

## Up and down: stamen movements in *Ruta graveolens* (Rutaceae) enhance both outcrossing and delayed selfing

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- **Background and Aims** Stamen movements directly determine pollen fates and mating patterns by altering positions of female and male organs. However, the implications of such movements in terms of pollination are not well understood. Recently, complex patterns of stamen movements have been identified in Loasaceae, Parnassiaceae, Rutaceae and Tropaeolaceae. In this study the stamen movements in *Ruta graveolens* (Rutaceae) and their impact on pollination are determined.
- **Methods** Pollination effects of stamen movements were studied in *Ruta graveolens*, in which one-by-one uplifting and falling back is followed by simultaneous movement of all stamens in some flowers. Using 30 flowers, one stamen was manipulated either to be immobilized or to be allowed to move freely towards the centre of the flower but be prevented from falling back. Pollen loads on stigmas and ovule fertilization in flowers with or without simultaneous stamen movement were determined.
- **Results** Pollen removal decreased dramatically ( $P < 0.001$ ) when the stamen was stopped from uplifting because its anther was seldom contacted by pollinators. When a stamen stayed at the flower's centre, pollen removal of the next freely moved anther decreased significantly ( $P < 0.005$ ) because of fewer touches by pollinators and quick leaving of pollinators that were discouraged by the empty anther. Simultaneous stamen movement occurred only in flowers with low pollen load on the stigma and the remaining pollen in anthers dropped onto stigma surfaces after stamens moved to the flower's centre.
- **Conclusions** In *R. graveolens* pollen removal is promoted through one-by-one movement of the stamen, which presents pollen in doses to pollinators by successive uplifting of the stamen and avoids interference of two consecutively dehiscent anthers by falling back of the former stamen before the next one moves into the flower's centre. Simultaneous stamen movement at the end of anthesis probably reflects an adaptation for late-acting self-pollination.

**Key words:** Dichogamy, experimental manipulation, pollen presentation, pollen removal, pollination, reproductive assurance, *Ruta graveolens*, stamen movement.

### INTRODUCTION

Much current research in pollination ecology has emphasized the importance of male reproductive success (pollen was successfully removed and transferred to the conspecific stigma) in shaping floral structures, particularly in outcrossing, animal-pollinated species (Thomson *et al.*, 2000; Kudo, 2003; Castellanos *et al.*, 2006; Conner *et al.*, 2009; Weigend *et al.*, 2010). Among the floral traits affecting male reproductive success, stamen movement is a key factor as it directly determines the contact frequency and precision of anther/pollen with pollinators (Schlindwein and Wittmann, 1997; Taylor *et al.*, 2006; Ren, 2010). Despite adaptations for the promotion of outcrossing, stamen movement is also found in some species to be capable of facilitating selfing when anthers move to the stigma (Nagy *et al.*, 1999; Liu *et al.*, 2006). Ren (2010) has summarized four main types of stamen movement: stimulated movement (Schlindwein and Wittmann, 1997), simultaneous and slow movement (Du *et al.*, 2012), quick and explosive movement (Taylor *et al.*, 2006;

Whitaker *et al.*, 2007), and cascade (successive) movement (Weigend *et al.*, 2010; Ren, 2010). These different types are not mutually exclusive (Ren, 2010).

Successive stamen movements, which involve one-by-one or group-by-group movements, are probably one of the most complex of such movements (Ren, 2010). It is found in most species of Loasaceae and Parnassiaceae, genus *Ruta* in Rutaceae and *Tropaeolum majus* in Tropaeolaceae (Ren, 2010). In these species, stamens are reflexed in the hooded petals (in Parnassiaceae, anthers aggregate at the centre of the flower) at the beginning of anthesis. The stamens then elevate one-by-one or group-by-group slowly from the petals to the centre of the flower for the anther to dehisce (in Parnassiaceae, the filament elongate and anthers dehisce individually). Of greater significance is that the anther-dehiscent stamen leaves the flower's centre before the next stamen moves into the flower's centre (Ren, 2010; Weigend *et al.*, 2010; Tang and Ren, 2011). Furthermore, in some flowers of *Ruta graveolens*, all of the stamens simultaneously elevate to enclose the pistil at the end of anthesis after they have finished

their one-by-one movement (Ren, 2010). These one-by-one and back-and-forth movements of stamens between petals and the flower's centre followed by simultaneous stamen movement in *R. graveolens* probably represent the most complex mode of stamen movement currently known in angiosperms (Ren, 2010; Tang and Ren, 2011).

Although successive stamen movements have been described in Loasaceae (Brown and Kaul, 1981; Wittmann and Schlindwein, 1995; Weigend *et al.*, 2010), their importance in terms of pollination has not yet been clarified, especially the adaptive significance of the former stamen leaving the flower's centre before the next one moves into it. Furthermore, the simultaneous movement of all of the stamens to the flower's centre after their successive movements at the end of anthesis in *R. graveolens* has received little, if any, attention. Successive movements of stamens and sequential dehiscences of anthers in *R. graveolens* allow direct measurement of pollen presentation and removal in this species. It thus provides an excellent opportunity to measure the detailed interactions of individual anthers with pollinators and to test the 'pollen presentation theory', which suggests that the scheduling and control of pollen release to visitors probably reflect selections to maximize pollen output (Harder and Thomson, 1989; Harder and Wilson, 1994; Thomson *et al.*, 2000).

Here we conducted two experimental manipulations, both in two different years, to determine the effects on pollen removal of successive stamen movements in *R. graveolens*. The pollination effect of simultaneous movement of stamens at the end of anthesis in some flowers was also explored by comparing the pollen loads on stigma and ovule fertilization for flowers with or without this movement. We hypothesized that: (1) the one-by-one movement of stamens might be a particular mechanism to present pollen in doses gradually to pollinators by successively presenting one mature anther at the flower's centre, maximizing the probability of contact with pollinators; (2) leaving of the former dehiscent anther from the flower's centre probably decreases interference of pollen removal between two consecutively moving stamens; (3) the simultaneous movement of all stamens to the flower's centre at the end of anthesis could be a mechanism for reproductive assurance by delayed self-pollination. Study of such extremely complex modes of stamen movements, successive stamen movements followed by simultaneous stamen movement, will be helpful in understanding the adaptations of stamen movements and the role of male reproductive success in floral evolution.

## MATERIALS AND METHODS

### *Study species and experimental populations*

*Ruta graveolens* L. is naturally distributed in Eurasia (Tang and Ren, 2011). In China, this species is planted as a medicinal herb in gardens, especially in central and northern parts of the country (Tang and Ren, 2011).

*Ruta graveolens* blooms from March to June (Weryszko-Chmielewska *et al.*, 2003; M.-X. Ren, pers. observ.). Flowers are yellow and borne in dichasial cymes. Each inflorescence has a single central pentamerous flower (five petals and ten stamens) that open first. All subsequent flowers (>20) are tetramerous with four petals and eight

stamens (Tang and Ren, 2011). Our pollination survey and experiments were conducted in two *R. graveolens* populations in two different years: the nursery of the Institute of Medicinal Plant Development of the Chinese Academy of Medical Sciences (Beijing, northern China, 40°01'59.40"N, 116°16'27.18"E; altitude 54 m) in 2010; and the garden of the Institute of Botany, Chinese Academy of Sciences (Beijing, 39°59'07.78"N, 116°12'40.01"E; altitude 69 m) in 2011. Both populations consisted of over 30 individuals.

### *Pollination observation*

According to preliminary observations, the two populations were frequently visited by insects during the whole period of anthesis. We classified the floral visitors into five functional groups: honeybees, halictid bees, hoverflies, flies and wasps. Functional groups were used rather than species because they are easier and more accurate to record and normally in each functional group members foraged similarly in flowers, and interacted with floral parts in a similar way (Fenster *et al.*, 2004; Gong and Huang, 2009).

The visitation rates of each functional group were observed during 0830–1030 and 1400–1630 h on 20 sunny days in May and June 2011 and therefore a total of 90 h of observation were obtained. With the aid of a video camera (Sony HDR-SR7), we determined the details of visiting behaviours of insects and their contacts with the stigmas and anthers at different heights due to different movement steps. We collected five individuals for each functional group to count pollen grains on their bodies using a dissecting microscope ( $\times 200$ , Olympus SZX7; Olympus, Tokyo, Japan), by which we determined these insects as effective pollinators or not.

### *Patterns of successive stamen movements*

To determine the movement processes of stamens in pentamerous and tetramerous flowers, we observed 15 flowers *in situ* respectively for pentamerous and tetramerous flowers in two populations on sunny days. A preliminary observation found that the movement of each stamen is slow, normally taking more than 20 min for one stamen to move to the flower's centre or return back, and stamen stops moving during the night, so the entire time for all stamen movements in a flower will be 2 or 3 d (Tang and Ren, 2011). Therefore, we determined the movement patterns mainly by *in situ* observation with the help of a video camera (Sony HDR-SR7). The time for each of the following key processes was recorded and calculated: (1) between the first stamen beginning to uplift and full opening of the flower (all petals are extended to be in the same plane); (2) a stamen reaching the flower's centre; (3) a stamen beginning anther dehiscence after arriving at the flower's centre; (4) a stamen staying at the flower's centre; (5) falling back from the flower's centre to the stamen's original position; (6) the next stamen starting its movement after the former stamen has fallen back to its original position; (7) between the start of simultaneous movement and the end of successive movements of all stamens; and (8) simultaneous movement of stamens (all stamens arriving at the flower's centre).

We also measured style length and anther height for each moving stamen every hour during the processes of successive

stamen movements for ten tetramerous flowers. These flowers were chosen by their similar start time of stamen movements (at about 1000 h) to avoid the effects of night on the pattern of stamen movements.

#### Nectar production

The production of nectar was examined for ten bagged flowers (five for pentamerous and tetramerous flowers, respectively) on sunny, warm days. Nectar was sampled every hour after the flowers fully opened until the successive stamen movements finished. We used a 10- $\mu$ L hand-held pipettor (Eppendorf, Germany) to quickly collect the nectar, which was then preserved in a 200- $\mu$ L microcentrifuge tube (Eppendorf), and we then determined nectar volume using pipettors of different spans (10, 5 and 2  $\mu$ L).

#### Pollen and ovule numbers

We determined the number of pollen grains produced by each anther of the pentamerous and tetramerous flowers. Individual anthers were dissected and washed in 1 mL distilled water to dislodge pollen grains from the anther. The suspensions were stirred in a vortex mixer for 30 s and ten 1- $\mu$ L samples of suspension were drawn, from which the number of pollen grains was counted under a dissecting microscope. The pollen count of ten sub-samples of 1  $\mu$ L each were averaged and multiplied by the dilution factor (1000) to obtain the total number of pollen per anther. Thirty anthers respectively from pentamerous and tetramerous flowers were used to determine pollen production per anther ( $G_1$  and  $G_2$  for pentamerous and tetramerous flowers, respectively). Numbers of ovules in

15 pentamerous and tetramerous flowers respectively were also counted under the microscope.

#### Experimental manipulation of successive stamen movements

We chose 15 plants bearing not fewer than three inflorescences as experimental plants for manipulation experiments. On each plant, three inflorescences were chosen and bagged before flower opening to provide unvisited flowers. The pentamerous flower and one tetramerous flower from each inflorescence were selected as experimental flowers. To ensure the data were comparable, all the manipulation experiments were carried out on the second stamen to move (hereafter 2nd stamen).

One of the three inflorescences from each plant was selected as the control to measure pollen removal under natural conditions. The stamens in the flower were allowed to move freely to the centre of flowers and received pollinator visits naturally. When the 2nd stamen began to leave the flower's centre, we took the anther off and measured the number of remaining pollen grains in it ( $G_1'$  and  $G_2'$  for pentamerous and tetramerous flowers, respectively) under the dissecting microscope with the same method as above. Pollen removal under natural conditions was  $G_1 - G_1'$  and  $G_2 - G_2'$  for pentamerous and tetramerous flowers, respectively.

Flowers (one pentamerous and one tetramerous) on the second inflorescence of each plant were used to determine the effect of stamen uplift on pollen removal. To do this we prevented the 2nd stamen in each of the selected flowers from moving to the flower's centre by binding it with yellow thread (Fig. 1G). Thread and petal colour was matched to minimize the possible effect of the thread on pollinator visitation. With the help of the thread, the anther was immobilized and

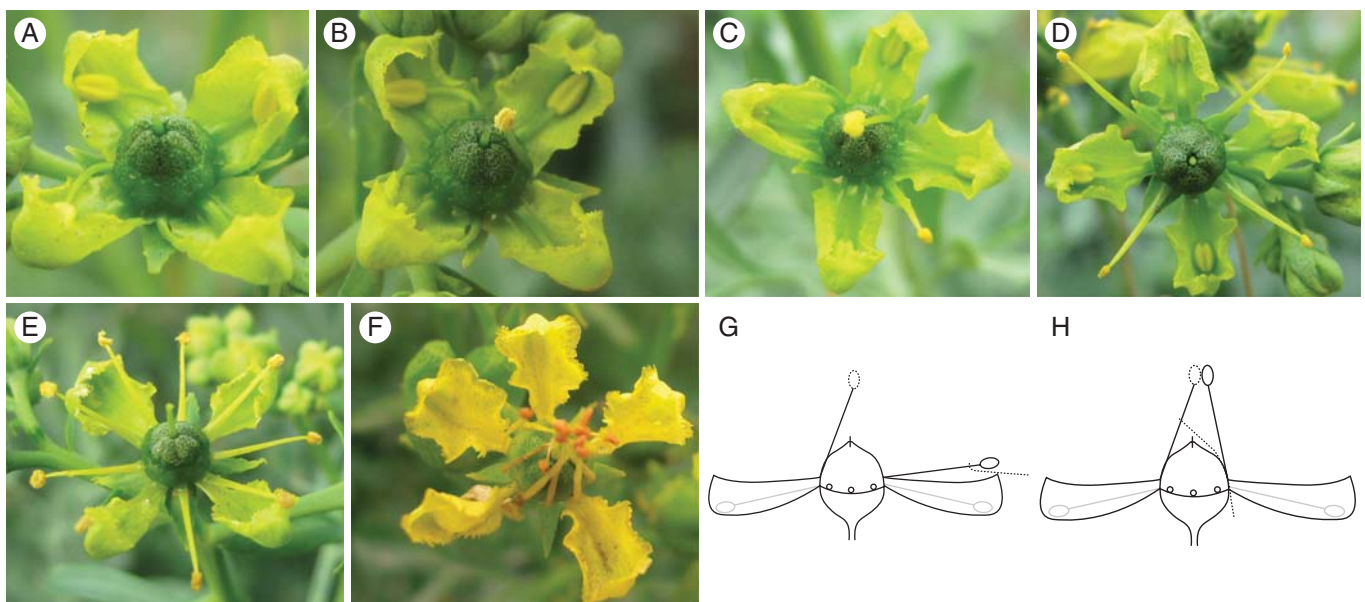


FIG. 1. Successive stamen movements and experimental manipulations in *Ruta graveolens* (tetramerous flowers as an example). (A) At the beginning of anthesis, all stamens are reflexed into hooded petals. (B) One antisepalous stamen uplifts slowly, moving its anther finally to the flower's centre, where the anther begins dehiscing. (C) This stamen falls back to the petal before the next stamen arrives at the flower's centre. (D) Four antisepalous stamens successively finishing their movements. (E) All the stamens have finished their movements successively and finally positioned at their original positions near petals, exposing the style to floral visitors. (F) In some flowers all the stamens will move to the centre of the flower and enclose the style after their successive movement. Manipulation experiments were carried out by using a thread to prevent one stamen's uplifting (G) or falling back (H) to examine their effects on pollination.



stayed near the petal. The flower was then allowed to be freely visited by insects. We recorded the visit rates of insects and their contacts with the manipulated stamen. When this stamen wilted, nearly 200 min later, the anther was taken to the laboratory to count pollen grains remaining in the anther ( $M_1$  and  $M_2$  for pentamerous and tetramerous flowers, respectively) under a dissecting microscope according to the methods above. Pollen removal was calculated as  $G_1 - M_1$  and  $G_2 - M_2$  for pentamerous and tetramerous flowers, respectively.

To determine the impact of stamens returning to their original position prior to uplift of subsequent stamens, we manipulated the first-moved stamen by binding it with a thread to stop it falling back (Fig. 1H) on the last inflorescence. The 2nd stamen was then allowed to uplift freely to the flower's centre and visited naturally by insects. The visit rates of insects and their contacts with the anthers of the 2nd stamen and former stamen were recorded. When the 2nd stamen began to leave the flower's centre we took it off to count pollen grains remaining in the anther ( $Q_1$  and  $Q_2$  for pentamerous and tetramerous flowers, respectively) under a dissecting microscope. Pollen removal was calculated as  $G_1 - Q_1$  and  $G_2 - Q_2$  in pentamerous and tetramerous flowers, respectively.

Simultaneous stamen movement at the end of anthesis

To examine the effect of simultaneous movement of stamens to the centre of the flower at the end of anthesis, we chose 30 flowers with this movement vs. 15 flowers without it in tetramerous flowers (pentamerous flowers were too few to do this experiment) in 2011. For the 15 flowers without simultaneous movement, we separated the sexual organs by forceps and counted the pollen grains remaining in anthers, pollen load on stigma surfaces, and numbers of fertilized and unfertilized ovules (the fertilized ovules were much larger and rounded than the unfertilized ovules, which can be easily distinguished under a dissecting microscope) in the laboratory. For the 30 flowers showing simultaneous movement of stamens, 15 were left to finish their movements and the other 15 were immediately removed, and the latter were also examined in the laboratory for pollen and ovule traits. After the last 15 flowers finished their simultaneous stamen movement and stamens surrounded the style for no less than 60 min, we collected these flowers and also examined pollen and ovule traits. The possible occurrence of reproductive

assurance could be estimated by increases in both self-pollen deposition on stigmas and numbers of fertilized ovules after simultaneous stamen movement had finished.

Statistical analyses

For data showing a normal distribution such as pollen and ovule numbers, we used Student's *t*-test to compare possible differences between pentamerous and tetramerous flowers. Pollen removal and the durations of different steps of successive stamen movements were also compared between tetramerous and pentamerous flowers by Student's *t*-tests. We used one-way ANOVA for data with random distribution such as pollinator visitation rate among manipulation treatments. All statistical analyses were performed in SPSS v.13.0.

RESULTS

Processes of successive stamen movements

Successive stamen movements were found in both pentamerous and tetramerous flowers in *Ruta graveolens*. For each type of flower, all the stamens were reflexed in the hooded petals at the beginning of anthesis (Fig. 1A). One antisepalous stamen then began to move out of the hooded petal about 1 h after the flower had fully opened, bringing the anther slowly to the flower's centre. It took approx. 15–20 min for the stamen to reach the flower's centre (complete stamen uplift) and after approx. 35–45 min the anther began to dehisce (Table 1, Fig. 1B). This stamen stayed at the flower's centre for more than 100 min and then began to fall back to the petal, which took approx. 20–25 min (Table 1). It took significantly longer for the first stamen of pentamerous flowers to start movement compared with tetramerous flowers ( $P < 0.01$ , Student's *t*-test,  $n_{tetra} = n_{penta} = 15$ ), while there were no significant differences for the movement processes between pentamerous and tetramerous flowers (Table 1).

Normally, the next antisepalous stamen starts moving only after the former one falls back to its original position near the petal (Fig. 1C). However, our observations found that movement of the stamen can be triggered by a large number of insect visits over a short time. Most of the triggered movements occurred soon after an insect visit (85 % in the first 10 min) and in 28 % of the studied flowers (60 of 214) two stamens simultaneously presented their anthers at the flower's centre. When all the antisepalous stamens finished

TABLE 1. Patterns of successive stamen movements followed by simultaneous stamen movement in *Ruta graveolens* flowers (min)

Floral type	T0*	T1	T2	T3	T4	T5	Ts0	Ts1
Pentamerous	79.0 (6.9)	16.7 (0.7)	44.7 (4.7)	114.5 (6.4)	25.7 (2.4)	95.0 (9.6)	4965.3 (245.5)	1305.1 (307.5)
Tetramerous	51.3 (12.0)	17.1 (0.9)	35.2 (2.9)	139.7 (7.9)	21.2 (2.3)	90.0 (10.9)	5689.2 (337.1)	1175.4 (733.8)

Data are mean (s.d.). T0, time for the first stamen to begin uplifting after the flower has fully opened. T1, time for a stamen to move from its original position to the flower's centre. T2, time for a stamen to begin anther dehiscence after arriving at the flower's centre. T3, duration of a stamen staying at the flower's centre (including T2). T4, time for a stamen to fall back to its original position. T5, time for the next stamen to start its movement after the former stamen has fallen back. Ts0, time between the start of simultaneous movement and the end of successive movements. Ts1, time for completion of simultaneous movement.

\* Significant difference at  $P < 0.01$  (Student's *t*-test). The times for all the other steps showed no significant differences ( $P > 0.05$ ) between the two types of flowers.

their movements successively (Fig. 1D), the antipetalous stamens began to elevate one-by-one only after  $152 \pm 108$  min (tetramerous flowers) or  $109.4 \pm 47.5$  min (pentamerous flower) ( $P > 0.05$ , Student's *t*-test,  $n_{\text{tetra}} = n_{\text{penta}} = 15$ ). The total time for all stamens to finish their successive movements was more than 2 d ( $3062.8 \pm 63.0$  and  $2951.9 \pm 32.1$  min for pentamerous and tetramerous flowers respectively;  $P > 0.05$ , Student's *t*-test,  $n_{\text{tetra}} = n_{\text{penta}} = 15$ ).

In both types of flowers, a large quantity of nectar was produced by the nectaries around the base of the ovary and the nectar soon emerged after it was removed (Fig. 2A). Pentamerous and tetramerous flowers produced similar nectar volume ( $205.83 \pm 37.06$  vs.  $186.27 \pm 26.32$   $\mu\text{L}$ , respectively;  $n_{\text{tetra}} = n_{\text{penta}} = 10$ ) for the whole period of successive stamen movements (2 or 3 d). Production of nectar did not differ significantly among the six stages (1–6).

During the period of stamen movements the style gradually increased in length, reaching its maximum after all stamens had moved back to petals (Fig. 2B). Therefore, *R. graveolens* showed a clear protandrous pattern.

#### Pollen and ovule production

In 2010, pollen per anther in tetramerous flowers was significantly higher than in pentamerous flowers ( $15\,293.33 \pm 4504.09$  vs.  $7393.94 \pm 1834.49$ , mean  $\pm$  s.d.;  $P < 0.0001$ ,

Student's *t*-test,  $n_{\text{tetra}} = n_{\text{penta}} = 15$ ). In 2011, although both types of flowers showed decreases in pollen production, tetramerous flowers still produced much more pollen in each anther ( $7019.90 \pm 1827.09$ ) than pentamerous flowers ( $5554 \pm 1166.59$ ;  $P < 0.001$ , Student's *t*-test,  $n_{\text{tetra}} = n_{\text{penta}} = 15$ ).

In contrast to the pattern of pollen production, pentamerous flowers produced far more ovules than tetramerous flowers ( $39.53 \pm 7.30$  vs.  $28.75 \pm 11.62$  and  $31.27 \pm 5.43$  vs.  $22.98 \pm 8.38$ , respectively, in 2010 and 2011,  $P < 0.001$ , Student's *t*-test,  $n_{\text{tetra}} = n_{\text{penta}} = 15$ ).

#### Pollination system

A total of 90 h of observation was carried out on 20 plants at the blooming peak in 2011. Honeybees (*Apis* sp.) made 1490 visits (52.35 %; total visits = 2846), halictid bees (*Lasioglossum* sp.) made 535 visits (18.80 %), hoverflies (Syrphidae) made 310 visits (10.89 %), wasps (*Vespa* spp.) made 275 visits (9.66 %) and flesh flies (Sarcophagidae) made 236 visits (8.29 %). They often spent a very long time on the flower (Table 2) and sought nectar secreted from the base of the ovary. They sometimes collected pollen from the dehiscing anther, which was located at the flower's centre (above the nectar). Because the nectar is produced by the disc at the base of the ovary (Fig. 1), the pollinators foraged mainly around the centre of the flower, thus touching the

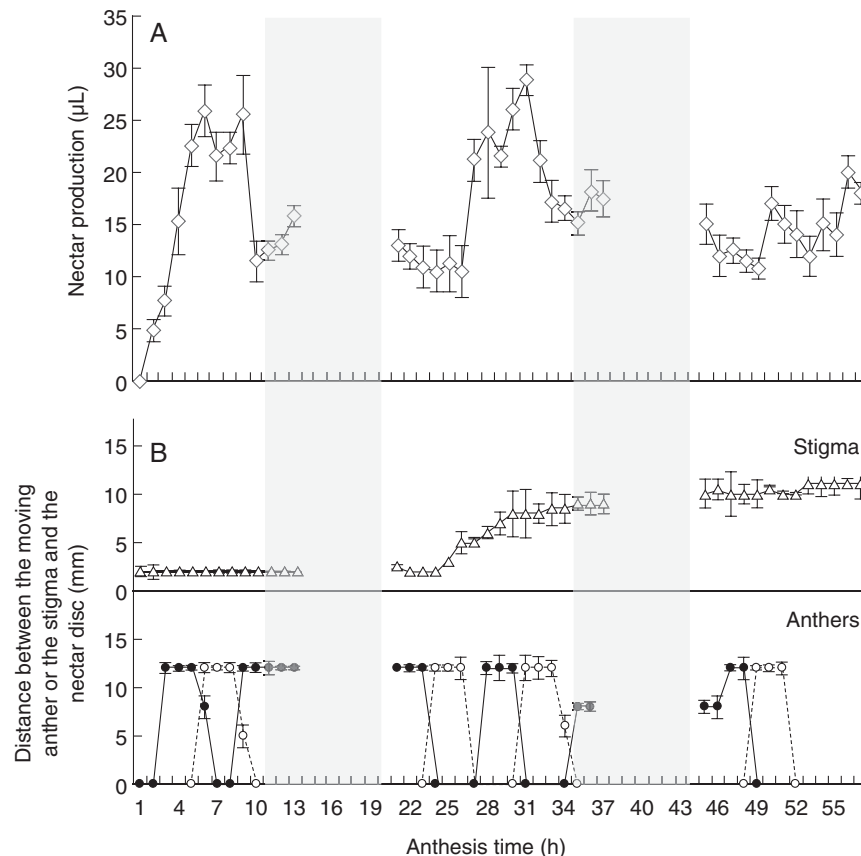


FIG. 2. Nectar production (A) during successive stamen movements and style elongation (B) in ten tetramerous flowers of *Ruta graveolens*. Data at the bottom of (B) indicate the anther height of the moving stamen measured from the nectar disc (ovary base) every hour: different symbols and lines are used for two consecutively moving stamens for clarity. Nectar is produced mainly during the day and the style gradually elongates, reaching its maximum after all stamens have fallen back. Shaded areas highlight night-time.

TABLE 2. Pollinators and their behaviours on flowers of *Ruta graveolens*

Pollinator	Visitation rate*	Mean visit duration (s) <sup>†</sup>	Reward	Percentage of visits contacting the dehiscing anther
Honeybees	16.56 ± 6.03	33.15 ± 2.55	Nectar	95 % (all by insects' dorsal part)
Halictid bees	5.94 ± 1.56	25.72 ± 6.08	Pollen	100 % (dorsal part)
Hoverflies	3.44 ± 2.14	11.85 ± 4.71	Pollen	90 % (dorsal and ventral parts)
Wasps	3.06 ± 0.75	7.53 ± 1.33	Nectar, pollen	55 % (dorsal part only)
Fleshflies	2.62 ± 1.53	26.18 ± 3.63	Nectar, pollen	85 % (dorsal part only)

\* Number of visits per hour (mean ± s.d.).

<sup>†</sup> Mean time for each visit on an individual flower (mean ± s.d.).

dehiscing anther located at the flower's centre more frequently (95 % of honeybee visits touched the anther, Table 2) than the anthers near the petals (43 % of honeybee visits touched the anther). Pollinators such as halictid bees, flies and hoverflies also mainly gathered pollen from the anther at the centre of the flower because the anther dehisces only after reaching the centre (Fig. 1). *R. graveolens* thus showed a generalized pollination system with very high visiting frequencies of pollinators (Table 2).

#### Effects of successive stamen movements on pollen removal

Under natural conditions, stamens uplifted freely from the petal to the centre of the flower, and anthers dispersed pollen very efficiently. In pentamerous flowers,  $6310.61 \pm 158.02$  and  $5258.76 \pm 43.15$  pollen grains (both more than 80 % of the total) were dispersed in 2010 and 2011, respectively. In tetramerous flowers,  $14\,740.56 \pm 92.30$  and  $6866.05 \pm 16.23$  pollen grains (both over 90 % of the total) were dispersed in 2010 and 2011, respectively (Fig. 3). Tetramerous flowers dispersed far more pollen than pentamerous flowers ( $P < 0.005$ , Student's *t*-test,  $n_{\text{tetra}} = n_{\text{penta}} = 15$ ).

When the stamen was artificially retained immobilized and stayed near the petal, visiting frequency and visit duration of floral visitors were not affected (one-way ANOVA,  $P = 0.15$ ). However, the anther was touched much less by floral visitors (one-way ANOVA,  $P = 0.022$ ) because most of the visiting insects were seeking nectar only at the flower's centre. For all experiments carried out in 2010, pollen removal from this immobilized stamen ( $4112.27 \pm 219.57$  and  $13\,098.89 \pm 307.92$  in pentamerous and tetramerous flowers, respectively) was dramatically lower than that of the stamen that naturally moved to the flower's centre ( $P < 0.001$ , Student's *t*-test,  $n_{\text{tetra}} = n_{\text{penta}} = 15$ ). The percentage of pollen removal also showed similar differences (Fig. 3). In 2011, pollen removal from the immobilized stamen also decreased significantly in both pentamerous ( $3398.68 \pm 305.93$ ) and tetramerous flowers ( $4561.20 \pm 455.01$ ) ( $P < 0.001$ , Student's *t*-test,  $n_{\text{tetra}} = n_{\text{penta}} = 15$ ), with percentages of pollen removal showing a similar pattern (Fig. 3).

When a stamen was manipulated staying at the flower's centre after its free uplift and dehiscence, visitation frequency and duration of floral visitors were not affected (one-way ANOVA,  $P = 0.33$ ). The next stamen was allowed to move freely to the flower's centre and its anther became very close to the former dehiscing anther, sometimes even contacting

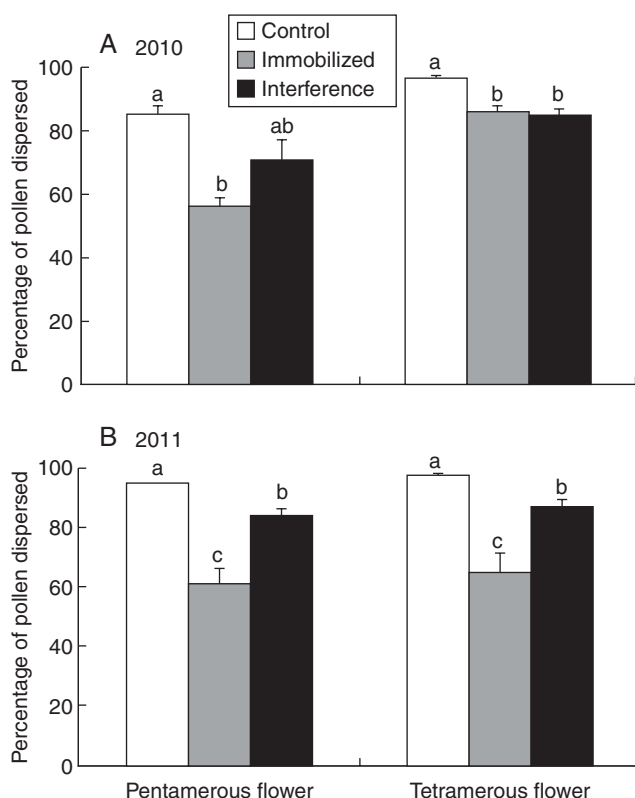


FIG. 3. Effects of successive stamen movements on pollen removal in *Ruta graveolens* in two different years. Different lower-case letters indicate a significant difference ( $P < 0.005$ ).

each other (Fig. 1H). As a consequence, floral visitors often touched these anthers simultaneously. Under these circumstances (interference by the former dehiscing anther), pentamerous flowers dispersed fewer pollen grains ( $5210.61 \pm 489.22$ ) although the difference was not significant ( $P = 0.098$ , Student's *t*-test,  $n_{\text{penta}} = 15$ ), while tetramerous flowers showed a dramatic decrease in pollen removal ( $P < 0.001$ , Student's *t*-test,  $n_{\text{tetra}} = 15$ ) in 2010 (Fig. 3). In 2011, both pentamerous and tetramerous flowers showed a significant decrease in pollen removal ( $4685.67 \pm 118.64$  vs.  $6131.28 \pm 176.27$ ,  $P = 0.001$  and  $0.002$  for pentamerous and tetramerous flowers, respectively, Student's *t*-test,  $n_{\text{tetra}} = n_{\text{penta}} = 15$ ; Fig. 3).

### Simultaneous stamen movement at the end of anthesis

Simultaneous movement of all stamens occurred normally at least 3 d later when their successive movements had finished. Our results showed that this simultaneous stamen movement occurred only in flowers with very low pollen loads on stigmas (Fig. 4A) and most ovules were not full developed (Fig. 4B). When the stamens moved simultaneously to the flower's centre they surrounded the style and their anthers touched the stigma (Fig. 4). During this process, the remaining pollen in anthers dropped to the stigma surface (Fig. 4A) and as a result more ovules were further fertilized (Fig. 4B), although no significant difference was detected ( $P > 0.05$ ).

## DISCUSSION

Stamen movements in *Ruta graveolens* flowers are the most complex currently known. They involve successive stamens moving to the centre of the flower and back to their original position as well as the simultaneous uplift of all stamens at the end of anthesis. These movements provide a mechanism to maximize successful pollination and reproductive assurance in this generalist-pollinated species.

### Successive stamen movements: one-by-one uplifts

In *R. graveolens*, one-by-one uplift and sequential dehiscence of anthers provided pollen in doses to the pollinators

only at the flower's centre. Those could be regarded as special forms of 'pollen packaging' and 'pollen dispensing' mechanisms (Lloyd and Yates, 1982; Harder and Wilson, 1994; Castellanos *et al.*, 2006), which should be favoured when pollinators are abundant but inefficient at delivering the pollen they remove, such as honeybees and halictid bees (Lloyd and Yates, 1982; Thomson *et al.*, 2000). Pollen loss during the pollination processes is very common in angiosperms and normally less than 1 % of the pollen removed from the anthers can reach conspecific stigmas (Harder and Wilson, 1994; Thomson *et al.*, 2000). Given this significant wastage, many plants present their pollen in small doses rather than all at once to maximize the amount of pollen donated to stigmas, especially for plants receiving enough pollinator visits as predicted by the 'pollen presentation theory' (Lloyd and Yates, 1982; Thomson *et al.*, 2000; Castellanos *et al.*, 2006). Therefore, the successive stamen movements in *R. graveolens* can be seen as a mechanism to maximize pollen removal (Harder and Thomson, 1989; Thomson *et al.*, 2000). When *R. graveolens* flowers were visited by a large number of insects over a short time, the movement of stamens could be triggered and sometimes two stamens presented their anthers simultaneously at the flower's centre. This pattern further revealed the adaptation of successive stamen movements regarding pollen presentation.

When the stamen was manipulated to be immobilized (staying near the petal) its anther was less likely to be contacted by the insects and pollen removal decreased dramatically (Fig. 3, Table 3). Sometimes the anther of this artificially immobilized stamen was touched by the abdomen of pollinators; however, these parts seldom touch the stigma located at the flower's centre and thus the pollen is less likely to be transferred to a conspecific stigma. Consequently, the movement of stamens to the flower's centre for anther dehiscence probably reflects an adaptation to increase both the probability and the precision in the contact of pollen grains with pollinators, increasing successful pollen removal and transfer.

### Successive stamen movements: one-by-one fall backs

When the former stamen was manipulated to stop falling back and to stay at the flower's centre, pollen removal of the next stamen decreased significantly (Fig. 3). Two scenarios could explain such a pattern. First, the former dehiscent anther would decrease the possibility and frequency of contact of the dehiscing anther with pollinators. Secondly, many pollen-collecting pollinators were 'hungry' and hurried to search for fresh pollen for their own energy needs (Harder and Thomson, 1989). The empty anther(s) at the flower's centre frequently discouraged these pollinators from leaving the flower quickly. Consequently, pollen removal of the nearby dehiscing anther would decrease due to insufficient contacts with pollinators.

These results suggest that 'anther-anther interference' is also possible in a single flower possessing numerous stamens, in addition to the widely recognized 'anther-stigma interference', i.e. sexual interference (Lloyd and Yates, 1982; Fetscher, 2001; Barrett, 2002; Rodriguez-Riano and Dafni, 2007). In other species with successive stamen movements, such as Parnassiaceae and Loasaceae, the anther-dehiscent stamens

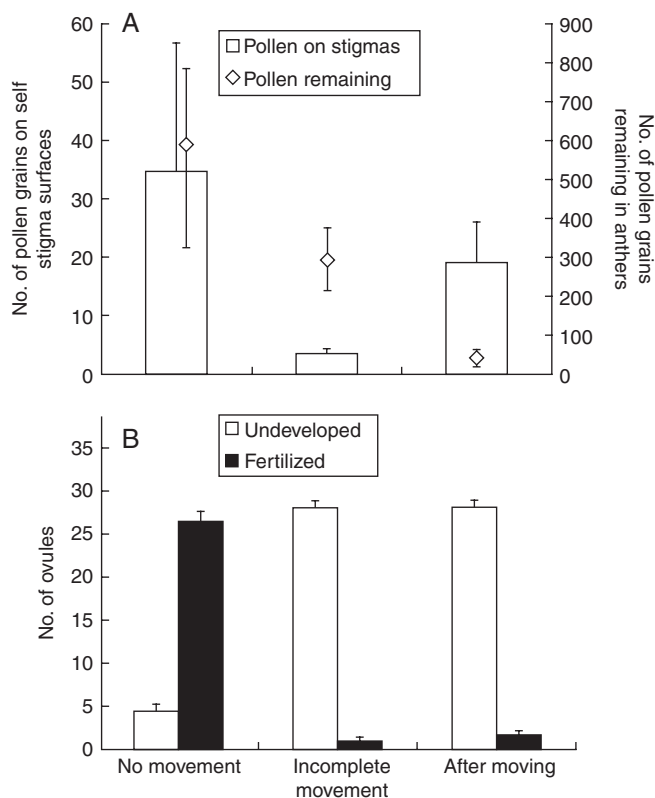


FIG. 4. Self-pollination effects of simultaneous stamen movement at the end of anthesis in *Ruta graveolens* flowers. (A) Pollen remaining in anthers and pollen deposition on the stigma surfaces; (B) ovule fertilization.



TABLE 3. Effects on pollen removal of successive stamen movements in *Ruta graveolens* flowers

Floral type	Manipulations on the stamen movement	No. of pollen removed (mean ± s.d.)*	
		2010	2011
Pentamerous	Control (naturally moved)	6310.61 ± 158.02a	5258.76 ± 43.15a
	Immobilized	4112.27 ± 219.57b	3398.68 ± 305.93c
	Interference by the former stamen	5210.61 ± 489.22ab	4685.67 ± 118.64b
Tetramerous	Control (naturally moved)	14 740.56 ± 92.30a	6866.05 ± 16.23a
	Immobilized	13 098.89 ± 307.92b	4561.20 ± 455.01c
	Interference by the former stamen	12 955.33 ± 300.01b	6131.28 ± 176.27b

\* Different letters indicate significant differences at  $P < 0.0001$  (Student's  $t$ -test).

will also leave the flower's centre or wilt before the next one moves into the flower's centre or begins anther dehiscence (Brown and Kaul, 1981; Wittmann and Schlindwein, 1995; Ren, 2010). These can be best explained as mechanisms to reduce interference between dehisced and dehiscing anthers, rather than avoiding obstruction of movement of the next stamens (Weigend *et al.*, 2010).

Therefore, we speculated that avoiding anther–anther interference is probably one of the selective forces acting on floral traits. For example, two anther levels in the same flower are found in many species, such as didynamous stamens in Lamiales (Foerste, 1888; Ren and Tang, 2010), tetradynamous stamens in Cruciferae (Kudo, 2003; Conner *et al.*, 2009) and tristly (populations contain three floral morphs that differ reciprocally in positions of stigma and anthers, in which each floral morph has two anther levels) (Harder and Barrett, 1993). They all have been proven to be effective in minimizing interference between two sets of anther(s) through temporally and spatially separated presentations of pollen (Harder and Barrett, 1993; Kudo, 2003; Ren and Tang, 2010) and/or interacting with different pollinators (Harder and Barrett, 1993; Ren and Tang, 2010). It seems reasonable to recognize anther–anther interference as a selective force in floral evolution, which is possible in flowers bearing a crowd of stamens.

Simultaneous stamen movement at the end of anthesis

The simultaneous movement of all stamens to the flower's centre after their successive movements occurs only at the very end of anthesis (Table 1) and was found only in flowers with low pollen loads on stigma and few ovules fertilized (Fig. 4). When stamens moved to the flower's centre the remaining pollen grains in anthers were deposited on stigmas (Fig. 4A). Because *R. graveolens* is highly self-compatible (Tang and Ren, 2011), this synchronous movement of stamens is probably selected for to achieve reproductive assurance through deposition of self-pollen to the stigma. As a result, we detected a trend of further fertilization of ovules 1 h after movement finished (Fig. 4B) although no significant differences were found, possibly due to the limited time interval to measure ovule fertilization.

Stamen movements for self-pollination at the end of anthesis are not rare in insect-pollinated plants, e.g. *Holcoglossum amesianum* (Orchidaceae) (Liu *et al.*, 2006), *Kalmia latifolia* (Ericaceae) (Nagy *et al.*, 1999) and *Opuntia spinosissima* (Opuntiaceae) (Negrón-Ortiz, 1998). Compared with selfing

mechanisms achieved by movements of other floral organs such as style curvature in Malvaceae (Ruan *et al.*, 2009) and a sensitive stigma with perianth movement in Bignoniaceae (Qu *et al.*, 2007), stamen movement is more accurate and efficient in delivering pollen to the self-stigma because it directly alters the spatial position of anther/pollen (Liu *et al.*, 2006; Ren, 2010). Moreover, stamen movement perhaps involves less energy demands as compared with the movements of style and perianth because the uplifting and bending of a filament is easily induced by an osmotic fluctuation in motor cells resulting simply from a decrease in  $Ca^{2+}$  concentration (Lechowski and Bialczyk, 1992) or different cell growth rates on opposite surfaces of the filament (Du *et al.*, 2012).

Conclusions

We have examined adaptations of successive stamen movements and simultaneous stamen movement in the generalist-pollinated *Ruta graveolens*. Pollen removal in this protandrous flower is promoted via one-by-one uplifting and falling back of stamens, which presents pollen in doses to the pollinators and avoids interference between the dehisced and dehiscing anthers. Reproductive assurance may be achieved by delayed self-pollination via simultaneous stamen movement at the end of anthesis. In particular, we provide the first experimental evidence for the existence of anther–anther conflicts within a single flower, which probably plays a role in shaping floral structures in flowers with numerous stamens.

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