

# Too Low to Kill: Concentration of the Secondary Metabolite Ranunculin in Buttercup Pollen does not Affect Bee Larval Survival

Claudio Sedyv · Rafal Piskorski · Andreas Müller ·  
Silvia Dorn

Received: 6 September 2011 / Revised: 19 April 2012 / Accepted: 3 June 2012 / Published online: 19 June 2012  
© Springer Science+Business Media, LLC 2012

**Abstract** Growing evidence suggests that the freely accessible pollen of some plants is chemically protected against pollen-feeding flower visitors. For example, a diet of pollen from buttercup plants (*Ranunculus*) recently was shown to have a deleterious effect on developing larvae of several bee species not specialized on *Ranunculus*. Numerous *Ranunculus* species contain ranunculin, the glucosyl hydrate form of the highly reactive and toxic lactone protoanemonin, that causes the toxicity of these plants. We tested whether the presence of ranunculin is responsible for the lethal effects of *R. acris* pollen on the larvae of two bee species that are not *Ranunculus* specialists. To investigate the effect on bee larval development, we added ranunculin to the pollen provisions of the *Campanula* specialist bee *Chelostoma rapunculi* and the Asteraceae specialist bee *Heriades truncorum*, and allowed the larvae to feed on these provisions. We quantified ranunculin in pollen of *R. acris* and in brood cell provisions collected by the *Ranunculus* specialist bee *Chelostoma florissomme*. We demonstrated that although ranunculin was lethal to both tested bee species in high concentrations, the concentration in the pollen of *R. acris* was at least fourfold lower than that tolerated by the larvae of *C. rapunculi* and *H. truncorum* in the feeding experiments. Ranunculin concentration in the brood cells of *C.*

*florissomme* was on average even twentyfold lower than that in *Ranunculus* pollen, suggesting that a mechanism different from ranunculin intoxication accounts for the larval mortality reported for bees not specialized on *Ranunculus* pollen.

**Keywords** *Ranunculus* · Ranunculin · Protoanemonin · Pollen · Plant defense · Osmiini · *Chelostoma* · *Heriades* · Megachilidae

## Introduction

Bees, including solitary native species, provide important ecosystem services as pollinators of flowering plants (Kremen et al., 2007). However, they exact considerable costs on plants, because they require enormous quantities of pollen to feed their broods (Müller et al., 2006). Flowers are expected to balance the need to attract bees for pollination with the need to restrict extensive pollen losses (Praz et al., 2008a; Sedyv et al., 2011). Various mechanisms have evolved that limit pollen loss by narrowing the spectrum of pollen-collecting flower visitors (Westerkamp, 1997; Westerkamp and Classen-Bockhoff, 2007) or by reducing the pollen quantity withdrawn by pollinators per flower visit. Examples of these mechanisms include specialized anthers, pollen-concealing flower structures, and portioned pollen release over extended time periods (Vogel, 1993; Harder and Barclay, 1994; Müller, 1996; Castellanos et al., 2006).

Growing evidence suggests that some plants that possess freely accessible pollen might also chemically protect the pollen. For example, the pollen of *Stryphnodendron polyphyllum* (Mimosoideae) is poisonous to the larvae of the honeybee (De Carvalho and Message, 2004). Similarly, the pollen of *Ranunculus* (Ranunculaceae) did not support larval development of three strict pollen-specialist bees specialized on

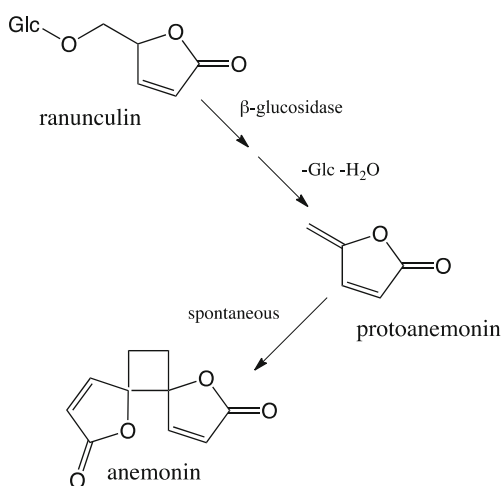
C. Sedyv · R. Piskorski · A. Müller (✉) · S. Dorn  
ETH Zurich, Institute of Agricultural Sciences,  
Applied Entomology,  
Schmelzbergstrasse 9/LFO,  
8092 Zurich, Switzerland  
e-mail: andreas.mueller@ipw.agrl.ethz.ch

### Present Address:

R. Piskorski  
Innovative Environmental Services (IES) Ltd.,  
Benkenstrasse 260,  
4108 Witterswil, Switzerland

*Campanula*, *Echium*, and Asteraceae, respectively, as well as one highly pollen-generalist bee (Praz et al., 2008a; Sedivy et al., 2011). The larval mortality pattern in these four species was characterized by rapid death upon onset of feeding, suggesting that *Ranunculus* pollen may contain secondary metabolites that are toxic to bee larvae. *Ranunculus* pollen also is known to be toxic to adult honeybees, which suffer high rates of mortality when feeding primarily on *Ranunculus* pollen, a phenomenon known as “Bettlacher May sickness” (Morgenthaler and Maurizio, 1941).

Fresh plants of the genus *Ranunculus* are known for their toxic effect on livestock (Kingsbury, 1964). This effect arises from high concentrations of the glucoside ranunculin, the precursor of the toxic protoanemonin, present in the plant tissue (Fig. 1) (Benn and Yelland, 1968). The content of ranunculin in *Ranunculus* species normally oscillates around 10 mg per g dry weight (d.w.) (Ruijgrok, 1966), but can reach nearly 200 mg/g d.w. in *R. cymbalaria* (Bai et al., 1996). Upon infliction of mechanical damage to plant tissue, the non-toxic ranunculin is hydrolyzed by endogenous  $\beta$ -glucosidase, an enzyme stored in the vacuole (Mauch and Staehelin, 1989), to yield the highly reactive anhydroaglycone protoanemonin (2,3-dihydro-5-methylidene-furan-2-one), a volatile lactone (Hill and Van Heyningen, 1951). When ingested, protoanemonin can cause gastric distress in livestock (Kingsbury, 1964). Applied to human skin, protoanemonin may produce erythema and blistering (Benn and Yelland, 1968). In addition, protoanemonin has antimicrobial properties (Campbell et al., 1979; Mares, 1987; Martin et al., 1990) and exhibits insecticidal effects on fly larvae of *Drosophila melanogaster* (Drosophilidae), adult beetles of *Tribolium castaneum* (Tenebrionidae), and ant workers of *Pheidole pallidula* (Formicidae) (Bhattacharya et al., 1993; Varitimidis et al., 2006).



**Fig. 1** The transformation of the glucoside ranunculin to the unstable and toxic lactone protoanemonin in plant tissue and spontaneous transformation to anemonin. Modified after Benn and Yelland (1968)

Some herbivorous insects are able to cope with high concentrations of ranunculin in their diet. Larvae of several leaf and stem mining species of agromyzid flies of the genus *Phytomyza* are specialized on *Ranunculus* and other ranunculin-containing genera of the Ranunculaceae, e.g., *Anemone*, *Clematis*, and *Helleborus* (Spencer, 1990). Larvae of *P. ranunculi* and *P. ranunculivora* often are found in leaves of *R. acris* (Pitkin et al., 2010), where they can be exposed to ranunculin concentrations of about 28 mg/g d.w. (Bai et al., 1996). The physiological basis of the ability of these herbivorous insects to tolerate ranunculin and/or protoanemonin remains unknown.

While stems, leaves, and the androecium of *Ranunculus* contain ranunculin in considerable amounts (Ruijgrok, 1966; Bonora et al., 1988; Bai et al., 1996), no attempt has been made to quantify ranunculin in *Ranunculus* pollen. The high relative amounts of protoanemonin released from pollen and anthers of some *Ranunculus* species (Bergström et al., 1995) as well as the high concentration of protoanemonin in the androecium of *R. ficaria*, which was found to be twice as high as the concentration measured in the whole plant (Bonora et al., 1988), may indicate the presence of substantial quantities of ranunculin in the pollen of *Ranunculus*. Furthermore, by thermal desorption of anthers of several Ranunculaceae, Jürgens and Dötterl (2004) detected high relative amounts of protoanemonin in three *Ranunculus* species, and protoanemonin from *Ranunculus* pollen seems to be used in host-plant recognition by the *Ranunculus* specialist bee *Chelostoma florisomne* (Dobson and Peng, 1997).

We hypothesized that protoanemonin released from the secondary metabolite ranunculin is responsible for the toxicity of *R. acris* pollen to the larvae of bee species that are not *Ranunculus* specialists. To investigate the effect of ranunculin on larval development, we selected two solitary bee species specialized on pollen of plants other than *Ranunculus*. We tested larval performance of *Chelostoma rapunculi*, a *Campanula* pollen specialist, and *Heriades truncorum*, an Asteraceae pollen specialist, on diets consisting of pollen from their natural host plants mixed with ranunculin in various concentrations. In addition, we quantified ranunculin in pollen and flower buds of *R. acris* as well as in brood cell provisions of *Chelostoma florisomne*, which is a pollen-specialist species that collects pollen exclusively from *Ranunculus* flowers (Sedivy et al., 2008).

## Material and Methods

**Bee Species** To assess the effect of ranunculin on larval development of solitary bees, we selected two species belonging to the same taxonomic group (Osmiini; Megachilidae) as the *Ranunculus* specialist *Chelostoma florisomne*. These species, *Chelostoma rapunculi* and *Heriades*

*truncorum*, are specialized on *Campanula* (Campanulaceae) and on Asteraceae, respectively (Westrich, 1989; Praz et al., 2008b; Sedivy et al., 2008). Neither can develop on a *Ranunculus* pollen diet (Praz et al., 2008a). All these species nest in pre-existing cavities such as insect-bored holes in dead wood and hollow stems, and they can easily be reared in hollow bamboo stalks. Once provisioning of the brood cells with pollen and nectar is complete, an egg is deposited onto the pollen diet, and the female bee closes the cell with a thin wall of clay or resin. Successful larval development ends with spinning a cocoon, in which the bee enters metamorphosis to the adult stage. For the experiments, we used eggs and brood cell provisions from bees in nesting stands on the campus of ETH Zurich.

**Bee Larval Performance** The larvae of *C. rapunculi* and *H. truncorum* were experimentally reared on a *Campanula* and Asteraceae pollen diet, respectively, obtained from conspecific nests and mixed with ranunculin in five increasing concentrations: 0 (control), 10, 20, 50, and 100 mg per g pollen dry weight (d.w.), henceforth referred to as the control treatment, 10, 20, 50, and 100 mg/g treatment. These concentrations are in line with natural ranunculin concentrations reported for *Ranunculus* plants (Ruijgrok, 1966; Bonora et al., 1988; Bai et al., 1996). Pollen dry weight of the brood cell provisions averages approximately 27% in *C. rapunculi* and 33% in *H. truncorum* (A. Bühler and A. Müller, unpublished). Ranunculin (>99% pure) originating from extractions of *Ranunculus* plants was obtained from Michael H. Benn (University of Calgary, Canada). To prepare the experimental pollen diet, ranunculin was ground to fine powder and thoroughly mixed with the brood cell provisions in a mortar. The mixing process was conducted in a careful and gentle way to prevent destruction of the pollen grains and, thus, to prevent the release of  $\beta$ -glucosidase followed by hydrolysis of ranunculin.

Rearing of bee larvae was conducted in individual artificial brood cells (for details see Sedivy et al., 2011). Freshly completed bee nests were collected daily from the nesting stands. Each egg was detached with a thin spatula from the brood cell provision and transferred onto 60 mg of the experimental pollen diet previously placed into the artificial cell. Larvae hatched and started feeding between 1 and 3 d after transfer onto the experimental pollen diet. Each larva was allowed to feed individually in a single artificial cell to mimic natural conditions. For each species and treatment, 24–31 eggs were transferred. Development took place in a climate chamber (E7/2; Conviron, Winnipeg, Canada) in darkness at  $25 \pm 0.5$  °C for 16 h followed by a 4 h gradual decrease to  $10 \pm 0.5$  °C, followed by a 4 h gradual increase back to  $25 \pm 0.5$  °C, at a constant  $70 \pm 0.5$  % relative humidity. Egg hatching, initiation of larval feeding, cocoon completion, and incidences of death were recorded every second

day. Survival time was considered the time between onset of feeding and either death or completion of the cocoon. Unhatched eggs were removed from statistical analyses.

**Statistical Analysis** Kaplan–Meier survival statistics was used to compare larval survival among the different treatments following Lee and Wang (2003). The number of days between hatching and completion of the cocoon was considered as ‘censored data’; individuals that died before the completion of the cocoon represented the exact observations for which the event (death) occurred, while those that completed the cocoon were the censored observations. The latter were considered survivors and were withdrawn from survival calculations. To test for differences among survival distributions, the *log-rank test* was applied with Bonferroni correction using the option ‘pairwise for each stratum’ implemented in the software when comparing two groups. For each species, we tested for differences in survival according to pollen-diet treatment, and larval survival of the two species was compared for each ranunculin concentration. For statistical analyses, SPSS 19.0.0 for Macintosh OS X (SPSS Inc., Chicago, IL, USA) was used.

**Ranunculin Recovery** To test whether the ranunculin concentration in the experimental pollen provisions remained stable during feeding experiments, we added 10 mg/g of ranunculin to brood cell provisions of *Heriades truncorum* and quantified the ranunculin content by LC-MS analysis (see below) immediately after mixing, after 8 d and after 22 d ( $N=5$  for each time interval). For this experiment, we used the same methodological procedure including climate chamber conditions as for the bee larval performance experiments.

**Plant Material** Ranunculin was quantified in pollen and flower buds of *Ranunculus acris* as well as in the brood cell provisions of the *Ranunculus* specialist *Chelostoma florissomme*. At each of 8 different locations in Switzerland, which spanned a geographic range of approximately 130 km (comprising locations around Neuchâtel, Solothurn, Aarau, and Zurich), 250 freshly opened flowers of *R. acris* were collected in April 2011. Flowers were bundled and fixed in an upside-down position over a parchment paper cone large enough to collect released pollen. After 24 h, the pollen that accumulated at the bottom of the cone was sieved through a 90  $\mu$ m-pore sieve and stored at  $-80$ °C until extraction and analysis. Our pollen collection procedure closely matched the pollen collecting behavior of bees, which harvest pollen from dehisced anthers before they deposit it in the brood cells. At each of the 8 locations, a single flower bud (close to blooming) was collected from each of 5 different plants, was immediately frozen in liquid nitrogen, and stored at  $-80$ °C until extraction and analysis.

At a large nesting site of *C. florissomme* in the surroundings of Neuchâtel (Gletterens), where *R. acris* was the near exclusive pollen source for this species, we collected 11 freshly completed nests. From each nest, the provision of the outermost (i.e., the most recently completed) brood cell was removed and immediately stored at  $-80^{\circ}\text{C}$  until extraction and analysis.

**Extractions of Plant Material** For extraction of ranunculin we basically followed the method described by Bai et al. (1996). All samples, i.e., pollen, flower buds, and brood cell provisions, were freeze-dried and individually extracted with methanol ( $3 \times 15$  ml) by repeatedly and thoroughly grinding in a mortar. To ensure that the mechanical damage inflicted on the plant material did not lead to a significant loss of ranunculin due to the action of  $\beta$ -glucosidase, the grinding was conducted in methanol. To test whether the hard pollen exine was successfully disrupted in order to extract the complete contents of the pollen grains, the ground pollen was examined microscopically. Extracts were filtered through a cotton plug, evaporated to dryness, and stored at  $-60^{\circ}\text{C}$  until LC-MS analysis.

**LC-MS Analysis** For the LC-MS analysis, the total dried extract was quantitatively dissolved in the mobile phase, and if necessary, an aliquot of the solution was further diluted. High-performance liquid chromatography (HPLC) was performed on an Agilent 1200 HPLC system (Agilent Ltd., Santa Clara, CA, USA) equipped with a binary solvent pump. Separation was performed on a reversed-phase 4.6-mm  $\times$  250-mm, 5  $\mu\text{m}$ , Phenomenex ODS Aqua column (Phenomenex, Torrance, CA, USA). An isocratic mode with MeOH/H<sub>2</sub>O (0.05% ammonium acetate) (6:4) at a flow rate of 1 ml/min (total run time, 5 min) was employed. The sample injection volume was 5  $\mu\text{l}$ .

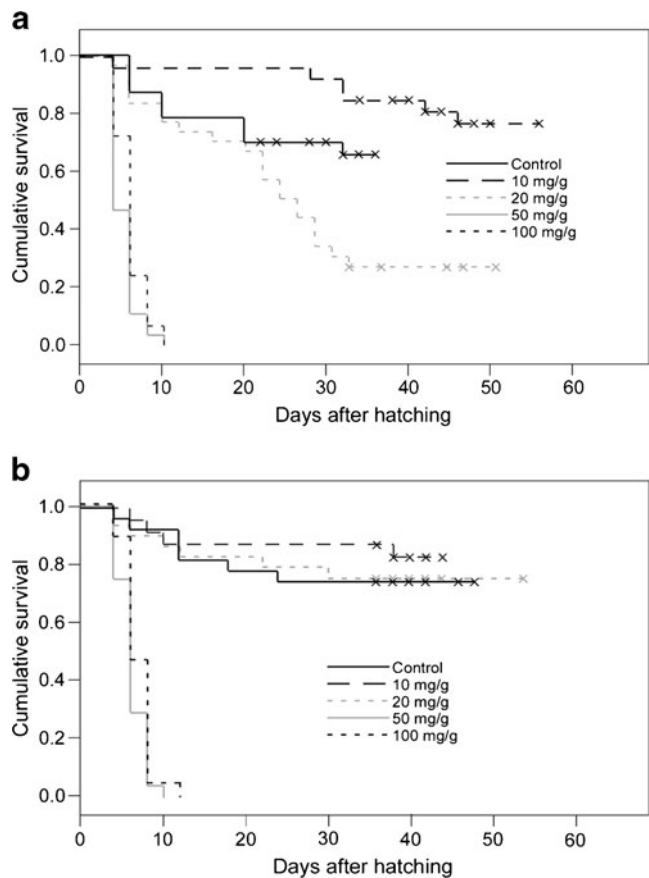
Mass spectrometry (MS) was performed on an electrospray ionization-quadrupole-time of flight (ESI-Q-TOF) MS system (maXis, Bruker Daltonics, Bexhill-on-Sea, UK). The instrument was operated in a wide-pass quadrupole mode, and the TOF data was collected for  $m/z$  50–1300 with low-collision energy of 8 eV. The optimized ion source and mass analyzer conditions were as follows: drying gas, N<sub>2</sub> (99.99%) at 8.0 l/h and temperature of 200  $^{\circ}\text{C}$ ; nebulizer pressure 1.6 bar; capillary and endplate voltages 500 V and 4,500 V, respectively; TOF tube voltage 9,880 V; reflection voltage 2,004 V; pusher voltage 1,640 V; MCP detector voltage 2,927 V. The system was mass calibrated in the positive-ion mode using a methanol solution of sodium formate on the enhanced quadratic algorithmic mode.

The signal of the extracted ion chromatogram at  $m/z$  299.1 ( $[\text{M}+\text{Na}]^{+}$ ) was employed for the quantification of ranunculin as sodium adduct. Quantification was performed using a five-point calibration curve obtained with pure

ranunculin using the Data Analysis 4.0 and Quant Analysis 2.0 software (Bruker Daltonics, Bexhill-on-Sea, UK).

## Results

**Bee Larval Performance** All larvae of *Chelostoma rapunculi* died within 4–10 d (median, 6 d) when feeding on the 50 mg/g and the 100 mg/g ranunculin treatment (Fig. 2a), while 29 % of the larvae survived on the 20 mg/g treatment (Table 1). Larval survival did not differ significantly between the control and the 10 mg/g and 20 mg/g treatments, respectively (*log-rank test*,  $\chi^2=5.688$ ,  $P=0.17$  and  $\chi^2=3.682$ ,  $P=0.55$  after Bonferroni correction), but differed significantly between the 10 mg/g and the 20 mg/g treatment (*log-rank test*,  $\chi^2=17.998$ ,  $P<0.001$ ). The larvae feeding on the control treatment required 20–34 d (median, 28 d) until completion of the cocoons compared to 34–56 d (median, 42 d) on the 10 mg/g treatment and 32–50 d (median, 44 d) on the 20 mg/g treatment.



**Fig. 2** Cumulative survival of larvae of bees specialized on plants other than *Ranunculus* when reared on their host pollen diet admixed with different quantities of ranunculin. Crosses indicate the days after hatching at which at least one individual reached the cocoon stage (censored data). **a** the *Campanula* specialist *Chelostoma rapunculi*, and **b** the Asteraceae specialist *Heriades truncorum*

**Table 1** Larval survival of the two bee species *Chelostoma rapunculi* and *Heriades truncorum* when reared on their host pollen diet mixed with ranunculin

Bee species	Ranunculin concentration (mg/g d.w.)	Hatched eggs (unhatched) <i>N</i>	Surviving larvae			Group heterogeneity	
			<i>N</i>	%	Survival time (days) <sup>a</sup>	<i>P</i>	Groups
<i>C. rapunculi</i>	0 (control)	23 (1)	15	65.2	26.30±2.35	<0.001	a, b
	10	28 (2)	22	78.6	49.17±2.59		a
	20	31 (0)	9	29.0	26.71±2.99		b
	50	28 (2)	0	0	5.21±0.30		c
	100	30 (1)	0	0	6.07±0.32		c
<i>H. truncorum</i>	0 (control)	29 (1)	23	79.3	35.52±2.39	<0.001	a
	10	25 (5)	21	84.0	39.37±2.34		a
	20	29 (1)	22	75.9	43.93±3.43		a
	50	24 (6)	0	0	6.17±0.34		b
	100	28 (2)	0	0	6.86±0.32		b

<sup>a</sup>Survival time gives the Kaplan-Meier survival time in days (mean±SEM) of the larvae on each pollen diet. Group heterogeneity was tested with the pairwise *log-rank test* between all treatments. Diets sharing the same letter did not differ significantly at  $P<0.05$  (*post hoc test*: pairwise *log-rank test* using Bonferroni corrections)

All larvae of *Heriades truncorum* died within 4–12 d (median, 6 d) when feeding on the 50 mg/g and 100 mg/g ranunculin treatment (Fig. 2b). Larval survival neither differed significantly between the control and the 10 mg/g and 20 mg/g treatments, respectively (*log-rank test*,  $\chi^2=0.294$ ,  $P=0.59$  and  $\chi^2=0.118$ ,  $P=0.73$  after Bonferroni correction), nor between the 10 mg/g and 20 mg/g treatment (*log-rank test*,  $\chi^2=0.549$ ,  $P=0.459$ ; Table 1). Larvae feeding on the control treatment required 30–42 d (median, 34 d) until completion of the cocoons compared to 36–44 d (median, 38 d) on the 10 mg/g treatment and 36–54 d (median, 42 d) on the 20 mg/g treatment.

A comparison between the two bee species tested revealed largely parallel performances across treatments; no significant differences were observed between the survival of the two species in the control, the 10 mg/g, the 50 mg/g, or the 100 mg/g treatments (*log-rank test*,  $\chi^2=1.48$ ,  $P=0.224$ ;  $\chi^2=0.995$ ,  $P=0.895$ ;  $\chi^2=3.833$ ,  $P=0.050$ ;  $\chi^2=3.032$ ,  $P=0.082$ ). In the 20 mg/g treatment, survival of *H. truncorum* larvae was greater than that of *C. rapunculi* larvae (*log-rank test*,  $\chi^2=11.347$ ,  $P<0.001$ ).

**Ranunculin Recovery** Recovery rate of ranunculin amounted to 62.5–71.0% (mean, 66.0%,  $N=5$ ) immediately after its addition to the experimental pollen provision, to 55.4–70.5% (mean, 60.8%,  $N=5$ ) after 8 d, and to 56.5–72.5% (mean, 63.2%,  $N=5$ ) after 22 d. These results indicate that the concentration of ranunculin mixed to the pollen provisions remained constant over a substantial period of time, and that about 60% of the added 10 mg/g ranunculin was biologically available.

**Ranunculin Content** To quantify the range of ranunculin in the field-collected samples, we assessed its concentration in pollen, flower buds, and brood cells (Table 2). Ranunculin concentration in the pollen of *Ranunculus acris* (mean,

0.55 mg/g) was almost forty times lower than that in the flower buds (mean, 19.45 mg/g), but almost twenty times greater than that in the brood cell provisions of *Chelostoma florissomne* (mean, 0.03 mg/g). The maximum concentration of ranunculin found in the pollen of *R. acris* was 1.34 mg/g (Table 2). Neither protoanemonin, the ranunculin anhydroglycone, nor anemonin, the product of spontaneous dimerization of protoanemonin, were detected in any of the samples.

## Discussion

Results of the feeding experiments provide evidence that the two tested solitary bee species not specialized on *Ranunculus* tolerated the 10 mg/g ranunculin treatment without any measurable effects on larval survival. Based on the ranunculin recovery experiment, the 10 mg/g ranunculin treatment corresponds to an approximate concentration of biologically available ranunculin of at least 5.54 mg/g d.w.

**Table 2** Content of ranunculin quantified by LC-MS in pollen and flower buds of *Ranunculus acris* and in brood cell provisions of the *Ranunculus* specialist bee *Chelostoma florissomne*

Source	<i>N</i>	Mean±SEM [mg/g]	Range [mg/g]
Pollen	8	0.55±0.18	0.03–1.34
Flower buds	8	19.45±5.20	3.72–53.93
Brood cell provisions <sup>a</sup>	11	0.03±0.01	0.003–0.12

<sup>a</sup>The *Ranunculus* pollen in the brood cell provisions was collected by foraging *C. florissomne* females from *R. acris*, which was the nearly exclusive pollen source for this species at the location where the brood cells were collected. Ranunculin amounts were calculated for pollen dry weight by subtracting average water and nectar contents in the brood cells

However, ranunculin in the pollen of *R. acris* quantified by LC-MS analysis amounted to maximally 1.34 mg/g d.w., a concentration that is at least fourfold lower than that tolerated by *Chelostoma rapunculi* and *Heriades truncorum*. The maximum ranunculin concentration in the brood cell provisions of the *Ranunculus* specialist *Chelostoma florissomme* was one order of magnitude lower (0.12 mg/g d.w.) than in the freshly collected pollen of *R. acris*, although the pollen in the analyzed brood cell provisions was derived from this *Ranunculus* species. Ranunculin is lethal when added to the natural pollen provisions of *C. rapunculi* and *H. truncorum* at very high concentrations (50 mg/g and 100 mg/g treatments). However, these concentrations greatly exceed natural concentrations of ranunculin found in *R. acris* pollen. Hence, the presence of ranunculin cannot explain the mortality of these bees when reared on a *Ranunculus* pollen diet, contrary to hypotheses proposed previously (Praz et al., 2008a; Sedivy et al., 2011).

Survival of *C. rapunculi* larvae feeding on the still high 10 mg/g and 20 mg/g ranunculin treatments was not significantly affected compared to the control treatment. However, mean survival values at the 20 mg/g treatment were low, and difference to the survival at the 10 mg/g treatment was significant, pointing to some adverse effects of the 20 mg/g treatment on larval survival. A sublethal effect (Piskorski et al., 2011a) of both the 20 mg/g and 10 mg/g treatments on *C. rapunculi* was noted as development times of the larvae were prolonged compared to the larvae in the control treatment.

Ranunculin admixed to the pollen provision at 10 mg/g could be recovered at a range of approximately 60%, irrespective of whether the incubation period lasted 0, 8, or 22 d. This finding indicates that a minor proportion of the ranunculin was deactivated during mixing, either through an adsorption or a chemical degradation. This result further indicates that the biologically available ranunculin concentration remained constant over long periods after mixing, thus underlining the validity of the conclusions drawn here.

Two previous studies described protoanemonin, the compound derived from hydrolysis of ranunculin, as the most prominent volatile in the headspace of pollen samples of *R. acris* (Bergström et al., 1995) and after thermal desorption of anthers of *R. acris* and other Ranunculaceae species (Jürgens and Dötterl, 2004). In both cases, only relative amounts were provided, and no information was given regarding absolute quantities of this lactone present in a volatile profile poor in other compounds. In another study, in which protoanemonin in different organs of *R. ficaria* was quantified after steam distillation, the androeceum was found to emit almost twice as much protoanemonin as the whole plant (Bonora et al., 1988). In that study, however, ranunculin was not quantified in the pollen itself. Thus, the high amounts of protoanemonin measured in the headspace of the androeceum of *R. ficaria* might have been derived from a high concentration of ranunculin in

the anther filaments, or in the anther tissue surrounding the pollen sacs prior to pollen release, rather than directly from the pollen. The low concentrations of ranunculin found in the pollen of *R. acris* in the current study is in line with the trace amounts of protoanemonin recently reported from an analysis of pollen volatiles of *R. bulbosus* (Piskorski et al. 2011b). The ranunculin concentrations we measured in the flower buds of *R. acris*, amounting to up to 54 mg/g d.w., corresponds to published levels of ranunculin in other ranunculin-containing Ranunculaceae species (Ruijgrok, 1966; Bai et al., 1996), thus validating our extraction and quantification methods.

Surprisingly, we found that the level of ranunculin in the pollen provision collected by *C. florissomme* was on average twentyfold lower than in pure pollen. Pollen in the cell provisions is diluted with nectar admixed by the foraging females at a ratio of approximately 1:1 (based on dry weight; A. Bühler and A. Müller, unpublished), explaining some but not all of the discrepancy noted between ranunculin concentrations in the flower pollen and in the bees' brood cell provisions. One process that substantially reduces ranunculin content in cut *Ranunculus* plants is drying, which triggers  $\beta$ -glucosidase-mediated autolysis of ranunculin with release of protoanemonin, yielding hay that is non-toxic to livestock (Majak, 2001). A similar process can likely be ruled out in the pollen harvested by *C. florissomme*, since the pollen is neither dried nor mechanically damaged during pollen collection, deposition, and storage in the brood cell. We hypothesize that the high sugar concentration originating from the nectar surrounding the pollen grains in the brood cell provisions may lead to an osmotic stress that provokes the release of protoanemonin from its precursor ranunculin, similar to the situation in drying *Ranunculus* plants.

In summary, the pollen of *R. acris* contains the secondary metabolite ranunculin in concentrations considerably below the lethal threshold for the tested bee larvae. Thus, we found no evidence that the incapability of several bee species to develop on a *Ranunculus* pollen diet is caused by ranunculin (Praz et al., 2008a; Sedivy et al., 2011). Hence, a different mechanism likely causes larval mortality of bees not specialized on *Ranunculus* pollen, such as the presence of another still unknown toxic pollen compound or the lack of essential nutrients in the pollen, e.g. certain sterols or amino acids.

**Acknowledgments** We thank Michael H. Benn (University of Calgary, Canada) for the gift of a substantial sample of ranunculin; Renato Zenobi and Louis Bertschi (MS service of the Laboratory of Organic Chemistry, ETH Zurich) for support and chemical analyses; the staff of the Neolithic village of Gletterens for providing bee nests; Bettina Gutbrodt and Mare Haider for technical support, and Heather Kirk and three anonymous reviewers for useful comments on the manuscript.

## References

- BAI, Y., BENN, M. H., MAJAK, W., and MCDIARMID, R. 1996. Extraction and HPLC determination of ranunculin in species of the buttercup family. *J. Agr. Food Chem.* 44:2235–2238.
- BENN, M. H. and YELLAND, L. J. 1968. Ranunculin. *Can. J. Chem.* 46:729–732.
- BERGSTRÖM, G., DOBSON, H. E. M., and GROTH, I. 1995. Spatial fragrance patterns within the flowers of *Ranunculus acris* (Ranunculaceae). *Plant. Syst. Evol.* 195:221–242.
- BHATTACHARYA, P. R., NATH, S. C., and BORDOLOI, D. N. 1993. Insecticidal activity of *Ranunculus sceleratus* (L.) against *Drosophila melanogaster* and *Tribolium castaneum*. *Indian J. Exp. Biol.* 31:85–86.
- BONORA, A., BOTTA, B., MENZIANI-ANDREOLI, E., and BRUNI, A. 1988. Organ-specific distribution and accumulation of protoanemonin in *Ranunculus ficaria* L. *Biochem. Physiol. Pfl.* 183:443–447.
- CAMPBELL, W. E., CRAGG, G. M. L., and POWRIE, A. H. 1979. Anemonin, protoanemonin and ranunculin from *Knowltonia capensis*. *Phytochemistry* 18:323–324.
- CASTELLANOS, M. C., WILSON, P., KELLER, S. J., WOLFE, A. D., and THOMSON, J. D. 2006. Anther evolution: pollen presentation strategies when pollinators differ. *Am. Nat.* 167:288–296.
- DE CARVALHO, A. C. P. and MESSAGE, D. 2004. A scientific note on the toxic pollen of *Stryphnodendron polyphyllum* (Fabaceae, Mimosoideae) which causes sacbrood-like symptoms. *Apidologie* 35:89–90.
- DOBSON, H. E. M. and PENG, Y. S. 1997. Digestion of pollen components by larvae of the flower-specialist bee *Chelostoma florissomme* (Hymenoptera: Megachilidae). *J. Insect Physiol.* 43:89–100.
- HARDER, L. D. and BARCLAY, R. M. R. 1994. The functional significance of poricidal anthers and buzz pollination: controlled pollen removal from *Dodecatheon*. *Funct. Ecol.* 8:509–517.
- HILL, R. and VAN HEYNINGEN, R. 1951. Ranunculin: the precursor of the vesicant substance of the buttercup. *Biochem. J.* 49:332–335.
- JÜRGENS, A. and DÖTTERL, S. 2004. Chemical composition of anther volatiles in Ranunculaceae: genera-specific profiles in *Anemone*, *Aquilegia*, *Caltha*, *Pulsatilla*, *Ranunculus*, and *Trollius* species. *Am. J. Bot.* 91:1969–1980.
- KINGSBURY, J. M. 1964. Poisonous Plants of the United States and Canada. Prentice-Hall, Englewood Cliffs, NJ.
- KREMEN, C., WILLIAMS, N. M., AIZEN, M. A., GEMMILL-HERREN, B., LEBUHN, G., MINCKLEY, R., PACKER, L., POTTS, C. G., ROULSTON, T., STEFFAN-DEWENTER, I., VAZQUEZ, P., WINFREE, R., ADAMS, L., CRONE, E. E., GREENLEAF, S. S., KEITT, T. H., KLEIN, A., REGETZ, J., and RICKETTS, T. H. 2007. Pollination and other ecosystem services produced by mobile organisms: a conceptual framework for the effects of land-use change. *Ecol. Lett.* 10:299–314.
- LEE, E. T. and WANG, J. W. 2003. Statistical Methods for Survival Data Analysis. Wiley and Sons, New York, NY.
- MAJAK, W. 2001. Review of toxic glycosides in rangeland and pasture forages. *J. Range Manage* 54:494–498.
- MARES, D. 1987. Antimicrobial activity of protoanemonin, a lactone from ranunculaceous plants. *Mycopathologia* 98:133–140.
- MARTIN, M. L., ROMAN, L. S., and DOMINGUEZ, A. 1990. In vitro activity of protoanemonin, an antifungal agent. *Planta Med.* 56:66–69.
- MAUCH, F. and STAEHELIN, L. A. 1989. Functional implications of the subcellular localization of ethylene-induced chitinase and  $\beta$ -1,3-glucanase in bean leaves. *Plant Cell* 1:447–457.
- MORGENTHALER, O. and MAURIZIO, A. 1941. Die “Bettlacher Maikrankheit”, eine Vergiftung durch Hahnenfuss-Pollen. *Schweizerische Bienenzeitung* 1:447–457.
- MÜLLER, A. 1996. Host-plant specialization in western palearctic anthidiine bees (Hymenoptera: Apoidea: Megachilidae). *Ecol. Monogr.* 66:235–257.
- MÜLLER, A., DIENER, S., SCHNYDER, S., STUTZ, K., SEDIVY, C., and DORN, S. 2006. Quantitative pollen requirements of solitary bees: Implications for bee conservation and the evolution of bee-flower relationships. *Biol. Conserv.* 130:604–615.
- PISKORSKI, R., INEICHEN, S., and DORN, S. 2011a. Ability of the oriental fruit moth *Grapholita molesta* (Lepidoptera: Tortricidae) to detoxify juglone, the main secondary metabolite in the non-host plant walnut. *J. Chem. Ecol.* 37:1110–1116.
- PISKORSKI, R., KRODER, S., and DORN, S. 2011b. Can pollen head-space volatiles and pollenkitt lipids serve as reliable chemical cues for bee pollinators? *Chem. Biodivers.* 8:577–586.
- PITKIN, B., ELLIS, W., PLANT, C., and EDMUNDS, R. 2010. The leaf and stem mines of British flies and other insects. <<http://www.ukflymines.co.uk>>.
- PAZ, C. J., MÜLLER, A., and DORN, S. 2008a. Specialized bees fail to develop on non-host pollen: do plants chemically protect their pollen? *Ecology* 89:795–804.
- PAZ, C. J., MÜLLER, A., and DORN, S. 2008b. Host recognition in a pollen-specialist bee: evidence for a genetic basis. *Apidologie* 39:547–557.
- RUIJGROK, H. W. L. 1966. The distribution of ranunculin and cyanogenetic compounds in the Ranunculaceae. *Meeting of the Phytochemical Group. Comparative Phytochemistry*, Cambridge UK, 1965:175–186.
- SEDIVY, C., PAZ, C. J., MÜLLER, A., WIDMER, A., and DORN, S. 2008. Patterns of host-plant choice in bees of the genus *Chelostoma*: the constraint hypothesis of host-range evolution in bees. *Evolution* 62:2487–2507.
- SEDIVY, C., MÜLLER, A., and DORN, S. 2011. Closely related pollen generalist bees differ in their ability to develop on the same pollen diet: evidence for physiological adaptations to digest pollen. *Funct. Ecol.* 25:718–725.
- SPENCER, K. A. 1990. Host Specialization in the World Agromyzidae (Diptera). Kluwer Academic Publishers, Dordrecht, Netherlands.
- VARITIMIDIS, C., PETRAKIS, P. V., VAGIAS, C., and ROUSSIS, V. 2006. Secondary metabolites and insecticidal activity of *Anemone pavonina*. *J. Biosciences* 61:521–526.
- VOGEL, S. 1993. Betrug bei Pflanzen: Die Täuschblumen. *Abhandlungen der Akademie der Wissenschaften und der Literatur in Mainz* 1:5–48.
- WESTERKAMP, C. 1997. Keel blossoms: bee flowers with adaptations against bees. *Flora* 192:125–132.
- WESTERKAMP, C. and CLASSEN-BOCKHOFF, R. 2007. Bilabiate flowers: the ultimate response to bees? *Ann. Bot.* 100:361–374.
- WESTRICH, P. 1989. Die Wildbienen Baden-Württembergs. Ulmer, Stuttgart, Germany.