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Does *Aconitum septentrionale* chemically protect floral rewards to the advantage of specialist bumblebees?

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Abstract. 1. Chemical protection of plants against herbivory is a well-studied phenomenon. However, chemical protection of floral rewards remains relatively unexplored. As with herbivore–plant interactions, toxic rewards may impact generalist and specialist foragers in different ways.

2. This study focuses on the toxic plant *Aconitum septentrionale* (Ranunculaceae). This plant is visited by specialist and generalist bumblebees. Alkaloid concentrations and profiles for the different parts of *A. septentrionale* were analysed to detect a potential chemical toxicity of floral rewards. In the same way, sequestration of alkaloids was tested on a pollen specialist species *Bombus consobrinus* and a generalist species *Bombus wurflenii*.

3. A liquid chromatography-quadrupole time-of-flight mass spectrometry method was developed to discriminate 16 major compounds in the plant. These alkaloids were present in all parts of the plant, but in different ratios. The concentration was high in the roots but also in pollen, providing evidence of chemical protection of this reward. By contrast, nectar had the lowest concentration of alkaloids. Only six alkaloids were detected in *B. consobrinus* tissues, at trace levels. For the generalist bumblebee *B. wurflenii*, no traces of alkaloids were detected.

4. Lappaconitine was the major alkaloid compound in pollen, nectar and *B. consobrinus* tissues. Low accumulation of alkaloids in *B. consobrinus* tissues could be an ecological advantage for this specialist species in terms of pathogen and predatory avoidance.

Key words. *Aconitum septentrionale*, alkaloids, bumblebees, entomotoxicology, specialized pollinators, UHPLC-(ESI)-Q-ToF/MS

Introduction

Chemical defence by secondary metabolites is a widespread feature in plants that develop under herbivore pressures (Stephenson, 1982; Wink 1988, 2003; Bennett & Wallsgrove, 1994; Adler *et al*., 2006). This chemical protection is costly in the form of energy, carbon and nitrogen, but it reduces the range of herbivores associated with the plants (Mithöfer & Boland, 2012). By contrast, animal-pollinated plants must attract visitors (generally bees) by producing floral rewards, mainly nectar and additionally pollen (Ollerton *et al*., 2011). Like herbivore–plant interactions, bee–plant interactions are clearly shaped by chemical compounds that might be different among floral rewards (Roulston & Cane, 2000; Manson *et al*., 2010; Cook *et al*., 2013). The study of secondary metabolites of floral rewards is particularly interesting, as they may play a dual role as a non-toxic compound for effective pollinators and a toxic repellent for herbivores, robbers or non-effective visitors (Irwin & Adler, 2006; Gegear *et al*., 2007; Sedivy *et al*., 2011, 2012; Adler & Irwin, 2012). A general overview on how plants chemically face this dilemma 'attraction versus protection' is very difficult because chemical composition of floral rewards is still poorly described (Praz *et al*., 2008).

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Consequently, little is known about the impact of toxic rewards on pollinators' development, behaviour and evolution (Sedivy *et al*., 2012).

Male and female bees feed on floral rewards and females provide larval brood cells with a mixture of pollen and nectar (Michener, 2007). Different foraging strategies have been described from specialist (i.e. oligolecty) to generalist behaviour (i.e. polylecty) (Robertson, 1925; Müller & Kuhlman, 2008). The origin and drivers of host–plant specialisation in bees are not well understood, but specialisation on toxic plants may be selected because competition with generalist visitors is reduced (Elliott *et al*., 2008). In plant–herbivore interactions, specialized foragers tend to be less affected by the chemical defences of the host plant than are generalist foragers.

This is due to an evolutionary adaptation to particular plant chemicals, whereby developing mechanisms detoxify, excrete or sequester plant toxic compounds (Mithöfer & Boland, 2012). Sequestration is common in specialist herbivores such as leaf beetles (Pasteels *et al*., 1990). It has the additional advantage of protecting them from predators, even if sequestration of toxins presents a physiological cost offset (Glendinning, 2002; Manson & Thomson, 2009). According to Nishida (1995), this mechanism could be the first evolutionary pressure for the adaptation between toxic plant and pollinators. Secondary metabolite sequestration, to our knowledge, has never been tested in bees specialized on toxic plants.

Our study focused on the toxic plant *Aconitum septentrionale* Koelle (Ranunculaceae) and two major foragers: the specialist *Bombus consobrinus* Dahlbom and the generalist *Bombus wurflenii* Radoszkowski (Fig. 1). We aimed: (i) to detect chemically protected rewards; (ii) to evaluate alkaloids sequestration in foragers; and (iii) to compare alkaloids in tissues of specialist versus generalist foragers.

Material and methods

Study species

The genus *Aconitum* comprises about 300 species throughout Eurasia and North America (excluding arctic and tropical regions) (Hess *et al*., 1977; Luo *et al*., 2005; Yuan & Yang, 2006). Monkshoods are typical 'bee-pollinated' plants with large inflorescences, bilateral symmetry and tubular flowers (Fig. 1) (Løken, 1973; Fukuda *et al*., 2001). Floral morphology makes monkshood pollen easily accessible only for long-tongued visitors (e.g. Darwin, 1876; Utelli & Roy, 2000; Duan *et al*., 2009).

Two species of bumblebee are specialized on monkshood pollen: *B. consobrinus* Morawitz, distributed in the north of the Palaearctic region; and *Bombus gerstaeckeri*, occurring in southern European mountains (Løken, 1973; Ponchau *et al*., 2006; Rasmont & Iserbyt, 2012). Their phenologies match monkshood blooming (i.e. from early July to mid-September) and their long tongue is adapted to easily reach the nectaries in the bottom of the flowers (Thøstesen & Olesen, 1996; Pekkarinen, 1997). Monkshoods are also visited by pollen generalist bumblebees such as *Bombus hortorum* and

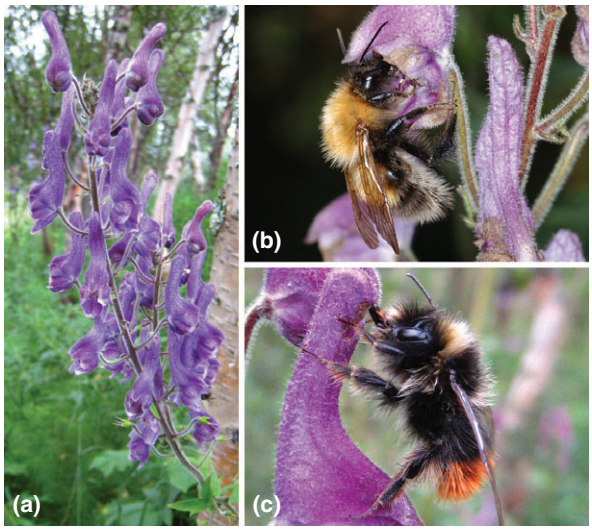

Fig. 1. (a) *Aconitum septentrionale* inflorescence (photograph: D. Roelants); (b) *Bombus consobrinus* on *A. septentrionale* flower (photograph: P. Rasmont); (c) *Bombus wurflenii* robbing nectar at the top of an *A. septentrionale* flower (photograph: D. Roelants).

B. wurflenii (L.) (e.g. Reinig & Rasmont, 1988; Bosch *et al*., 1997; Utelli & Roy, 2000). *B. wurflenii* and *B. consobrinus* co-occur in Scandinavia in patches of *A. septentrionale*. This species has violet flowers and a helmet with a wide base, tapering abruptly into a narrow hood (Federov *et al*., 1999; Utelli *et al*., 2000). It occurs in Scandinavia and Russia in wetlands (Utelli *et al*., 2000). *B. wurflenii* has a shorter tongue than *B. consobrinus* and the workers start flying before the blooming of *A. septentrionale*. The mandibulae of *B. wurflenii* are adapted to nectar-robbing behaviour. *Aconitum septentrionale* can be an important resource for *B. wurflenii*. During spring, this bumblebee forages on other flowers, but during the summer, it can rely almost exclusively on *Aconitum* pollen and nectar (Reinig & Rasmont, 1988). Floral composition of pollen loads (determination based on pollen grain morphology) confirmed the collection of *Aconitum* pollen by both *B. wurflenii* and *B. consobrinus* (D. Roelants, unpublished).

As far as is known, all the 54 phytochemically investigated species of *Aconitum* contain aconitine-like alkaloids, which are neurotoxic and cardiotoxic for mammals and insects (e.g. Ameri, 1998; Mizugaki & Ito, 2005; Singhuber *et al*., 2009). Variations of composition and concentration have been observed between species, between conspecific individuals and through different parts of the same plant (e.g. root, leaf and flowers) (Xiao *et al*., 2006; Singhuber *et al*., 2009). Considering the chemical structure of the aconitine-like alkaloids as well as their effects, three groups are distinguished in *Aconitum* spp. (Ameri, 1998). *Aconitum septentrionale* is considered as middle toxic, based on the analyses published by Zinurova *et al*. (2000) on seeds and those by Goncharov *et al*. (2006) on roots, because it contains important concentrations of lappaconitine. The LD50 is 5.9 mg kg^{-1} per intravenous administration (Ameri, 1998).

Chemical analyses

Sample preparation. Plants and bumblebees were sampled in August 2010 in two localities: Funäsdalen (Sweden, 62°35′43″N, 12°10′54″E, 790 m) and Røros (Norway, 62°38′42″N, 11°51′22″E, 720 m). Bumblebee workers of *B. consobrinus* and *B. wurflenii* were collected by net and killed by freezing after 48 h of a sugar diet. Roots, leaves and flowers were sampled from single specimens. Pollen and nectar were collected by paintbrush and by microcapillarity from each flower, respectively, and were pooled to have a sufficient amount for analyses. Plant samples and insect tissues were stored at −20 °C until extraction and chromatographic analysis. Samples were kept in the freezer (−80 °C) for 2 h prior to lyophilisation. Dry samples were ground to a fine powder, and an accurately weighed amount was placed in a 1.5 ml microcentrifuge tube (Eppendorf, Hamburg, Germany) with five to 10 glass beads (diameter 2 mm).

Alkaloids were extracted using a tissue homogenizer (Retsch Mixer Mill MM300, Düsseldorf, Germany) at 30 Hz during 4 min in the presence of aqueous methanol (50%) and 0.5% formic acid. Following centrifugation at 14 000 rpm for 2.5 min (Centrifuge 5424, Eppendorf), 100 µl of the clear solution was transferred to a vial. The samples were diluted 2.5 times with the extraction solvent prior to injection of 5 µl in the analytical system.

Ultra-high liquid chromatography–quadrupole time of flight mass spectrometry (UHPLC-(ESI)-Q-ToF/MS). Chemical analyses were carried out using UHPLC-(ESI)-Q-ToF/MS. A detailed description of the method is provided in Document S1.

This robust method covered the mass range of all alkaloids [Molecular Weight (MW) from 329 to 673]. Alkaloids from *A. septentrionale* and bumblebees were detected and tentatively identified by fragmentation Collision Induced Dissociation (CID) with the high resolution Mass Spectrometry (MS) (Document S2).

Statistical analyses

Differences between total alkaloid concentrations of the different samples were compared using non-parametric tests equivalent to one-way ANOVA (Kruskall–Wallis test). When the P-value was significant ($P < 0.05$), a Steel–Dwass test was conducted on the data. It is a multiple pairwise comparison (*post hoc* test) similar to the Wilcoxon test, but P-values were adjusted to avoid increases of type error I due to multiple testing.

Alkaloid profiles (relative abundances) in the different parts of the plant and the bumblebee tissues were visually assessed using non-metric multidimensional scaling (nMDS) ordinations. This method preserves the rank order of the inter-samples distance, as opposed to the linear relationship of classical metric scaling (i.e. principal component analysis, PCA). Moreover, nMDS has the advantage of robustness, e.g. not being sensitive to outliers and to normality and homoscedascity assumptions of classical metric scaling (Clark *et al*., 1996). The nMDS is based on the Bray–Curtis similarity matrix calculated on log-transformed relative abundances of alkaloids. This ordination is often more representative of true distances than the PCA ordination currently used. All nMDS plots were generated in R employing two dimensions (applying a conventional cut-off of < 0.2 for the stress value) and 50 runs; using functions from ECODIST, ELLIPSE and BIODIVERSITYR.

The minimum stress solution from this was used to produce the nMDS plots in which each spatial distance between samples can be interpreted as the relative difference in alkaloid composition (points that are closer are more similar than points that are more distant).

To test differences between samples, PERMANOVA was performed on log-transformed relative abundances. This is a permutation-based version of the MANOVA using the Bray–Curtis distance between samples to partition variance and randomizations or permutations of the data to produce the P-value for the hypothesis test ('Adonis' command, R-package VEGAN). To identify the compounds that were uniquely present in each sample, we performed an indicator compound analysis (INDVAL, Dufrêne & Legendre, 1997). This test calculates the probability of a compound being found in association with one particular sample (Holm's correction).

An indicator value–which is a function of a compound relative abundance and relative frequency–was calculated for each alkaloid and used to identify the sample with the highest indicator value for each compound. Univarite analyses (Wilcoxon test) were performed to detect a difference in relative abundance of quantifiable alkaloids in bumblebee tissues. All data analyses were performed in R version 2.2.1 with SCIVIEWS R console (version 0.9.2).

Results

Total concentration and profile of alkaloids in plant

The different parts of *A. septentrionale* displayed significantly different concentrations of total alkaloids ($H = 37.41$, $P < 0.001$). Pollen and roots had significantly higher concentrations than the other parts of the plant. Nectar had the lowest concentration of alkaloids (Fig. 2).

Lappaconitine (polar alkaloid) was one of the major alkaloids (from 19.6% to 40.1%) in all parts of *A. septentrionale*. Septenine was also a major alkaloid (from 12.8% to 24.4%) except for nectar, which had a high percentage of leucostine A (21.3 ± 10.9%). Oreaconine was also found in some parts as a major compound, especially in roots (31.9 ± 5.8%). 6-O-Acetylacosepticine was among the three most abundant alkaloids in flowers and nectar (13.0 ± 5.9% and 10.1 ± 10.2%, respectively).

According to composition in alkaloids, nMDS ordination arranged the different parts of the plant along NMDS1 and NMDS2 into two groups that were slightly separated: one including leaves and roots, and one including flowers, pollen and nectar (stress value = 0.18; Fig. 3).

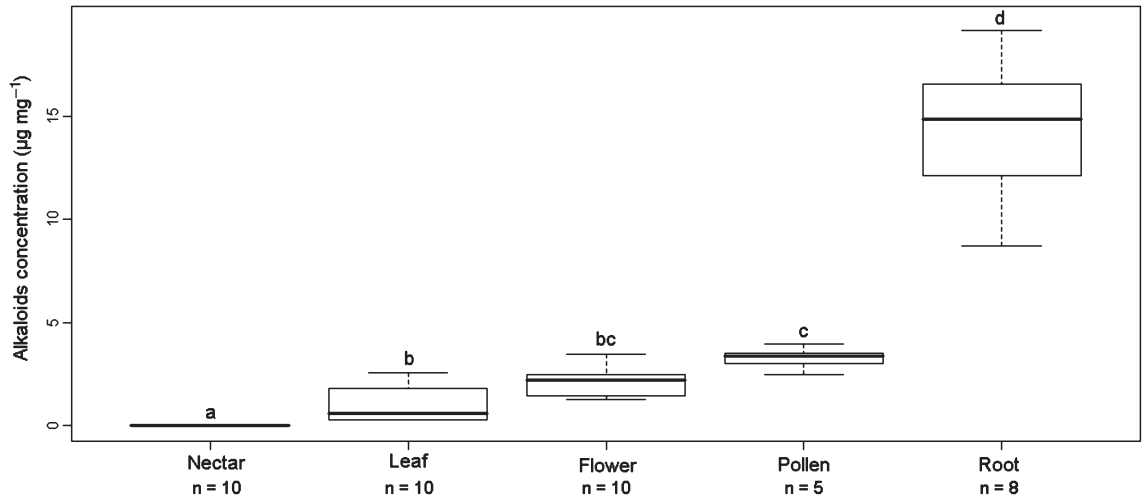

Fig. 2. Total alkaloid concentration in the different parts of *Aconitum septentrionale*. Letters indicate data that are significantly different according to a Steel–Dwass multiple comparison test ($\alpha = 0.05$).

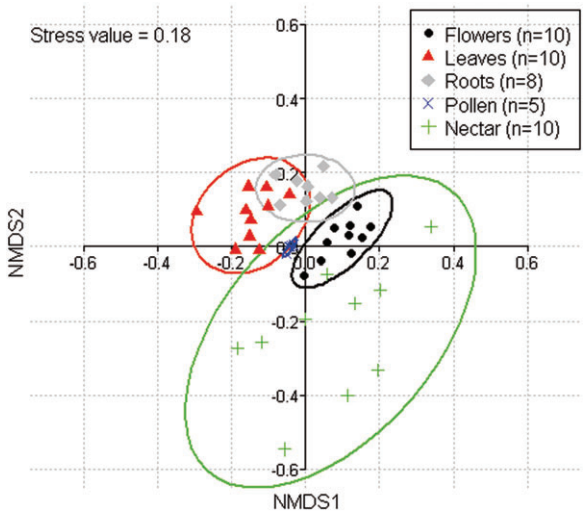

Fig. 3. Non-metric multidimensional scaling (nMDS) analysis of alkaloid profiles from the different parts of the plant (stress value = 0.18). Ellipses delineate the clusters that are significantly different from each other (multiple pairwise PERMANOVA, $P < 0.05$). Analyses were performed on log-transformed relative abundances of alkaloids.

The density of point cloud highlighted the constancy of pollen alkaloid profiles, while the point cloud of nectar was much more scattered. Despite overlaps of points, the PERMANOVA detected a significant difference in alkaloid composition between the different parts of the plant ($F = 11.39$, $P < 0.001$***). Multiple pairwise analyses showed that all parts are different from each other. However, INDVAL did not detect an indicative compound associated with one of the parts (indicator values < 50). All parts of *A. septentrionale* contained the same major alkaloids, but in different ratios.

Alkaloids in floral rewards and bumblebee tissues

Significant differences were detected between the bumblebee tissues and the floral rewards in the total concentration of alkaloids ($H = 21.15$, $P < 0.001$). Pollen had significantly higher alkaloid concentration than the *Bombus* specimens. The three major compounds for pollen were lappaconitine ($24.9 \pm 10.7\%$), septenine ($24.4 \pm 1.4\%$) and oreaconine ($14.1 \pm 1.0\%$); and for nectar were lappaconitine ($32.5 \pm 12.4\%$), leucostine A ($21.3 \pm 10.9\%$) and 6-O-acetylacosepticine ($10.1 \pm 10.2\%$). Due to the low concentrations of alkaloids and high interindividual variability in *B. consobrinus*, no significant difference could be detected between the two species of bumblebee (Fig. 4, Steel–Dwass *post hoc* test). Because no traces of alkaloids were present in *B. wurflenii*, dissimilarities between samples could not be calculated with the Bray–Curtis method, and PERMANOVA and nMDS ordination were not performed. The comparisons between floral rewards and bumblebees were only conducted on *B. consobrinus* data. The nMDS ordination highlighted two groups: (i) one group consisting of nectar (profile variable as shown by the sparse cloud) and pollen (profile highly conserved); and (ii) the other consisting of *B. consobrinus* (stress value = 0.11, Fig. 5). The analysis of PERMANOVA detected a significant difference in the profile of alkaloids between *B. consobrinus* and floral rewards ($F = 12.02$, $P < 0.001$***).

INDVAL showed that: (i) lappaconitine ($P = 0.016$, indicator value = 53.13) was significantly associated with *B. consobrinus*; (ii) 6-demethyldelphatine ($P = 0.030$, indicator value = 81.70) and leucostine A ($P = 0.016$, indicator value = 67.09) had a significant association with nectar; and (iii) septatisine ($P = 0.016$, indicator value = 100), acoseptine ($P = 0.016$, indicator value = 100), 10-hydroxymethyllycaconitine ($P = 0.016$, indicator value = 93.24), oreaconine ($P = 0.030$, indicator value = 60.79), septentriosine ($P = 0.030$, indicator value = 60.25) and N-acetylsepaconitine ($P = 0.022$, indicator

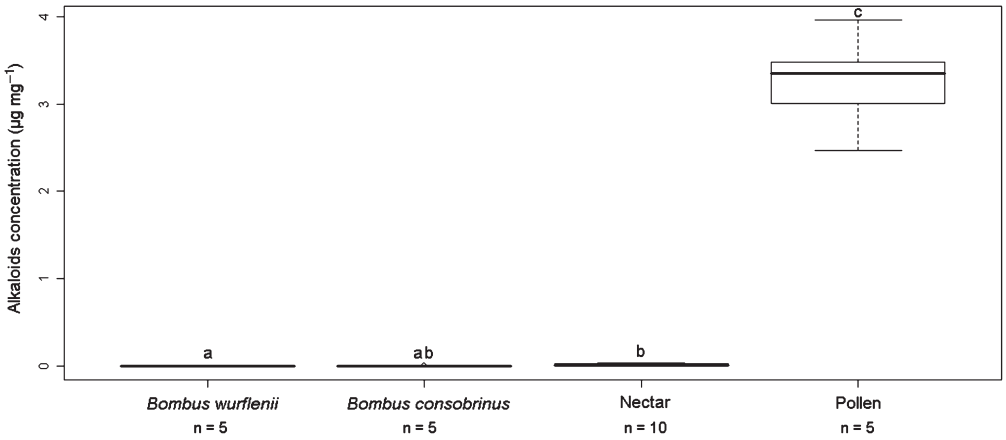

Fig. 4. Total alkaloid concentration in bumblebee and floral rewards tissues. Letters indicate samples that are significantly different according to a Steel–Dwass multiple comparison test ($\alpha = 0.05$).

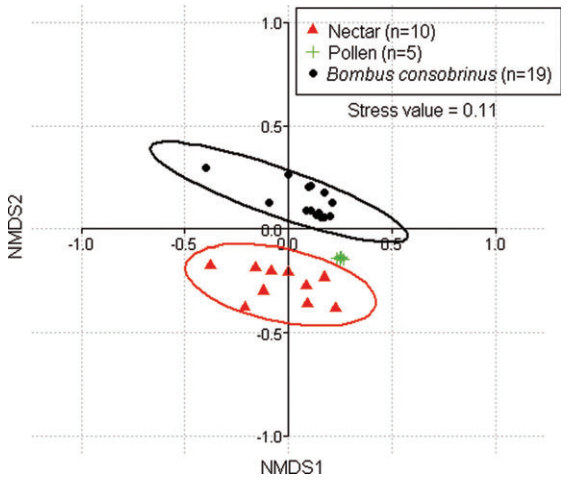

Fig. 5. Non-metric multidimensional scaling (nMDS) analysis of alkaloid profiles from nectar, pollen and *Bombus consobrinus* (stress value = 0.11). Ellipses delineate the clusters that are significantly different from each other (multiple pairwise PERMANOVA, $P < 0.05$). Analyses were performed on log-transformed relative abundances of alkaloids.

value = 58.28) were significantly associated with pollen. Moreover, lappaconitine was indicative for *B. consobrinus* in pairwise analyses with floral rewards ($P = 0.016$, indicator values = 66.68 and 72.34 for comparison with nectar and pollen, respectively).

Only traces of alkaloids (six components) were detected in bumblebee tissues (Fig. 6). Although no significant difference was detected between the two species with regards to the total concentration of alkaloids ($W = 14$, $P = 0.841$, Wilcoxon's test) and to the profile of alkaloids ($F = 1.462$, $P = 0.179$, PERMANOVA) between the two species, univariate tests were conducted on the compounds that are quantifiable (2-acetyl-septentriosine and lappaconitine). No difference was detected for the total concentration of 2-acetyl-septentriosine ($W = 78$, $P = 0.099\,58$), but the two species had different concentrations of lappaconitine, which was more abundant in *B. consobrinus* ($W = 102$, $P = 0.003\,435$).

Discussion

Protection of Aconitum septentrionale rewards

Toxicity of *A. septentrionale* rewards is difficult to assess without experimenting on growing larvae or on the imago survival of bumblebees. However, we can extrapolate some hypotheses from our results and the data in the literature. On the one hand, we found that *A. septentrionale* has a high level of aconitine-type alkaloids in all parts except the nectar. Alkaloid concentration in pollen of *A. septentrionale* (0.33%) is above the ED50 value of all the alkaloids tested by Detzel and Wink (1993) for *Apis mellifera* (values from 0.007% for cinchonine to 0.2% for tropine). On the other hand, the major alkaloid in pollen and nectar of *A. septentrionale* is the middle toxic alkaloid lappaconitine (Ameri, 1998). The pollen toxicity threshold of *A. septentrionale* for bumblebees is therefore difficult to assess, but the level of alkaloid concentration is potentially lethal or sublethal, at least for *A. mellifera*.

The potential toxicity of pollen due to alkaloids might be interpreted as a chemical defence to limit losses resulting from excessive pollen harvest or herbivory. This protective role has been already suggested by Praz *et al*. (2008) for plants of the genus *Ranunculus*. Praz *et al*. showed that the larvae of some non-*Ranunculus* specialist bees failed to develop on this pollen. By contrast, nectar of *A. septentrionale* has a low concentration of alkaloids and should be suitable for a large range of foragers. But the low toxicity of nectar is balanced by the difficult access to nectaries hidden in the bottom of the flowers. The consumption of nectar by visitors with non-adapted mandibulates (i.e. syrphid flies or short-tongued bees) is therefore limited.

We can hypothesize that the high alkaloid concentration (for pollen) and the low accessibility (for nectar) of *A. septentrionale* floral rewards discourage generalist visitors and herbivores, and could promote evolution of specialized behaviour,

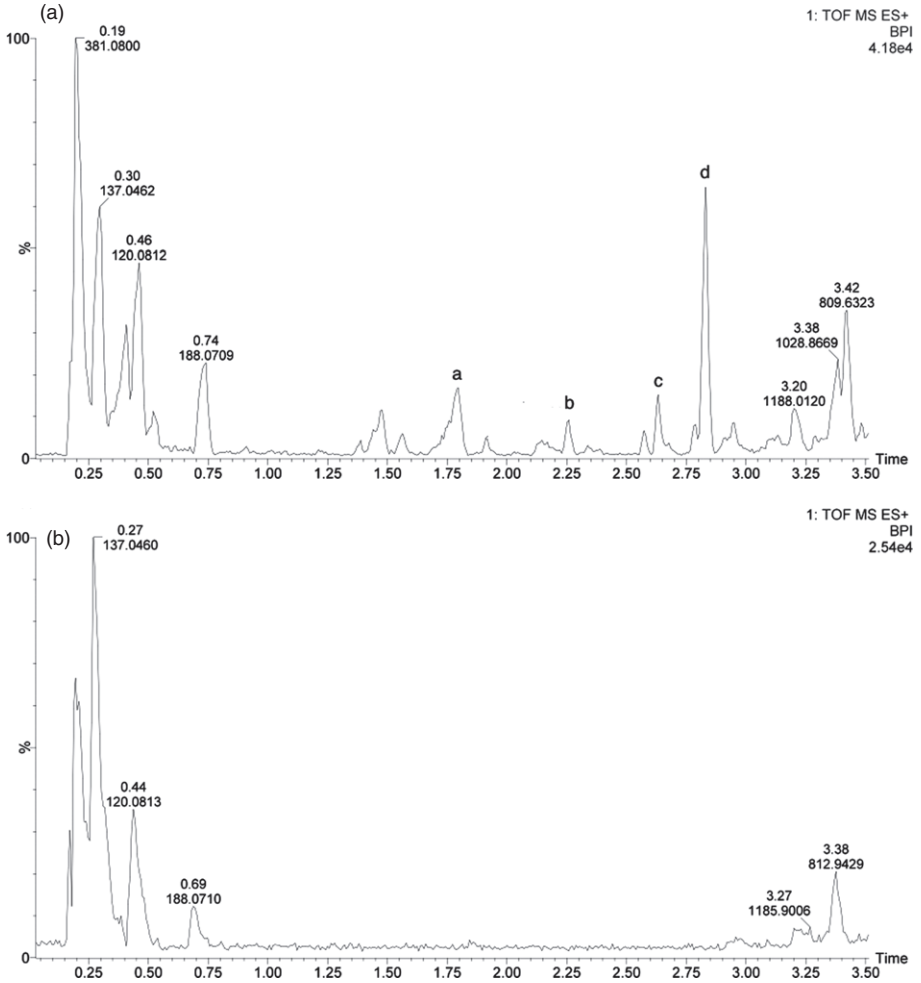

Fig. 6. Chromatograms of alkaloids detected in bumblebees, *Bombus consobrinus* (a) and *B. wurflenii* (b). Letters indicate alkaloids: a, 2-acetylseptentriosine/septenine; b, 6-O-acetylacosepticine; c, N-acetylsepaconitine; d, Lappaconitine.

such as in *B. consobrinus*. Evolution of pollinator specialisation in *A. septentrionale* was probably positively selected, as it is likely to improve pollination quality (Suzuki *et al*., 2007).

Alkaloid sequestration in bumblebees

The amount of alkaloid in the tissues of the two bumblebee species, specialist and generalist, is too low to hypothesize any sequestration of alkaloids. However, lappaconitine was detected in the specialist *B. consobrinus*, while no alkaloid was detected in *B. wurflenii* (Fig. 6). Evidence is that the presence of alkaloids (even in traces) in *B. consobrinus* does not result from a contamination by the digestive tract content. Studies on blowflies have reported that *Calliphora vicina* takes 65 min to clear its gut (Greenberg & Kunich, 2002). Thus, we can expect that a 48-h sugar diet for the bumblebees must have eliminated the alkaloids present in the insects' digestive tract.

The difference in alkaloid concentration between the more generalist *B. wurflenii* and the specialist *B. consobrinus* may be explained by the breadth of diet. *Bombus consobrinus* forages on pollen of *Aconitum* only when its phenology matches the blooming of *Aconitum* (Løken, 1973), while *B. wurflenii* forages on other pollen before, during and after *Aconitum* blooming (Reinig & Rasmont, 1988). All larvae of *B. consobrinus* have been fed with a high concentration of alkaloid. The uptake of alkaloids by larvae could be transmitted in adult tissues. Following this pilot work, future studies should be conducted at the larval stage to detect and to localise potential alkaloid accumulation.

In blowflies, while opioids are excreted by larval organs (via Malpighian tubules or nephrocytes), a quantity of synthetic alkaloids is assimilated into the insect tissues and can be retained within the adult after emergence (Bourel *et al*., 2001; Gosselin *et al*., 2010, 2011; Parry *et al*., 2010). By contrast, the generalist diet of *B. wurflenii* during spring may have diluted the alkaloid concentration ingested from *Aconitum* rewards in its tissues. We can hypothesize that for its whole life cycle, *B. wurflenii* is less likely than *B. consobrinus* to accumulate these compounds in higher concentrations.

Moreover, imago of generalist and specialist species could have different metabolisms of excretion or detoxification (e.g. lower excretion in *B. consobrinus*). An investigation of the cytochrome P450 in bumblebee metabolism could shed light on potential detoxification pathways highlighted in other insects (e.g. Dow & Davies, 2006; Dow, 2009; Parry *et al*., 2010). Bioassays should also be applied to predict the lethal and sublethal effects of toxic pollen on different generalist or specialist species.

Higher concentration of alkaloids in *B. consobrinus* tissues may increase its fitness, as alkaloids protect against microbial and/or predator attacks (especially in the larval stage), potentially without having an effect on offspring performance (Elliot *et al*., 2008; Manson *et al*., 2010). This positive characteristic could be added to the advantage of low foraging competition (Duan *et al*., 2009).

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/een.12032

Document S1. Description of the analytical method.

Table S1. List of major diterpenoïd alkaloids (16) tentatively identified in *Aconitum septentrionale* and bumblebee tissues.

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