief, 20 flowers from each gender phase were sampled at each of the studied populations. Flowers were brought to the laboratory for analysis in a cool container, and were used within 60-120 minutes of picking. Pistils were cleared in 10% chloral hydrate at 65°C for 5 minutes and washed with H₂O, softened

with 5M NaOH at 65°C for 5 minutes and washed again with H₂O. Pistils were then incubated with 0.1% Aniline Blue in 0.1 M K₃PO₄ buffer pH 8.3 for 3 hours in darkness and then washed with 0.1 M K₃PO₄ buffer. A drop of glycerol was used to mount the pistils onto a microscope slide. They were then carefully squashed under a cover slip. Germinating pollen tubes 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).

<u>Viability of pollen grains</u> during flower different gender phases was determined by Methylthiazolyldiphenyl-tetrazolium bromide (MTT) staining as described previously (Rodriguez and Dafni 2000, Khatun and Flowers 1995). Eight anthers from four plants were sampled at anthesis and two days later - during the female phase, and were brought to the lab in cooled containers. Pollen grains were treated with 0.9 % MTT (SIGMA, USA) for 15 minutes, then inspected under a light microscope. The number of viable grains (identified by deep-red staining) was determined among ~ 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^oC for 1 hour prior to MTT treatment.

RESULTS

Nectar production

Nectar production during the flower's female stage was higher than during the male stage at all seasons and locations: During 2004 and 2005 in Mount Scopus the female phase production rate was 0.89 ± 0.15 and $1.07 \pm 0.17 \mu$ l/h respectively, i.e. 3.3- 2.9 times higher than the male phase production rate. The same pattern was observed in Mount Meron during 2003 and 2004: the female phase production rates were 0.43 ± 0.13 and $0.77 \pm 0.12 \mu$ l/h respectively, 1.5-3.1 times higher than the male phase the male phase (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 sexphase: $F_{1, 175}=242.86$, P<0.001). Nectar sugar concentrations did not differ between the flower phases, and varied between 28-32 % (Table 1).

				Nectar Production (mL/Hour)		Sugar Concentration (%)	
	Date	Location	Hour	Male	Female	Male	Female
_	1.4.05	Mount Scopus	7:00-11:00	0.31 ± 0.08 a	$0.89\pm0.15~\mathrm{b}$	29.9 ± 1.49 a	28.3 ± 0.69 a
	29.3.04	Mount Scopus	6:30-9:30	0.32 ± 0.07 a	$1.07\pm0.17~\mathrm{b}$	31.1 ± 1.38 a	$32.3\pm0.98~\mathrm{a}$
	14.4.04	Mount Meron	6:00-10:00	$0.25\pm0.07a$	$0.77\pm0.12~\mathrm{b}$	30.4 ± 1.91 a	31.03 ± 1.13 a
	31.4.03	Mount Meron	6:00-10:00	0.28 ± 0.08 a	$0.43 \pm 0.13 \text{ b}$	28.1 ± 1.03 a	29.2 ± 1.18 a

 Table 1 . Differential Nectar production and sugar concentration in Salvia heirosolymitana flowers during male/female phases.

- Different letters within the rows indicate statistical significance at p<0.01 after performing students T-test

- In each sampling period, 20 flowers from each gender phase were sampled from 4 to 6 different plants \pm SE

At full bloom, most flowers along the inflorescence base were at their female phase, but this percentage dropped towards the inflorescence top (Fig. 2A). The distribution of male-phase flowers complemented this pattern, as they were more abundant at the top part of the inflorescences than at their bottom. The inflorescence base at that time had an average number of 4.6 open flowers, more than twice than its head (Fig. 2B). Integration of the above results indicates the existence of a nectar production gradient along the inflorescence, in which nectar production at the inflorescence base is approximately 2.5 fold higher than its head.



Figure 1

Figure 1- Male and female phases in *Salvia heirosolymitana* **flower**, stigma receptivity, pollen viability and germination during these phases. Vertical inflorescence at full bloom A), anthesis B), male phase C), female phase D). Representative pictures of DAB (3,3'-diaminobenzidine) stain of the stigma prior to anthesis E) and during male F) and female G) phases. Note the increase in brown-dark precipitate, which indicates increase in reactive oxygen species (ROS) activity. Scale bar for E-G = 1mm.

Representative pictures of MTT (Methylthiazolyldiphenyl-tetrazolium bromide) staining of pollen grains during anthesis H), two days after anthesis I) and pollen grains sampled during anthesis and boiled at 80° c for 1 hour prior to MTT treatment J). Scale bar for H-J = 50μ m.

Representative pictures of Aniline blue staining of germinating pollen tubes in pistils during male K) and female L) phases. White arrows in K) indicate Xylem (Xy) location in the pistil while white arrows in L) indicate Callose (Ca) deposits of pollen tubes. Scale bar for K-L = $60\mu m$.

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 (SE) during anthesis to 4 % \pm 0.7 two days later. Pollen grains that were collected at anthesis and boiled for 1h in 80°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 (10.1-12.6) in all sampled populations. However, it was still much higher (approximately 10-fold) than the number of pollen grains in male-phase flowers (1.1-1.5) (Table 2). The number of germinating pollen tubes at the pistils' base during its female phase was very low in all populations (4.2-6.4). 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 (3.6-6.5) to the numbers observed in the sampled female-phase pistils.

Table 2. Number of pollen grains on stigma and germinating pollen tubes in style base during flowers'male/female phases at various natural populations of *Salvia heirosolymitana*. For representative images of germinating pollen tubes in pistils see fig. 1 K-L.

	Number grains	r of pollen on stigma	Number of germinating pollen tubes in style base			
Location			Attache	Fallen flowers		
	male phase	female phase	male phase	female phase		
Mt.scopus	1.3 ± 0.60	11.2 ± 1.54	0.36 ± 0.16	6.4 ± 0.44	6.5 ± 0.71	
givat-ram	1.4 ± 0.53	13.7 ± 2.10	0.18 ± 0.13	5.5 ± 0.66	5.2 ± 0.60	
evan-sapir	1.1 ± 0.29	10.6 ± 1.65	0.17 ± 0.12	4.4 ± 0.62	3.4 ± 0.37	
nes-harim	1.5 ± 0.41	10.1 ± 1.14	0.21 ± 0.16	4.6 ± 0.52	4.7 ± 0.44	
nahal-refaim	1.1 ± 0.54	12.6 ± 1.37	0.10 ± 0.09	4.2 ± 0.46	n.d.	
Average	1.3 ± 0.47	11.6 ± 1.56	0.20 ± 0.13	5.02 ± 0.54	4.95 ± 0.55	

- n=20 flowers from each population \pm SE

- n.d. - not detected

To assess the possibility of self pollination in *S. hierosolymita*, 6-8 inflorescences were covered by nets in Givat-Ram and Mount Scopus populations, and the number of pollen grains was determined in female-stage stigmas. Most of the observed stigmas were pollen free (94% [n=36/38] in Givat-Ram and 85% [n=17/20] in M. Scopus). Since handling of the nets under field conditions may cause some artificial pollen spread, the same protocol was performed on flowers grown in controlled room. 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 self pollination can occur in *S. hierosolymita* but at a very low rate, and that the majority of the pollen grains recorded on stigmas of non-covered plants are due to pollinator activity.





DISCUSSION

Differential gender nectar production in hermaphrodite flowers has been recently reviewed (Carlson and Harms, 2006). In many species the male phase produces more nectar than the female phase, but several examples of the opposite trend have also been reported. 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. From a more general point of view, our findings contribute to the important issue of the implication of sexual selection theory in plants (Charnov, 1979., Charlesworth et al., 1987; Lyons et al., 1989; Grant, 1995, Klinkhamer et al., 1994, Winsor et al., 2000; Thomson 2006). The results presented in this study contradict predictions derived from this theory: Flower nectar production, a measure of investment in attraction, was significantly lower in male-phase flowers than in the female phase. Additionally, the incomplete viability of fresh pollen, the low pollen load on stigmas during their female phase and the observed ratio of germinating pollen tubes/ovules that was close to 1 (Table 2), suggest that female choice in *S. hierosolymitana* is very limited.

We investigated one possible reason for the discrepancy between theoretical prediction and empirical observation, namely the possibility that female fitness is limited by fertilization opportunities, rather than by resources, in our study species. Pollen availability is a major factor effecting reproductive success in many plants (Ashman et al., 2004; Wesselingh 2007). Laboratory pollen measurements located only 12 grains, on average, on *S. hierosolymitana* stigmas during their female phase. Only 5-6 germinating pollen tubes 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 the viability of the remaining 60% decreases dramatically 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 four ovules to be

fertilized (as most members in the *Lamiaceae* family, Heywood VH, 1978), and only 3.2 of them develop into seeds. A possible interpretation for this observation is that pollen quantity and/or quality may limit fertilization in insect-pollinated *S. hierosolymitana*

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 reward for pollinators than malephase flowers. The higher pollen loads on female-phase stigmas as compared with male-phase flowers suggest that female-phase flowers indeed 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). An alternative (and trivial) explanation for the higher pollen loads in femalephase flowers is that these flowers are older than male flowers, and have therefore provided more time for pollen accumulation on the stigmas. However, the morphology of male-phase stigmas (facing upwards with clustered lobes) seems to reduce the ability of pollen to accumulate. In the female phase, on the other hand, the stigma bends down and its lobes bifurcate. This morphology increases the likelyhood of stigma-pollinator touch events, which may lead to pollen accumulation.

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. Within this context, it is interesting to note that self pollination in *S. hierosolymitana's* flowers is rare, even though pollen may be a limiting factor in this species. This

suggests that the selective benefits of outcrossing may be higher than that of selfing, although crosspollinating plants bear the risk of not receiving sufficient pollen.

Pollen removal rate from anthers by visiting pollinators may also explain the 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 few visits are needed for complete pollen release from the anthers, no further benefit arise from producing extra nectar for male function. Pollinator activity and behavior were not studied in this research and further work is needed to clarify these essential features in *S. hierosolymitana*.

An additional implication of *S. hierosolymitana's* female-biased nectar production is in contributing to the formation of nectar gradient along the inflorescence. 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. Foraging bees in vertical inflorescences tend to arrive at the inflorescence base, climb upward, and leave the inflorescence before reaching the top flowers (Waddington & Heinrich, 1979, Best & Bierzychudek 1982, Orth, 1997). We speculate that the nectar production gradient recorded in our study may reinforce the tendency of insect foragers to move from bottom to top. By following the nectar gradient, foraging insects are expected to move from female-phase to 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 geitonogamous pollen transfer.

We conclude that the selective advantage of female-biased nectar production in *S*. *hierosolymitana*'s may be twofold: enhancement of female reproductive success under pollen limitation, and enhancement of offspring quality though increased outcrossing. Additional research is needed for evaluating the relative importance of these putative benefits.

ACKNOWLDEGEMENTS

The authors wish to thank Mimi Ron and Ori Fragman-Sapir from the Hebrew University botanical gardens at Mount Scopus and Givat-Ram respectively for essential assistance, Alex Levine from the department of Plant Sciences at the Hebrew University for help with laboratory facilities and Yaron Shaul for assistance in field work. We are grateful to Tom de Jong from Leiden University for critical reading of the manuscript. Sadly, Prof. Tzvi Sachs from the department of Plant Sciences in the Hebrew University who encouraged this study, passed away before completion of the work. The study was supported by the Center for Rationality and by the Institute for Advanced Studies at the Hebrew University

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