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Spatial fragrance patterns within the flowers of *Ranunculus acris (Ranunculaceae)*

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Abstract: Floral scents emitted from different flower parts of *Ranunculus acris* were investigated by trapping headspace volatiles onto Porapak Q followed by solvent desorption and GC-MS analysis. Isoprenoids, strongly dominated by *trans*-β-ocimene, constituted the principal class of volatiles in all flower parts except pollen; sesquiterpenes were especially diverse. Odors collected separately from petals, stamens, and sepals + gynoecium comprised the same volatiles, but these were present in disparate proportions among the flower parts, thereby creating subtle contrasts within the flower. The main sources of volatiles were the petals and stamens, which made equal contributions to the whole-flower fragrance. Emissions from the petals differed quantitatively between the apical and basal petal regions, thereby paralleling optical nectar-guide patterns. Pollen odor was markedly unlike that of other flower parts, with only few volatiles, a high representation of 5-methylene-2(5H)-furanone (protoanemonin), and no detectable *trans*-β-ocimene. The distinctiveness of the pollen's volatile profile suggests that it may serve a signalling role to pollen-feeding insects.

Flowers attract insects from varying distances through an interplay of visual and chemical stimuli, which by virtue of their species-specific patterns also allow insects to discriminate between flowers of different species (Sprengel 1793; Kevan 1978; Faegri & van der Pijl 1979; Williams 1983; Menzel 1985, 1990; Menzel & Shmida 1993; Dobson 1994). Floral stimuli may differ not only between species, but also between different organs within a single flower. Such localized, intrafloral contrasts have been studied especially with respect to color patterns (e.g., Vogel 1950, Daumer 1958, Rosen & Barthlott 1991, Lunau 1992 a). These increase the attractiveness of flowers visited by food-seeking animals by visually advertising the sites of food rewards and guiding insects to them, often acting in combination with tactile or olfactory stimuli (Osche 1983; Waser 1983; Gori 1983; Casper & La Pine 1984; Weiss 1991; Lunau 1991, 1992 b). Visual patterns are generally recognized by humans, with the exception of those falling into the ultraviolet spectral range. Less evident, however, are the spatial variations in a flower's odor, which often require careful close-up examination and chemical

analysis to be detected (LEX 1954, VON AUFSESS 1960, DOBSON & al. 1990). The potentially infinite diversity of flower odors, made possible by variations in both the chemical identities and relative proportions of the volatiles making up a floral odor blend (WILLIAMS 1983, KAISER 1993 a, KNUDSEN & al. 1993), together with the highly developed olfactory sense of insects suggest that volatile stimuli can provide more fine-tuned identification labels for flowers than visual cues. For insects, whose lives and activities are strongly governed by chemical stimuli, odor patterns within a flower may be of key importance for the successful location and recognition of a food source.

While petals are often considered the principle emitters of flower fragrances (e.g., VOGEL 1962, KUGLER 1970, WILLIAMS 1983), in many species the floral odors have been attributed mainly to the androecium (e.g., PORSCH 1954, 1956; ENDRESS 1986; THIEN & al. 1975; COLEMAN & COLEMAN 1982; PELLMYR 1984, 1985; GROTH & al. 1987; D'ARCY & al. 1990). These reports, however, are based largely on subjective evaluation with the human nose and need chemical verification. Indeed, the differing abilities of humans to perceive individual volatiles may lead to erroneous conclusions regarding the actual intensity or chemical composition of an odor (e.g., NILSSON 1985). For example, through comparative chemical analyses of separate flower parts, it has been shown that the characteristic "spicy" component of the flower fragrance in *Rosa rugosa* THUNB. (*Rosaceae*), which arises exclusively from the androecium and is generally perceived as strong to humans, constitutes only a minor proportion of the flower's total odor, with most of the flower volatiles (namely, the rose-like components) being released from the petals (DOBSON & al. 1990). Similar situations where pollen makes quantitatively small but chemically distinctive contributions to the whole-flower odor are evident from chemical comparisons of whole flowers and pollen in other unrelated species (DOBSON, BERGSTRÖM, & GROTH, unpubl.) This underscores the importance of objective methodological evaluations, attained through chemical analyses, in studies investigating flower odors and their possible effects in pollination and plant reproduction.

Evidence from behavioral studies demonstrates the ability of honey bees and bumble bees to olfactorily distinguish different regions of a flower, including the pollen and visual food guides (VON FRISCH 1923, LEX 1954, BOLWIG 1954, MANNING 1956, VON AUFSESS 1960, LUNAU 1992 b), and studies of other flower-visiting insects suggest similar discriminative behavior (BRANTJES 1978, CHARPENTIER 1985). This points to the possible importance of these intrafloral scent patterns in orienting insects that seek food or even oviposition sites within flowers. Chemical studies addressing the comparative analysis of volatiles emitted from different flower parts, however, are few. They include analyses of corolla regions in *Cycnoches* and *Ophrys* (*Orchidaceae*) to locate osmophore regions (GREGG 1983, BORG-KARLSON 1990), and comparisons of different flower organs, such as in *Ophrys* (BORG-KARLSON 1990), staminodes versus the rest of the flower in *Cypripedium calceolus* L. (*Orchidaceae*) (NILSSON 1979), calyx versus corolla + stamens in *Majorana syriaca* L. (*Lamiaceae*) (DUDAI & al. 1988), corolla versus stamens in several *Pyrolaceae* species (KNUDSEN & TOLLSTEN 1991), whole flowers versus pollen in several species from different families (DOBSON & al. 1987, DOBSON 1991 a, unpubl.), and comparisons of calyx + gynoecium (green flower parts), corolla, and stamens in

Rosa rugosa (*Rosaceae*) (Dobson & al. 1990). The results indicate that odor differentiation between flower parts may be brought about primarily by either variation in the quantitative relationships of individual compounds, as in *Moneses uniflora* (L.) A. Gray (*Pyrolaceae*) (Knudsen & Tollsten 1991) and *Majorana syriaca* (Dudai & al. 1988), or emission of chemically different volatiles, as in *Pyrola* species (Knudsen & Tollsten 1991) and *R. rugosa* (Dobson & al. 1990).

In one detailed intrafloral study, conducted on the strongly scented flowers of *R. rugosa*, all flower organs were found to have sharply differentiated volatile profiles (Dobson & al. 1990). The flowers produce no nectar and are visited mainly by bumble bees, which use the distinct odor of the pollen, and to a lesser extent that of the petals, in selecting which individual flower to visit (Dobson 1991 a, unpubl.). To determine whether flower odors of other species display similar intrafloral patterns, which might in turn be correlated with the behavior of pollinating insects, the present study was conducted on the flowers of *Ranunculus acris* L. (*Ranunculaceae*). This species is the main host plant of the flower-specialist bee, *Chelostoma florisomne* (L.) (*Megachilidae*), whose behavior towards visual and olfactory floral stimuli is under investigation (H.Dobson, unpubl.); it also provides a contrast with *R. rugosa* in having a less prominent fragrance. The methodological procedures here followed a scheme similar to that used for *R. rugosa*, where odors of green flower organs (sepals + gynoecium), petals, and androecium were investigated by collecting volatiles from both isolated flower parts and from flowers with the specified parts removed; this was facilitated by both species having shallow dish-shaped flowers with easily removed petals and abundant, exposed pollen. *R. acris* is further characterized by offering small amounts of nectar (in nectaries partly concealed under flap-like scales at the base of each petal), and a comparison was thus also made of the basal (nectariferous) and distal portions of the petals.

Ranunculus acris, commonly known as the meadow buttercup, is a perennial herb that grows as a frequent and often abundant component of moist meadows and pastures. It is native to Eurasia, where it has a wide distribution in temperature and arctic regions and where it comprises several subspecies (Harper 1957, Clapham & al. 1962, Tutin & al. 1964). It has also spread to other areas, including North America (Hickman 1993). The flowers, many per plant and borne on branched stems, measure 15–25 mm in diameter and have bright yellow, glossy petals. Although the genus *Ranunculus* includes self-compatible species (Rendle & Murray 1988), *R. acris* is self-incompatible (Østerbye 1975) and thus depends on cross-pollination by insects for seed set. The flowers are visited by a variety of small insects, especially flies, micropterigid *Lepidoptera*, beetles, and small bees (Knuth 1898, Harper 1957, Olesen & Warncke 1989, Totland 1993, Dobson unpubl.), which feed on both the pollen and nectar. The flowers emit a fragrance that is sweet, but at times almost imperceptible, to the human nose.

Material and methods

Flower material was gathered in the field from a large population near the Ecological Research Station on the island of Öland, in southeastern Sweden, during June and July 1988–1990; additional pollen samples were collected in 1991 and 1992. Flowering stems from which the various flower samples were obtained were brought into the laboratory

and placed in water. Under these conditions, anthesis of individual flowers spanned three to four days and pollen was shed over three days, based on 47 marked flowers (1990). The amount of pollen was much lower on the first day of bloom ($\chi = 0.11 \pm 0.10$ mg/flower), corresponding to the female phase, than on the second and third days ($\chi = 0.39 \pm 0.27$ mg/flower and $\chi = 0.31 \pm 0.22$ mg/flower, respectively), with an overall average of 0.81 mg/flower; on the fourth day the petals and stamens dropped off. Day two was the main bloom stage used for collecting floral volatiles.

Samples. Ten types of samples were investigated. Each type comprised either similar or dissimilar flower parts and each (except green foliage) was represented by 2 or more replicate samples; samples varied in the number of flowers they contained. The sample types were as follows. 1) Whole flowers: flowering stems placed in water (1 sample, with an undetermined flower number), and flowers cut off at the top of the pedicel (2 samples: 80, 200 fls). 2) Flowers with petals removed (3 samples: 80, 25, 100 fls). 3) Flowers with stamens removed (3 samples: 50, 30, 31 fls). 4) Sepals and gynoecium, namely flowers with both petals and stamens removed (2 samples: 100, 130 fls). 5) Stamens alone, removed from flowers (2 samples: 90, 100 fls). 6) Petals alone, removed from flowers (2 samples: 85, 50 fls). 7) Basal portion of petals, encompassing the nectariferous tissue (2 samples: 30, 70 fls). 8) Distal portion of petals, external to the nectariferous tissue (2 samples: 30, 35 fls). 9) Pollen (12 samples: 75–187 mg, with $\chi = 109$ mg; flower number, $\chi = 135$, based on the average amount of pollen per flower as calculated above). For sample preparation, fresh pollen was hand-collected for 1–6 days (usually 2–3) and stored in the freezer (– 22°C, in vials or wrapped in aluminum foil) until the quantities were sufficient for volatile collection. 10) Green plant parts consisting of stems and leaves (1 sample). Flowers incurred little damage during the removal of petals or stamens, since the area of attachment is very small (i.e., < 1 mm for petals) and the parts came away with relative ease; however, to compare the distal and basal petal regions, petals were cut along the boundary of the nectariferous tissue into 2 segments.

Volatile collection. Volatiles were collected onto Porapak Q using headspace sorption-concentration methods (DOBSON 1991 b). Fresh flower material was placed in cylinder-shaped glass containers, ranging in volume from 40 ml to 2 l, which had a small opening at each end to allow air to flow through the container. Glass cartridges (50 × 5 mm) filled with the polymer Porapak Q (150 mg, 80–100 mesh) were attached to both ends. Pressurized air was passed through the system at 15–60 ml/min, depending on the cylinder size. Air entering the cylinder was filtered through the first cartridge, while the second cartridge trapped the flower volatiles from the air exiting the sample-filled cylinder. Collection of volatiles was carried out for 24 h (in the case of some pollen and stamens samples for 48 h, cut-up petals for 18 h). For pollen samples obtained in 1987, 1988, and 1989, pollen was packed into a 15 × 5 mm glass tube with silanized glass wool at both ends, and air flow was driven by pressure; in 1991 and 1992, the pollen was packed more loosely into 40 × 5 mm glass tubes containing small amounts of silanized glass wool both inside (to suspend the pollen) and at both ends, and air flow was driven by suction using a battery-run pump. Volatiles from empty glass cylinders served as control samples and were collected for each set-up.

Analysis. Immediately following odor collection, volatiles were eluted from the Porapak cartridges using 2 ml distilled pentane and the liquid eluates stored at – 18 °C. Because the volatile samples were weak, all were gently concentrated to 10 μl on a water-bath at 42 °C (VON KLIMETZEK & al. 1989) prior to analysis by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). GC conditions: Hewlett Packard 5880A, CP-Wax 52 fused silica column (CB, 25 m, 0.25 mm ID, coating 0.2 μm), temperature programmed at 50 °C for 5 min, then increased by 5 °C/min to 250 °C and isothermal for 20 min. GC-MS conditions: Finnigan 4021, fused silica capillary column coated with Superox-FA (25 m, 0.15 mm ID), temperature programmed at 50 °C for 5 min, then

increased by 5 °C/min to 220 °C and isothermal for 20 min. Also used for GC-MS were a Finnigan TSQ and a Finnigan ITD, both equipped with a CP-Wax 52 column.

Volatiles were identified by the correspondence of both their GC retention times and mass spectra with those of known compounds, obtained from personal libraries and from authentic samples. The composition of volatiles in each floral sample, expressed in percent representation of individual volatiles, was based on the relative peak areas of each compound. To determine the absolute quantities of volatiles per flower (1989 samples only), 1000 ng pentyl acetate was added as a standard to each sample prior to the concentration steps; the quantity of each compound was calculated by comparing its chromatogram-peak area with that of the standard.

Results

General patterns. The composition of volatiles from each sample type, expressed as the average percent value of each constituent volatile over all replicate samples of a given type, is shown in Table 1. Variation between replicate samples in the percent representation of individual compounds differed among compounds. With respect to the main volatiles, variation coefficients among replicate samples (corrected for small samples, SOKAL & ROHLF 1981) were lowest for *trans*-β-ocimene (range: 1.1% in isolated stamens to 13.2% in flowers-minus-stamens), moderate for α-farnesene (range: 14.8% in flowers-minus-stamens to 54.2% in whole flowers) and linalool oxide (range: 12.5% in isolated petals to 71.9% in isolated stamens), and exceptionally high for 2-phenylethanol (range: 29.4% in isolated stamens to 137.9% in flowers-minus-petals). For those volatiles showing high variation among replicates, conclusions regarding their distribution patterns among sample types can be only tentatively reached.

The whole flowers and each of the different floral parts, with the exception of pollen, had overall similar volatile profiles. Furthermore, the composition of volatiles in flowers from which either the petals or the stamens had been removed followed trends predicted from comparisons between whole flowers and isolated petals or stamens, respectively. Whole flowers, petals, sepals-plus-gynoecium, and stamens were strongly dominated by a single compound, *trans*-β-ocimene, which constituted 74–86% of their emitted volatiles. Among the other compounds, sesquiterpenes were relatively abundant and diverse. Approximately 30 compounds were detected in each of the flower parts, but only 6–8 were present in relative abundances greater than 1.0%. These included mainly linalool oxide, 2-phenylethanol, and the sesquiterpenes β-selinene, α-farnesene, and *trans*-α-bergamotene.

Vegetative green plant parts differed from flower samples in having three, nearly equally represented, dominant compounds rather than only one. These were *trans*-β-ocimene, α-farnesene, and *cis*-3-hexenyl acetate. In addition, there was a greater representation of several sesquiterpenes and "green leaf" volatiles (i.e., *cis*-3-hexenyl acetate and *cis*-3-hexenol). It should be noted that volatiles collected from flowers that were still attached to the stem, thereby including green foliage, were similar in composition to those collected from flowers that had been cut off at the pedicel (these two samples were placed together under whole-flowers in Table 1), suggesting that the contribution of volatiles by the green plant parts is small compared to that of the flowers.

Table1. Mean percent composition of volatiles in different flower parts and in green foliage of *Ranunculus acris* (*n* number of replicate samples). Compounds within each group listed in order of GC retention time

Compound	Flower (n = 3)	Petal (n = 2)	Stamen (n = 2)	Se+gyn[1] (n = 2)	Fl-pe[1] (n = 3)	Fl-st[1] (n = 3)	Green (n = 1)
Fatty acid derivatives							
Hexyl acetate							0.1
Cis-3-hexenyl isovalerate[2]				0.6	tr[3]		3.3
Cis-3-hexenyl acetate	< 0.1	0.4	< 0.1	0.7	< 0.1	0.2	25.0
2-hexenyl acetate							0.2
Cis-3-hexenol	0.3	0.4	0.4	1.5	0.2	0.6	2.8
Heptanol	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Octanol	tr						
Protoanemonin[4]	< 0.1	0.2	0.2	0.7	< 0.1	0.2	
Isoprenoids							
Monoterpenes							
Cis-β-ocimene	0.3	0.5	0.6	0.4	0.6	0.5	0.3
Trans-β-ocimene	84.0	79.8	82.5	75.4	85.5	73.6	29.0
Trans-β-ocimene epoxide	0.5	0.4	0.5	0.3	0.5	0.6	tr
Linalool	0.4	0.1	1.0	0.9	0.9	0.2	0.1
Linalool oxide (furanoid) 1	2.0	0.8	3.6	0.6	2.0	0.7	tr
Linalool oxide (furanoid) 2	0.1	< 0.1	0.2	< 0.1	0.1	< 0.1	
Sesquiterpenes							
Sesquiterpene 1	0.6	1.1	0.7	1.7	0.6	1.2	tr
Sesquiterpene 2	0.1	0.2	0.2	0.4	< 0.1	0.2	0.2
Cis-α-bergamotene	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	1.0
Trans-α-bergamotene	1.9	1.3	1.3	1.0	1.1	2.7	7.0
Caryophyllene	0.1	0.3	0.3	0.3	< 0.1	0.4	2.2
Sesquiterpene 3	< 0.1	0.3	< 0.1	< 0.1	< 0.1	< 0.1	
Sesquiterpene 4	0.2	0.2	0.2	0.3	< 0.1	0.2	1.1
Sesquiterpene 5	< 0.1						1.4
Sesquiterpene 6							0.3
Sesquiterpene 7	1.2	1.8	1.5	1.9	1.3	2.6	0.5
Sesquiterpene 8	0.1	0.2	0.2	< 0.1	< 0.1	0.3	
Germacrene D	0.1	tr	tr	< 0.1	tr	tr	2.8
β-selinene	3.0	3.6	1.4	2.6	1.4	5.8	2.7
α-farnesene	2.6	5.1	1.4	8.7	3.2	4.4	18.5
Benzenoids							
Methoxybenzene[5]	< 0.1	0.2	< 0.1	< 0.1	< 0.1	0.2	
Methyl 2-hydroxybenzoate[6]	0.3	0.5	0.3	0.6	0.4	0.7	0.1
2-phenylethanol	1.1	0.8	2.3	0.4	1.2	2.7	< 0.1

Table 1 (continued)

Compound	Flower (n = 3)	Petal (n = 2)	Stamen (n = 2)	Se+gyn[1] (n = 2)	Fl-pe[1] (n = 3)	Fl-st[1] (n = 3)	Green (n = 1)
Other							
Unidentified 1	< 0.1	0.2	0.2	0.3	0.1	0.3	0.2
Unidentified 2	< 0.1	0.2	0.1	0.4	0.2	0.3	tr
Unidentified 3							0.2
Unidentified 4	0.2	0.2	0.2	< 0.1	< 0.1	< 0.1	tr
Unidentified 5							0.2

[1] *Se+gyn* flower without petals and without stamens; *Fl-pe* flower without petals; *Fl-st* flower without stamens
[2] *cis*-3-hexenyl 3-methylbutanoate
[3] *tr* trace quantities detected only after search for characteristic mass spectral ion fragments
[4] 5-methylene-2(5H)-furanone
[5] Anisole
[6] Methyl salicylate

Petals. The volatile profile of the petals (alone or as the prominent part in the flower-minus-stamens samples) was overall similar to that of whole flowers. However, the petals had higher percentages of α-farnesene and, to a lesser extent, of the sesquiterpenes 1 and 7, and lower percentages of linalool and its oxides. Compared to other flower parts, petals were generally characterized by a higher representation of β-selinene and methoxybenzene (anisole).

The composition of volatiles was not uniform over the petal, as indicated by comparison of the apical and basal (nectariferous) regions (Table 2). Considering the emitted quantities of the 14 principal compounds (i.e., detected as > 1.0% in at least one of the petal regions), the basal area contributed over 50% of the total amount of each compound, except *trans*-α-bergamotene, from the two regions. The basal nectariferous region also showed a higher diversity of compounds detected in more than trace amounts and was characterized by relatively high emissions of α-farnesene and 2-phenylethanol. On a per tissue wet-weight basis, this translates into the basal region emitting individual compounds in quantities that were 1–15 times higher than in the apical regions, since the wet weight of the petal apex exceeded that of the base by a factor of 1.8 ± 1.1 (N = 25). Considering the possible effects of damage incurred when the petals were cut, it can be seen in Table 2 that the summed emissions from the two cut petal regions differed from intact petals mainly in having lesser quantities (< 50%) of the dominant volatile *trans*-β-ocimene and of β-selinene, but greater amounts (≥ 200%) of linalool and its oxides, *cis*-3-hexenol and *cis*-3-hexenyl acetate, and (< 400%) 2-phenylethanol.

Sepals plus gynoecium. The volatile profile of the green flower parts (sepals-plus-gynoecium samples) showed similarities with the profiles of both the green foliage and the petals (Table 1). The green flower parts shared with the green foliage a higher representation of green volatiles (*cis*-3-hexenyl acetate, *cis*-3-hexenyl

Table 2. Quantities (ng/flower) of each volatile,[1] expressed as means and ranges, collected from the basal and apical regions of the petal, and comparison of the summed quantities from both regions with emissions from intact whole petals. Number of replicate samples, n = 2 for each type (30–85 flowers per sample)

	Basal region[2]		Apical region		Basal + apical
Compound	χ	(Range)	χ	(Range)	vs. intact[3]
Fatty acid derivatives					
cis-3-hexenyl acetate	4.1	(2.2–6.0)	4.0	(tr–7.9)	+
cis-3-hexenol	5.3	(4.5–6.1)	3.2	(tr–6.3)	+
Isoprenoids					
Trans-β-ocimene	188.2	(170.2–206.1)	99.6	(68.8–130.4)	–
Trans-ocimene epoxide	1.8	(1.5–2.1)	tr	–	–
Linalool	2.6	(1.9–3.4)	tr	–	+
Linalool oxide (furanoid) 1	8.2	(8.0–8.3)	6.6	(6.2–7.0)	+
Trans-α-bergamotene	4.0	(tr–8.1)	6.4	(3.7–9.2)	=
Caryophyllene	1.3	(1.0–1.6)	tr	–	–
Sesquiterpene 4	1.6	(1.0–2.3)	tr	–	=
Sesquiterpene 7	8.3	(8.0–8.6)	6.8	(6.3–7.2)	=
β-selinene	12.8	(10.4–15.2)	9.0	(8.3–9.7)	–
α-farnesene	53.6	(42.3–64.8)	6.2	(4.5–7.8)	+
Benzenoids					
Methyl 2-hydroxybenzoate	4.2	(3.0–5.3)	tr	–	=
2-phenylethanol	25.0	(18.2–31.8)	tr	–	+

[1] Excludes compounds detected as trace amounts in both petal regions
[2] Contains the nectary
[3] Summed quantities in basal and apical petal regions greater than 125% (+), less than 75% (–), or 75–125% (=) of the quantities in intact petals

Table 3. Percent composition of volatiles collected from pollen samples, grouped over different years

	1987–1989 (n = 4)		1991–1992 (n = 8)	
Compound	χ[1]	(Range)	χ[1]	(Range)
Protoanemonin	18.0	(5.6–32.4)	74.7	(49.1–95.8)
α-farnesene	68.8	(51.4–83.3)	6.4	(tr–12.8)
2-phenylethanol	6.1	(tr–13.5)	18.9	(4.2–39.1)
Heptanol	6.3	(2.7–11.9)	tr	(tr–0.1)
Octanol	0.6	(tr–2.3)	–	
Linalool oxide (furanoid) 1	0.3	(tr–1.0)	tr	
Total %	100.1		100.0	

[1] All trace quantities set at 0.05% for calculations of mean values

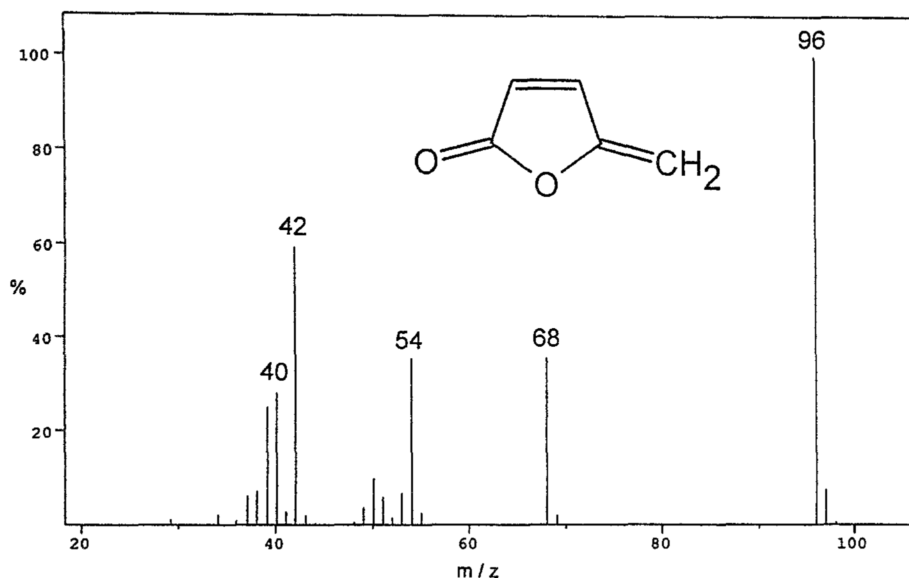

Fig.1. Mass spectrum of 5-methylene-2(5H)-furanone (protoanemonin)

isovalerate, and *cis*-3-hexenol) and α-fernesene, and a lower representation of the more typical floral compound 2-phenylethanol than was found in the whole flowers. However, they also resembled the petals in showing a lower percent of linalool oxide and a higher percent of α-farnesene, sesquiterpenes 1 and 7, and methyl 2-hydroxybenzoate (methyl salicylate). Noteworthy in the sepals plus gynoecium is the comparatively high representation of protoanemonin, a compound otherwise detected principally in pollen (Table 3).

Androecium. With respect to volatiles from the androecium, the stamens (alone or as the prominent parts in flower-minus-petals samples) were distinguished from both petals and sepals by having a higher representation of linalool oxide and a lower representation of α-farnesene and β-selinene (Table 1). They resembled green flower parts in their relatively high percent of linalool.

Pollen. The volatile profile of pollen contrasted sharply with those of all flower parts, including the stamens (Table 3). It displayed very few volatiles (5–6) and no *trans*-β-ocimene. Furthermore, one of its two principal constituents, protoanemonin, was detected almost exclusively in pollen. This major component in pollen volatiles (6–32% in the 1987–1989 material, 49–96% in the 1991-1992 material) was identified as 5-methylene-2(5H)-furanone, otherwise known as protoanemonin. We first identified it tentatively through its mass spectrum in its intact form (Fig. 1) and then following chemical ionization and hydrogenation; definitive identification was made after the mass spectra and gas chromatographic retention values had been compared with those of an authentic sample. The percent representation of the two most abundant pollen compounds, α-farnesene and protoanemonin, varied quite widely among samples. This variation formed a pattern that related to the sampling years, dividing them into two cohesive groups (Table 3). In the 1987–1989 samples, α-farnesene was dominant and comprised over 50% of the total volatiles; in the 1991–1992 samples, this dominant position was occupied

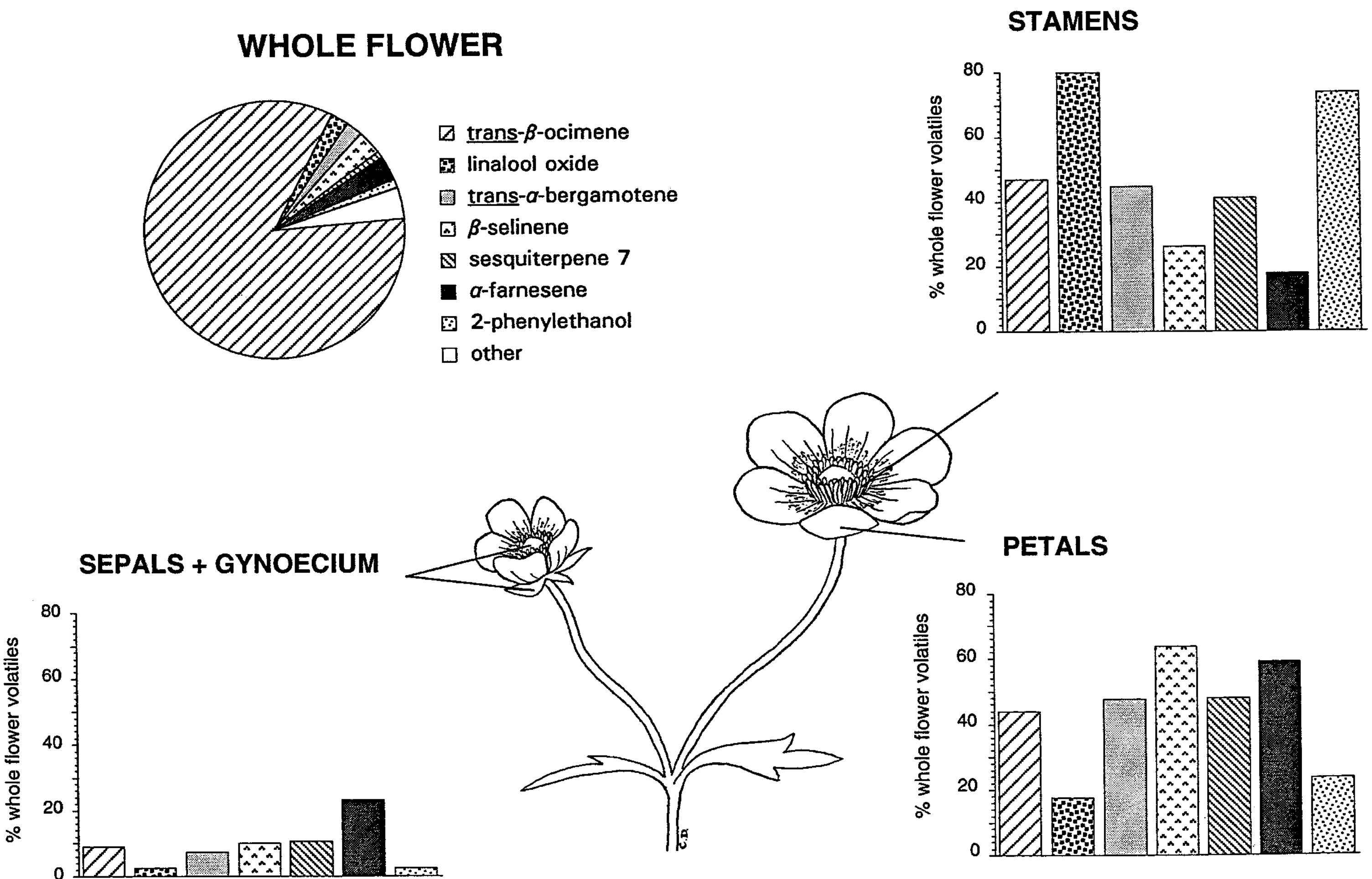

Fig. 2. Percentage contribution by different flower parts to the individual compounds in the whole-flower fragrance; included are only major volatiles, present as ≥ 1% of the fragrance. Based on quantitative determinations of volatile emissions per flower for each sample type

by protoanemonin. No causative effect for this pattern is apparent in relation to volatile-collection conditions, elution solvents (ether versus pentane), or pollen freshness (number of days pollen was stored prior to volatile collection). However, whereas pollen samples in 1987–1989 were stored at 0–5 °C in glass vials, those in 1991–1992 were stored by wrapping the pollen tightly in aluminum foil; greater exposure to air and water vapour within the vials may possibly have resulted in increased polymerization of the protoanemonin to its crystal (nonvolatile) dimer form, anemonin. In addition to protoanemonin and α-farnesene, the pollen odor contained 2-phenylethanol and small to trace amounts of heptanol, octanol, and linalool oxide.

Quantitative patterns. Quantitative determinations of volatile emissions, established by comparing GC peak areas of the floral compounds to that of the internal standard, showed that 44.3% of the volatiles in whole flowers were released from the petals, 45.8% from the androecium (i.e., stamens), and only 9.9% from the sepals plus gynoecium (Fig. 2). For each individual volatile that was detected at a > 1% level in the whole flowers, Fig. 2 shows the relative contribution made by the separate flower parts. The petals and androecium contributed equally to the whole flower with regards to the dominant compound *trans*-β-ocimene and several sesquiterpenes. However, linalool oxide and 2-phenylethanol came mainly from the androecium, and over half of the β-selinene and α-farnesene was released from the petals. Green flower parts made only small contributions in the case of each volatile, with α-farnesene being the most prominent. Noteworthy emission patterns in terms of the minor constituents in whole flowers (not shown in Fig. 2), are linalool with 86% from the androecium, and methyl 2-hydroxybenzoate with 81% from the petals. For many compounds, the summed quantities emitted from the three flower parts were similar to the amounts emitted from whole flowers; however, for *cis*-3-hexenol, *cis*-3-hexenyl acetate, most sesquiterpenes, and 2-phenylethanol, the summed quantities were higher. Based on the number of flowers per sample, whole flowers emitted an average of 1.89 μg/flower over the 24 h of odor sampling, or 25.7 ng/g flower (flower weight: $\chi = 73.6 \pm 10.7$ mg, N = 40).

Discussion

Flower volatiles. Results from the present study revealed only small variations in odor composition between the different flower parts of *Ranunculus acris*, with the noteworthy exception of pollen. In contrast to *Rosa rugosa*, where the floral organs differed in terms of both the identities of volatiles present and the percent representations of individual volatiles (Dobson & al. 1990), in *R. acris* the petals, stamen, and sepals plus gynoecium (green flower parts) all emitted the same volatiles, which were dominated strongly by the single monoterpene *trans*-β-ocimene. However, subtle scent patterns were displayed among these flower parts, as well as between the distal and basal regions of the petals, in the differing percent representations of certain individual compounds. Although not prominent quantitatively, sesquiterpenes were the most diverse group of volatiles in all plant samples except pollen, and much of the variation between flower parts occurred

within this chemical group. Against this generally uniform floral background, pollen formed a strong contrast by emitting a distinctive, simple odor that lacked *trans*-β-ocimene and prominently included the lactone protoanemonin. The sharp difference in odor between pollen and other flower parts is similar to the situation in *R. rugosa* (DOBSON & al. 1990), and suggests that it may influence interactions between the flowers and pollen-seeking insect visitors, especially by fine-tuning the insect's close-up orientation to the flower.

The emission rate of flower volatiles in *R. acris*, with 1.9 μg/flower over 24 h, or 26 ng/g flower (26 ppb), fell well into the low range among species documented to date. Compared to other collections carried out over 20–24 h, its fragrance emissions approximated those in *Bartsia alpina* L. (*Scrophulariaceae*, 100 ng/inflorescence) (BERGSTRÖM & BERGSTRÖM 1989), but they were less than one half those in *Nicotiana rustica* L. (*Solanaceae*), *Fragaria* × *ananassa* DUTCH. (*Rosaceae*), and *Medicago sativa* L. (*Fabaceae*), each with close to 100 ng/g flower (LOUGHRIN & al. 1990 a, HAMILTON-KEMP & al. 1990, BUTTERY & al. 1982). Forming even more of a contrast, *R. acris* emission rates were 10 to 100 times weaker than in apple (*Malus* × *domestica* BORKH., *Rosaceae*) (LOUGHRIN & al. 1990 b) and several other species of *Nicotiana*, with up to 2400 ng/g flower (LOUGHRIN & al. 1990 a, b), *Hoya carnosa* R. BR. (*Asclepiadaceae*), with 50 μg/-flower (MATILE & ALTENBURGER 1988), and *Platanthera stricta* LINDLEY (*Orchidaceae*),with up to 50 μg/inflorescence per h (PATT & al. 1988). Comparative chemical data with other species thus confirm the general human perception that buttercup flowers of *R. acris* have a weak fragrance.

High emissions of *trans*-β-ocimene characterized the whole above-ground plant of *R. acris*, but the compound's dominance in the volatile profile was most pronounced in flowers, where it represented over 75% of the total volatiles from both intact flowers and separate flower parts. A much higher representation of *trans*-β-ocimene in flowers compared to foliage has also been found in alfalfa, *Medicago sativa* (BUTTERY & al. 1982); in *Vicia faba* L. (*Fabaceae*) it was identified in the flowers to the exclusion of the foliage (SUTTON & al. 1992). However, the reverse pattern may occur, e.g., in *Trifolium pratense* L. (*Fabaceae*) (BUTTERY & al. 1984). *Trans*-β-ocimene is relatively common in flower fragrances (KNUDSEN & al. 1993), but as a dominant constituent it has been reported in the flowers of only a few species of the *Amaryllidaceae* (SURBURG & al. 1993), *Apocynaceae* (KNUDSEN & TOLLSTEN 1993), *Fabaceae* (BUTTERY & al. 1982, SUTTON & al. 1992), *Orchidaceae* (KAISER 1993 a, b), *Papaveraceae* DAHL & al. 1990), *Rosaceae* (ROBERTSON & al. 1993), and *Ranunculaceae* (GROTH & al. 1987). The principal chemical features distinguishing the flowers from the foliage of *R. acris* included the lesser dominance of *trans*-β-ocimene in the foliage (where it occurred in amounts equal to those of *cis*-3-hexenyl acetate and α-farnesene), the differing relative amounts of each of the individual sesquiterpenes (except β-selinene), and the greater quantities in the flowers of 2-phenylethanol, methyl salicylate, and of several other minor constituents, all of which are generally typical of flowers (HAMILTON-KEMP & al. 1990, KNUDSEN & al. 1993). As documented in other species (BUTTERY & al. 1984, BINDER & al. 1990), the "green leaf volatiles", which occur widely in green plant parts and consist mainly of six-carbon aliphatic compounds (VISSER & al. 1979), occurred in greater representation in the foliage of *R.*

acris. Among the flower samples, they showed variation that depended on the sample preparation, especially the degree of damage incurred to the floral tissue.

Detaching flowers of *R. acris* from the stem had little effect on the composition of emitted volatiles, since the volatile profile of isolated flowers was similar to that of attached flowers. Correspondence between volatiles collected from flowers still attached to the plant versus flowers cut at the pedicel has been reported in some species (SUTTON & al. 1992), but not in others (MOOKHERJEE & al. 1990, KITE & al. 1991, LOUGHRIN & al. 1991, BUCHBAUER & al. 1993). The available evidence suggests that changes in the quantity or rhythmicity of floral volatile emissions, brought about by uprooting the plants or detaching the flowers, vary widely with both the flower species and the volatile compound (MATILE & ALTENBURGER 1988, MOOKHERJEE & al. 1990, KAISER 1991). Unfortunately, no comparisons were made here with flowers on rooted plants to establish any effects caused by severing the stems. Along similar lines, the removal of petals or stamens from the flowers of *R. acris* did not notably affect the relative representations of component volatiles, and the volatile profiles of isolated flower parts were close to those expected based on comparison of intact flowers and flowers with parts removed. However, the more drastic cutting of the petals resulted in a marked decrease of collected *trans*-β-ocimene, but an increase in other volatiles, especially 2-phenylethanol and the two green leaf volatiles, *cis*-3-hexenol and *cis*-3-hexenyl acetate. Changes in the latter two compounds were most notable; increases in both their relative and absolute quantities were only slight in isolated flower organs as compared to intact flowers, whereas in cut petals they were larger and close to double that of intact petals. Reports that the release of these compounds is enhanced in leaves following plant injury (ANDERSEN & al. 1988, DICKE & al. 1990, WHITMAN & ELLER 1990) suggest that the increases observed here in cut petals may also be a response to wounding. Responses of flowers to damage appear to vary among species. Excision of labella petals in orchids can cause the release of aliphatic aldehydes in some *Ophrys* species (A.-K. BORG-KARLSON, pers. comm.) but not such effects were reported in *Cycnoches* (GREGG 1983). The excising method used may be critical. When flowers of *Rosa rugosa* were dismembered in a similar manner as in *R. acris*, they likewise did not show any marked changes in their volatile profiles (DOBSON & al. 1990). This suggests that in species where flower parts can be detached easily with little tissue tearing, as in *R. rugosa* and *R. acris*, such removal is a reliable method for studying the volatiles emitted from different flower parts.

Evolutionary significance of intrafloral volatile patterns. Spatial scent patterns within a flower probably function in a similar way as visual patterns (LEX 1954, VON AUFSESS 1960). Thus, differences in the intensity and/or quality of emitted volatiles between floral organs or even between regions within an organ may serve as guides to insects, assisting them in locating food rewards or leading them to position themselves appropriately on the flower for effective pollination. Quantitative determinations of floral volatiles in *R. acris* showed that the main sources are the petals and androecium. Furthermore, these two organs contributed equally to the whole-flower fragrance, thus resembling the situation in some *Pyrolaceae* species (KNUDSEN & TOLLSTEN 1991) but differing from that in *R. rugosa*

(Dobson & al. 1990). Contributions to the total flower scent by the green flower parts (sepals plus gynoecium) were small in both *R. acris* and *R. rugosa* (Dobson & al. 1990); however, the participation of the gynoecium alone has not been assessed. In *Pyrola norvegica* G.Knaben and *P. rotundifolia* L., where a significant portion of the whole fragrance cannot be accounted for by the petals or androecium alone, Knudsen & Tollsten (1991) point to the gynoecium as a probable major source.

Although the petals and androecium of *R. acris* emitted similar quantities of volatiles, they showed subtle differences in composition. This differentiation consisted primarily of the greater predominance of linalool and its oxides in the stamens, which offer the pollen rewards, and of α-farnesene and β-selinene in petals, which bear the nectaries. Even more distinctive were the patterns within the androecium; here the odor of pollen, although comparatively weak, differed sharply in the composition of its volatiles from the odors of all other flower parts, including the staminal tissue. The overall weaker odor of the sepals plus gynoecium showed chemical features in common with both the petals and green foliage and thus formed a lesser contrast than that provided by reward-bearing organs. Noteworthily, both the nectar and pollen sources showed chemical characteristics that may serve as olfactory flags within the flower, advertising the sites and the presence of food rewards.

Within the nectar-offering organs, or petals, clear contrasts were evident between the nectary and non-nectary portions. The shiny yellow petals of *R. acris* have a visually distinct basal nectariferous area, which is comparatively dull-colored and also absorbs ultraviolet light, thus forming a dark center in the flower (Daumer 1958, Rosen & Barthlott 1991). This visual differentiation, a general characteristic of yellow-flowered *Ranunculus* species, is often accompanied by fragrance changes perceptible to humans (von Aufsess 1960). In *R. acris* (*R. acer* L.), *R. ficaria* L., and *R. caucasicus* M. B., the basal region is described as having a stronger and qualitatively different scent compared to the distal region, whereas in other species (e.g., *R. lanuginosus* L., *R. repens* L., and even *R. ficaria*) only the odor intensity changes (von Aufsess 1960). The chemical analysis reported in this study of the two petal regions in *R. acris* confirmed these observations. Petal volatiles emanated primarily from the basal region containing the nectaries, forming a gradient of increasing odor intensity towards the flower center; and along this same gradient, the odor profile changed in a qualitative manner due to the differing relative amounts of certain compounds. It was not ascertained whether the nectar itself also contained volatiles. Although these scent gradients are subtle, von Aufsess (1960) demonstrated that they are clearly perceptible to bees. Indeed, honey bees trained to the odors of either petal region in *R. acris* distinguished between the two odors when offered both under test conditions, directing significantly more flights to the trained petal region: 85% of 265 flights when trained to the distal region, 74% of 435 flights when trained to the basal region. Such discriminatory ability could undoubtedly assist bees in locating the nectaries and thereby increase their foraging efficiency.

In a similar fashion, odor contrasts formed by the chemically distinct pollen odor in *R. acris* could direct pollen-seeking insects. Pollen of another *Ranunculus* species, *R. lingua* L., has been described as being both stronger and qualitatively

different from the rest of the flower, based on subjective human evaluation (VON AUFSESS 1960). This suggests that pollen distinctness may be a common feature in the genus *Ranunculus*. The few volatiles detected in the pollen of *R. acris* included α-farnesene and protoanemonin, as well as lesser amounts of 2-phenylethanol and three minor compounds. The lactone protoanemonin was the most abundant volatile, and its high representation characterized pollen from other flower parts. This compound has been reported in the above-ground plant parts (especially foliage) of many *Ranunculaceae* species (e.g., BAER & al. 1946, HERZ & al. 1951, BONORA & al. 1987, SOUTHWELL & TUCKER 1993), and its widespread occurrence in different genera suggests that it is characteristic of the family. It was first described as the dimer anemonin in *Anemone pratensis* L. (*Ranunculaceae*) by HEYER in 1780 (in KARRER 1976), but is found in the intact plants of *Ranunculaceae* predominantly in its glycosidic form, ranunculin, from which it is released by an enzymatic process upon maceration of the plant tissue (HILL & VAN HEYNINGEN 1951). Many of the poisonous, skin-irritant, and medicinal properties of *Ranunculaceae* have been attributed to protoanemonin (see HARPER 1957, BONORA & al. 1988, SOUTHWELL & TUCKER 1993). This compound has also been described as a component of the aroma of roasted food (see HARDT & BALTES 1987). Some of the biological and chemical aspects of protoanemonin are covered by ALONSO & al. (1991) and BONORA & al. (1987).

Protoanemonin has been reported in several species of *Ranunculus* (ASAHINA & FUJITA1922, KIPPING 1935), and *R. acris* is among those having the highest amounts (BONORA & al. 1987). In a detailed study of *R. ficaria* L. subsp. *bulbifer* (MARSDEN-J.) LAWALRÉE using HPLC, BONORA & al. (1988) found protoanemonin to be present in all parts of the plant, but it was especially high in stems and flowers. Furthermore, when they compared the different flower organs, they found the gynoecium to be the main site of accumulation (with 75% of the whole flower total, or 8800 μg/g fresh weight), followed by the androecium (23% whole flower total, or 2784 μg/g); petals and sepals, in contrast, had only minor amounts (< 100 μg/g). This rich concentration of protoanemonin in the reproductive flower organs is in general agreement with our findings reported here for *R. acris*, where the volatile profile of the sepals plus gynoecium had a greater relative representation of protoanemonin (0.7%) than did the profiles of the other non-pollen floral parts (≤ 0.2%). Although the representation in the stamens was low, that in the pollen varied considerably but reached up to 96%. The protoanemonin content of pollen was not quantified on a per g basis, due to the overall low amounts of volatiles that are collected and the difficulty of retrieving volatiles from the oily pollenkitt using headspace sorption. Protoanemonin is thought to be synthesized mainly in the leaves, from where it is translocated to the other plant parts (SOUTHWELL & TUCKER 1993). Notably high amounts (16%) of protoanemonin in flowers were obtained by SURBURG & al. (1993) when they concentrated the floral volatiles from *Anemone nemorosa* L. using vacuum headspace methods; it is conceivable that these quantities may have resulted from the high vacuum-forced emission of protoanemonin from the flowers. The variation observed in the protoanemonin content of *R. acris* pollen is not unexpected, given that the compound is very unstable and readily polymerizes to its nonvolatile dimer, anemonin (HILL & VAN HEYNINGEN 1951). It is thus possible that the figures obtained here are underrepresentations of the actual

content of protoanemonin in *R. acris* volatiles. Furthermore, levels of detected protoanemonin are known to vary readily in response to many factors, including methodological approaches, changes in environmental parameters, and due to natural population-based variation (BONORA & al. 1987).

Pollen appears to be the principal food sought by insects visiting *R. acris* flowers, but the species is used as both a nectar and pollen plant by a wide diversity of insects, representing six orders and at least 45 families (KNUTH 1898, HARPER 1957, PROCTOR & YEO 1973). Among these, *Diptera* seem to be especially numerous, and small bees, beetles, and *Lepidoptera* locally abundant (KNUTH 1898). Nectar production in *Ranunculus,* however, is generally low, and observations of bee visitors has led FAEGRI & VAN DER PIJL (1979) to suggest that the flowers produce only enough nectar to provide the energy needed by pollen foragers during their visits to *Ranunculus*, forcing them to use other nectar plants for more general sustenance. However, some bees rely on the flowers as both nectar and pollen sources (WESTRICH 1989). In Britain the most characteristic visitors are pollen-feeding insects, particularly chrysomelid beetles and the micropterigid moth *Micropterix calthella* (L.) (HARPER 1957). Analysis of the gut contents of syrphid flies at a site in Denmark indicated that several species feed on *R. acris* pollen, and some use it as their principal pollen source (OLESEN & WARNCKE 1989). Of special interest is the solitary bee, *Chelostoma florisomne* (*Megachilidae*), which throughout Europe forages exclusively on *Ranunculus* species for pollen and generally for nectar as well (WESTRICH 1989). Experiments on the bee's response to floral odors confirm the findings here that the whole flowers and isolated pollen differ in their volatile profiles. While naive adult bees showed no clear ability to recognize *Ranunculus* among other plant species when offered a choice of flower odors, they displayed a significant preference for *Ranunculus* when offered pollen odors (H. DOBSON, unpubl.). These results also indicate that key compounds used in the bee's olfactory recognition of its host plant are located specifically in the pollen; behavioral bioassays of protoanemonin are currently underway to identify its role in this recognition process.

The possibility that protoanemonin, which is known for its poisonous properties and supposed deterrent effects on herbivores (e.g., livestock) and microorganisms (BONORA & al. 1988), may also be used as an attractant to other animals, such as pollen-feeding pollinators, is not unusual in the realm of insect-plant interactions. PELLMYR & THIEN (1986) have suggested that flower volatiles used by insect pollinators as identifiers of food sources may have their evolutionary origins in flower defense, where the chemicals served to protect reproductive organs from the destructive feeding of phytophagous insects. This pathway of chemical interlinking between insects and flowers is thought to have played a major role in angiosperm diversification and evolution (PELLMYR & THIEN 1986). Accordingly, in one possible scenario, protoanemonin may have evolved in *Ranunculaceae* principally as a protection of the whole plants against herbivores and, in species that developed a dependency upon insects for pollination, over time became excluded from the nectar-producing organs (i.e., petals), leaving the flowers with their highest concentrations in the reproductive organs, namely pollen and ovules, as in *Ranunculus ficaria* (BONORA & al. 1988) and *R. acris*. Detailed studies of the distribution of protoanemonin in the flowers of other *Ranunculaceae* would provide

a more complete and clear picture of the functions this compound may serve in the reproductive biology within the family.

Evidence that pollen-seeking insects orient specifically to pollen volatiles, provided especially by studies of bees (VON FRISCH 1923; VON AUFSESS 1960; DOBSON 1987, 1991 a), leads to the conclusion that bees could use pollen odors to olfactorily determine if pollen is available in the flower. Bees could thereby restrict their visits to rewarding flowers. Although pollen volatiles may comprise only a small portion of the whole-flower fragrance, changes in their intensity may be readily perceptible to insects, as has been shown to be the case for bumble bees visiting *R. rugosa* (DOBSON 1991 a, unpubl.). In this species, where the pollen has a distinctly different odor from the rest of the flower (DOBSON & al. 1990), visitation by bumble bees was altered by manipulating (through application of chemicals) the amount and composition of pollen volatiles on the flowers. This phenomenon may actually be quite common in plants and would explain observations of bees discriminating between flowers with differing amounts of pollen reward (ZIMMERMAN 1982, THOMSON & al. 1989).

The advantage to *R. acris* in having a distinct pollen odor, however, is unclear, since the flowers are protogynous (HARPER 1957) and must therefore be visited during the initial, female stage (before pollen is shed) in order to produce seeds. In its protogyny, prominent or distinctive androecial (pollen) odor, as well as flower morphology and generalist entomophily that includes primitive pollen-feeding micropterigid moths, *R. acris* shares features with the typical flower of primitive extant angiosperms in the *Magnoliidae* (ENDRESS 1986, 1990; BERNHARDT & THIEN 1987; GOTTSBERGER 1988; FRIIS & ENDRESS 1990; PELLMYR & al. 1990; LLOYD & WELLS 1992). Among these are *Zygogynum* species (*Winteraceae*), which have the conflict of being protogynous and of offering food reward (i.e., pollen) only during the male phase. This problem seems to be overcome at least in part by the initiation of flower odor release during the female phase, when it may be critical in luring pollen-covered insects from the male-phase flowers and thereby promote pollination (THIEN & al. 1985). In *Eupomatia bennettii* F. MUELL. (*Eupomatiaceae*), small differences in the proportions of individual volatiles were observed between the female and male flower phases, but their effect on pollinator behavior remains to be established (BERGSTRÖM & al. 1991). Within the *Ranunculaceae, Actaea* and *Cimicifuga* species have flower odors that are thought to originate from the androecial staminodes, but in this case the male and female flower stages overlap in time (PELLMYR 1984, 1985; PELLMYR & al. 1984; GROTH & al. 1987). In regard to *R. acris*, it should be noted that although the most distinctive volatile in pollen, protoanemonin, was detected as a minor constituent in other flower parts, its greatest representation among these was in the sepals plus gynoecium, and according to BONORA & al. (1988) in *R. ficaria* the gynoecium can be a major accumulation site. This could lend to the gynoecium a more prominent role in attracting pollinating insects, especially during the flower's female phase. It could also provide a temporally consistent chemical identification label that would encourage flower (i.e., *Ranunculus*) constancy in pollinators, although it might then preclude bees from using protoanemonin in the pollen odor as a measure of pollen availability. Olfactory cues emanating from pollen and localized petal regions have been shown to be used in the orientation of bees to flow-

ers (von Frisch 1923; Lex 1954; von Aufsess 1960; Dobson 1987, 1991 a; Lunau 1992 b), but no studies to our knowledge have experimentally examined insect responses to gynoecial cues, which may in fact be of major importance in many plants. It is thus plausible that the protoanemonin emitted from the gynoecium of *R. acris* attracts pollen-seeking insects by deception to flowers in the female stage when only nectar rewards are available.

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