Distasteful nectar deters floral robbery

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Highlights

- *Aconitum* nectar alkaloids function as a defence against nectar robbing bumblebees
- Nectar alkaloids are more distasteful to nectar robbers than pollinating bees
- Pollinators are also deterred from feeding on *Aconitum* flowers with nectar alkaloids
- Nectar toxins function as a defence when floral nectar is infrequently encountered

eOTC Blurb/ In Brief

Barlow et al. show that nectar alkaloids in specialized *Aconitum* flowers deter nectar-robbing bumblebees. Nectar toxins may have co-evolved with other nectar traits to influence whether bees choose to rob flowers. Pollinating bees were also sensitive to nectar alkaloids suggesting that defence may come at a cost of fewer pollinator visits.

SUMMARY

Toxic nectar is an ecological paradox[1,2]. Plants divert substantial resources to produce nectar that attracts pollinators [3], but toxins in this reward could disrupt the mutualism and reduce plant fitness [4]. Alternatively, such compounds could protect nectar from robbers [2], provided they do not significantly alter pollinator visitation to the detriment of plant fitness [1,5–8]. Indeed, very few studies have investigated the role of plant toxins in nectar for defence against nectar robbers [4,9,10]. Here, we compared two *Aconitum* species (*A. napellus* and *A. lycoctonum*) that have flowers specialized for long-tongued bumblebee pollinators (*Bombus hortorum*) but are occasionally robbed by short-tongued bumblebees (*B. terrestris*) [6,11–13]. Pollinator visits to flowers were much more frequent than by robbers but visits correlated negatively with nectar alkaloid concentration and declined sharply between 200-380ppm. However, alkaloid concentrations of > 20ppm were deterrent to *B. terrestris* suggesting robbers were less tolerant of nectar alkaloids. Nectar of both plant species contained similar concentrations of carbohydrates and toxic alkaloids, but *A. lycoctonum* was more likely to secrete nectar in each flower and was also visited more frequently by pollinators and robbers. We conclude that alkaloids in *Aconitum sp.* nectar affect rates of both pollinator visitation and robbery but may have co-evolved with nectar availability to maintain the fitness benefits of specialized plant-pollinator relationships. Chemical defence of nectar is, however, ultimately constrained by pollinator gustatory sensitivity.

Keywords

Alkaloids, *Bombus*, *Aconitum*, bumblebees, chemical defense, nectar larceny, nectar toxin, specialized pollinators, Rana automated monitoring.

RESULTS

Robbery occurred more often on *A. lycoctonum*

We examined the incidence of nectar robbery (identified by characteristic holes in the corolla hood, or galea which are chewed out by robbing bees) on two species of *Aconitum* (*A. napellus* and *A. lycoctonum*) that were grown side-by-side at the Royal Botanic Gardens, Kew, UK. Both *Aconitum* species were robbed (Figure 1A, B), but the frequency depended on the year of observation (year, GLM, $\chi_1^2 = 65.8$, $P < 0.001$). In both years, flowers of *A. lycoctonum* (Year 1: 8%, Year 2: 41%) were more frequently robbed than flowers of *A. napellus* (Year 1: 2%, Year 2: 26%) (species, GLM, $\chi_1^2 = 12.0$, $P = 0.001$).

To quantify insect visits to both species and identify the robber(s), we used conventional manual monitoring together with a novel automated monitoring system (Rana, see methods). We recorded 1340 raceme visits by insects to *A. napellus* (Figure 1C) and *A. lycoctonum* (Figure 1D) over a 293 h observation period. Two generalist bumblebee species accounted for 96% of visits (*B. hortorum*, 92.5% and *B. terrestris*, 3.6%) (Movie S1). *B. hortorum,* the long-tongued pollinator, always landed on the flower, contacted the stamens/stigmas, and collected nectar by feeding from the nectar spurs in accordance with the highly adapted floral structure; it was 3 times more likely to visit *A. lycoctonum* (5.51 raceme visits hr^{-1}) than *A. napellus* (1.67 raceme visits hr^{-1}) (Figure 1E). It also investigated these species' flowers, by flying towards and hovering above them without alighting, at a similar rate (Figure 1E).

By contrast, while Rana recorded exploratory visits it did not capture *B. terrestris* successfully robbing *A. napellus*, although *ad hoc* observations of robbery were recorded or evidenced by the presence of holes in galeas. When *B. terrestris* visited *Aconitum lycoctonum*, it never accessed nectar in a way that would lead to pollination. Instead, it occasionally chewed a hole through the top of the floral galea near the two nectar spurs (Fig. 1B) to acquire nectar (*A. lycoctonum*: 3 of 27 visits, 11%, 0.02 visits hr^{-1}) (Figures 1A-B & F) or abandoned flowers before reaching the nectar (*A. lycoctonum*: 11 of 27 visits, 41%, 0.06 hr^{-1}). The most frequent behaviour exhibited by *B. terrestris* was investigation of the flowers

of both species without alighting (*A. napellus*: 21 of 21, 100%, 0.18 hr^{-1}; *A. lycoctonum*: 13 of 27 visits, 48%, 0.07 hr^{-1}) (Figure 1F).

Aconitum sp. have similar concentrations of nectar alkaloids and sugars

LC-HRESIMS analysis revealed that the galea and nectar of each *Aconitum* species contained a unique alkaloid profile (Table S1). We characterised 7 of the alkaloids in *A. lycoctonum* and *A. napellus* by mass spectral data analysis and comparison to compounds known from *Aconitum* spp. [14]. (Table S1). In both species, all compounds identified in the galea were identified in nectar (Figure 2A, Table S1) although the alkaloid concentration in the galea was 7 times greater than in the nectar (Table S1, 2-way ANOVA: location, $F_1 = 74.5$, P = 2.96e-11) in both species (2-way ANOVA interaction species x location: $F_1 = 0.518$, P = 0.475). While the total alkaloid concentration in both species did not differ significantly (2-way ANOVA: species, $F_1 = 1.85$, P = 0.180), the relative concentrations of individual alkaloids in the galea and nectar did (PERMANOVA: *A. lycoctonum*, $F_1 = 28.47$, $R^2 = 0.50$, P < 0.001; *A. napellus*, $F_1 = 9.02$, $R^2 = 0.33$, P <0.001; Tables S1 and S2 and Figure S1).

Alkaloids were always found in nectar but varied in concentration. The total concentration of the alkaloids was more variable in *A. lycoctonum* (CV = 0.82) than *A. napellus* (CV = 0.71, CV = coefficient of variation). The concentration of total alkaloids in nectar was positively correlated in the galea and nectar of *A. lycoctonum* (Pearson's correlation coefficient, $t_{12} = 3.89$, P < 0.010) (Figure 2A). This was also the case for the 5 most abundant compounds we characterised in *A. lycoctonum* nectar; 8-*0*-methyllycoctonine, leucostine A, 8-*0*-methyllycaconitine, lycaconitine and alkaloid-1AL (Table S1, Table S3). While total alkaloids were not correlated across galea and nectar for *A. napellus* ($t_6 = 0.47$, P = 0.658) (Figure 2A) the most abundant compound identified in this species, aconitine, was positively correlated so that high concentrations in nectar correlated with high concentrations in the galaea (Table S3).

We surveyed all flowers of up to 4 racemes of individual plants of *A. lycoctonum* (N = 7) or *A. napellus* (N = 5) for the presence and amount of nectar found in the nectar spurs. Nectar was present in 79%

of the flowers of *A. lycoctonum* (N = 183, including flowers damaged by larcenists) but only in 48% of the flowers of *A. napellus* (N = 123, Table 1, $\chi_1^2 = 25.6$, $P < 0.001$). When nectar was present, the volume of nectar per flower was significantly lower in *A. lycoctonum* than in *A. napellus* (Table 1A, t-test, $t_{51} = -2.41$, $P = 0.019$). Using HPLC, we found that the mean nectar concentration for *A. lycoctonum* was 61.3% wt vol^{-1} sugars and for *A. napellus* was 63.0% wt vol^{-1} sugars with nectar in both cases that was dominated by sucrose (Table 1A).

Alkaloids in *Aconitum* spp. nectar deter adult worker bumblebees

We examined how bee visitation to flowers of both *Aconitum* spp. was influenced by alkaloids found in a subset of plants. *B. hortorum* (pollinators) were more likely to visit flowers of *A. lycoctonum* containing nectar with low concentrations of total alkaloids ($n = 12$, R^2 (adj) = 0.27, $F_{12} = 5.8$, $P < 0.050$) (Figure 2B1) suggesting alkaloids at high concentrations might deter visits. Visitation rate was a negative function of the nectar concentrations of three of the five most prevalent alkaloids (Figure 2B2, Table S4). Extrapolation of our data indicates that visits by *B. hortorum* decline sharply above 200 ppm and cease at 380 ppm total alkaloid concentration (Figure 2B1). Overall, mean visitation rate by *B. hortorum* to *A. lycoctonum* was 16.3 visits/hr. There was a trend for *B. hortorum* to avoid flowers of *A. napellus* in which nectar contained high aconitine levels (Table S4), but visitation to flowers of this species was generally low compared to *A. lycoctonum* (2.45 visits/hr); because our statistical power was low, we were unable to observe the same relationship with total alkaloids in *A. napellus* ($n = 8$, R^2(adj) = 0.06, $F_6 = 1.44$, $P = 0.276$).

We were also unable to examine the relationship between nectar robbery by *B. terrestris* and alkaloid concentration in the galea or nectar of either plant species because these events were too rare in our observations. It was, therefore, not possible to make a direct comparison of correlations of visitation frequency and nectar alkaloids between robbers and pollinators from the field data.

To determine the concentrations of alkaloids in nectar that deter robbers we used a novel laboratory method that assessed bee gustatory responses at a range of ecologically relevant concentrations of

alkaloids [15]. Alkaloids were isolated from the flowers and dissolved in 100 mM sucrose solution. *B. terrestris* workers were deterred from consuming 100 mM sucrose solutions (i.e. 3.4% wt/vol) containing concentrations as low as 20 ppm of the alkaloids extracted from the galea of both *Aconitum* species (Figure 3, GLM, conc, $\chi_4^2 = 256$, $P < 0.001$). This concentration was between ten and twenty times lower than the concentration required to deter the pollinating species suggesting that the pollinating species while not immune has higher tolerance of the deterrent effects of the alkaloids. While *B. terrestris* was likely to consume equivalent concentrations of alkaloids from both *A. napellus* and *A. lycoctonum* (Figure 3, GLM, species x conc, $\chi_4^2 = 1.71$, $P = 0.789$, Figure S2A), detailed analysis of their behaviour from video recording revealed that they made more contacts with *A. napellus* extracts (Figure S2B). This indicates that the robbing bees detected the alkaloids of A. *napellus* at a lower concentration than those of *A. lycoctonum* suggesting they were more sensitive to them [15]. Furthermore, we compared the bees' responses to 20 ppm concentrations of the galea alkaloid mixtures and to the most prevalent alkaloid recorded in the nectar of both species (Figure 3). The most prevalent nectar alkaloid of *A. napellus*, aconitine, was as deterrent as the galeal mixture of total alkaloids; however, it was almost twice as deterrent to the bees as the most prevalent nectar alkaloid of *A. lycoctonum* (8-*O*-methyllycoctonine) (Figure 3, GLM, $\chi_4^2 = 94.7$, $P < 0.001$) suggesting alkaloids in *A. napellus* may be more deterrent than in *A. lycoctonum* and may explain differences in the visitation frequency between the two species. This relationship was also observed across a concentration range for aconitine and another alkaloid found in *A. lycoctonum* nectar (lycaconitine) (Figure S3). In addition, we tested *B. terrestris* workers with a mixture of fructose and sucrose mimicking nectar concentrations in both *Aconitum* species (as in Table 1); *B. terrestris* workers were also significantly repelled by 100 ppm (the mean concentration in the nectar of both *Aconitum* spp.) of the alkaloid mixtures in solutions mimicking nectar. (Note: unfortunately, we were unable to culture *B. hortorum* to conduct equivalent laboratory evaluations of their responses to the alkaloids). Thus, while we recognise the limits of direct comparison across field and laboratory measures, our data suggest that *B. terrestris* is more sensitive to *Aconitum* alkaloids than *B. hortorum*. Furthermore, our data indicate

that *B. terrestris* detects lower concentrations of the alkaloids of *A. napellus* nectar (aconitine) than those of *A. lycoctonum*.

Nectar pay-off from *Aconitum* spp. was equal for robbers but greater for pollinators on *A. napellus*

Many studies in the field and in the lab have established that profitability of foraging is ultimately what governs bee behaviour (e.g. [16]). Uncertainty in obtaining nectar from a given flower can affect bee foraging preferences; bees are more risk averse when they experience flowers with no nectar than when they experience flowers with nectar that varies only in volume or concentration [17]. In our experiments, nectar robbers of *Aconitum* sp. do not know *a priori* whether a given flower will contain nectar in its nectar spurs (unless avoiding flowers with scent of recent visitors [18,19]) because nectar is concealed deep within two nectary spurs that are hidden under an adapted sepaloid hood or galea. Another factor, therefore, that could influence the incidence of robbery in our experiments is the economics of foraging. To estimate the economic value of nectar robbery in our study site, we calculated the nectar pay-off: the probability of encountering nectar multiplied by the mean energetic value/flower. This value represents the mean nectar pay-off in a foraging site. To calculate the energetic value of nectar for each plant species, we multiplied the molar concentration of each sugar by the amount of ATP produced by each molecule (Table 1A, 38 ATP for glucose and fructose and 76 ATP for sucrose) (Note: we assumed that there were equal numbers of flowers of each species present in a foraging patch). The plant species most frequently robbed, *A. lycoctonum,* produced nectar of lower energetic value per unit volume and less nectar on average per flower (Table 1A). In fact, we estimated that the energetic value per flower was 1.6 times greater in *A. napellus* than *A. lycoctonum.* However, flowers of *A. lycoctonum* were more likely to contain nectar, so that for *B. terrestris* robbing nectar from *A. lycoctonum*, the probability of encountering nectar was 79%. This means that the pay-off would be 79% of the ATP per *A. lycoctonum* flower (15.9×10^{19}), or 12.6×10^{19} ATP/floral visit in this population. In contrast, only 48% of the flowers of *A. napellus* contained nectar. Assuming that both plant species had the same size population, robbery of *A. napellus*, provided an almost equal

pay-off (12.7 x 10^{19} ATP/floral visit) to *A. lycoctonum*. Because the pay-off was similar for these two plant species, we expected levels of robbery on both *Aconitum* spp. to be similar in this ecological context. However, we observed that bees were up to 4x more likely to rob *A. lycoctonum*.

We made the same calculations for pollinators (Table 1B). In the absence of robbers, the overall net energetic gain (pay-off) for pollinators was the same on both species. In the presence of robbers that take nectar from damaged flowers (Table 1B), this calculation changes. The total nectar pay-off to pollinators on *A. lycoctonum* in this situation was 1.1 times lower in Year 1 and 1.3 times lower in Year 2 (Table 1B). In the absence of information about the way toxins affect the palatability of nectar, these data lead us to expect *B. hortorum* to forage exclusively on *A. napellus* when nectar robbery occurs in a plant population. Instead, we found they were 3x more likely to visit *A. lycoctonum* (Figure 1). They did, however, continue to visit *A. napellus*, albeit at a lower rate (Figure 1).

DISCUSSION

Our data are the first to show that plant compounds in nectar may function in a natural ecological setting as a defence against nectar robbers. We found that nectar robbing bees (*B. terrestris*) were deterred in the laboratory by concentrations of *Aconitum* species alkaloids that were more than 10 times lower than those we recorded in nectar of flowers that were still visited by the pollinating, long-tongued bee species, *B. hortorum*. Indeed, extrapolation of our data suggest that pollinator visits cease when concentration of toxic alkaloids in nectar is between 200-400 ppm. Frequency of visits by pollinators and robbers was higher on *A. lycoctonum* than *A. napellus* and this may be explained by our laboratory feeding tests that showed *B. terrestris* was less sensitive to alkaloids from the more frequently visited plant, *A. lycoctonum*.

Additionally, we observed that *A. napellus* nectar occurred less frequently in flowers (i.e., was less predictable). Since this species was also less frequently robbed, the likelihood of encountering nectar

might also influence foraging behaviour and toxins in nectar may be more effective against robbers when the occurrence of nectar in each flower is hard to predict. Nectar in *Aconitum* species is concealed in nectar spurs that are themselves covered by an adapted sepaloid hood (galea) so it is not possible to see the nectar in the nectary or readily detect any volatiles it might emit. The fact that pollinating bees visited *A. napellus* less frequently even though foraging on it was, on average, a better economic strategy indicates that protecting nectar from robbers using distasteful compounds comes with the potential cost of fewer pollinator visits.

The evolution of specialized floral parts that exclude certain visitors in preference to specific pollinators is likely to have been caused in part by competition between pollinators for access to nectar. Pollinators with longer mouthparts for accessing nectar can rapidly drive traits in plant populations that favour floral parts that protect nectar from other visitors [20,21]. In response to this co-evolved specialization, pollinators with shorter mouthparts make holes in specialized floral structures to access nectar [22], depriving plants of pollen transfer and specialist pollinators of nectar.

Other studies have shown that nectar quality, volume per flower, and floral morphology are all features that influence frequency of robbery [23]. Our experiments are the first to directly show that the presence of alkaloids in nectar reduces rates of nectar robbing by bee species, but, similarly, influences the pollinators. For nectar robbers and pollinators, foraging has a metabolic cost and the economics of the energetic value of nectar governs floral choice [16,17,24–26]. Biting through the corolla and nectar spur to access nectar has an additional energetic cost in time, so the energy value of the nectar must be greater than the extraction cost [16]. Nectar robbers may also be deterred by the alkaloids found in floral parts (e.g., galea) and this is indicated in our studies where insects partly chewed the galea before abandoning the flowers. In *Nicotiana attenuata*, nicotine is concentrated at the site of robbery near the base of the corolla, although no explicit link has been established between behaviour of the nectar robber and corolla alkaloids [27]. Our data show that alkaloids in the corolla

are deterrent to bees although we did not explicitly test whether their presence prevents bees from biting holes.

In the case of *Aconitum* flowers, robbing bees cannot know whether a given floral nectary will contain nectar before biting a hole because the nectar occurs in two highly concealed nectary spurs which are hidden beneath the sepaloid hood or galea (Figure 1A). Given the energetic costs of robbery, the risk of finding empty flowers would likely affect their decision to forage in a patch of flowers, as it does pollinators [17]. For these reasons, bees are likely to rob only when the benefits outweigh foraging on other flowers (e.g. pollinating [1,23,24]). Alkaloids in *A. napellus* nectar were more deterrent than those in *A. lycoctonum,* but our data suggest that the greater frequency with which nectar occurs in *A. lycoctonum* may also explain why this species was preferred by the robber.

Toxins in nectar could harm or kill pollinators and nectar robbers [2,28,29]. The metabolic costs associated with detoxification, combined with the risk of death caused by poisoning, reduce the value of nectar [30]. For this reason, toxins should also alter the cost-benefit analysis bees determine while foraging. The alkaloid, gelsemine, reduced the value of sucrose solutions free-flying bees would otherwise choose [26]. Given a choice of two solutions containing detectable concentrations of gelsemine, bees chose to collect nectar with the lowest concentrations of this alkaloid [26]. We expect that the addition of alkaloids to nectar of *Aconitum* species, especially *A. napellus* on which bees had a lower probability of encountering nectar, reduced the value of nectar to an economic tipping point in favour of the pollinator and away from the robber. This economic balance depends on the environmental context (i.e., availability of other flowering plants) and competition between pollinators for access to nectar. In addition, *Aconitum* spp. flowers also have relatively high concentrations of sugars in nectar; high quality nectar, combined with a large volume of nectar, could be a mechanism to off-set costs to pollinators of foraging on *A. napellus* flowers (e.g., infrequently encountered nectar in concert with toxins).

In general, bee species may not be particularly sensitive to toxins in food [28,31], perhaps because they have few gustatory receptors for detection [32]. The fact that *B. terrestris* was more likely to reject solutions containing *Aconitum* alkaloids suggests that short-tongued generalists may be more sensitive to toxic compounds than long-tongued specialists like *B. hortorum*. Only a few studies have tested the gustatory sensitivity of *B. terrestris* to potentially toxic compounds. These have shown that *B. terrestris* is more sensitive to alkaloids than other types of plant compounds and that gustatory acuity for most compounds is generally limited to a range above 10 ppm [33,34]. None have reported detection thresholds for other *Bombus* species or how bees respond to mixtures of alkaloids. Our work shows that bumblebee species differ in their sensitivity to alkaloids in nectar and that mixtures of compounds can be more potent deterrents than single compounds.

Flower structures that suit specialist pollinators and protect nectar from non-pollinating species are a valuable trait that benefits plant reproduction. Nectar robbery reduces this advantage and can reduce plant fitness. Mechanisms to prevent nectar robbery would be expected to occur when gains made through specialization outweigh having flowers with nectar available to all floral visitors. Here we demonstrate for the first time that toxins act as a nectar defence in floral structures adapted for specialists. Our study indicates that non-nutrient plant compounds may have co-evolved with other nectar traits such as nectar carbohydrate concentration, volume per flower, and production in each flower (i.e., predictability) to deter nectar thieves by influencing the economics of robbery.

AUTHOR CONTRIBUTIONS

PCS conceptualised the research hypothesis with GAW. SEB, PCS and GAW designed experiments, wrote the first draft of the manuscript and analysed data. CM, MB conducted research experiments on taste and deterrency with GAW, ECM conducted field observations and data collection with SEB. AB, SEB, PCS and IWF sampled nectar, extracted plant material and conducted all chemical analysis

and quantification for bioassays. BMP helped design field experiments using Rana. All authors contributed to revisions of the manuscript.

ACKNOWLEDGEMENTS

The authors would like to thank Sebastien Kessler for assistance with lab assays; Emma Knight for nectar collection and analysis; and Mark O'Neill (Tumbling Dice Ltd) for technical assistance with Rana. MB was funded by an Erasmus+ Mobility training scheme. This work was funded in part by the following grants from the British Ecological Society (4870/5910) to SEB; and the Leverhulme Trust (RPG-2012-708) and the BBSRC (BB/M00709X/1) to GAW and in part by a grant from the BBSRC, NERC, the Wellcome Trust, Defra, and the Scottish Government under the Insect Pollinators Initiative (BB/I000968/1) to GAW and PCS.

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Figure legends

Figure 1. Pollinating and nectar robbing bumblebees prefer *Aconitum lycoctonum* over *A. napellus*. *A. lycoctonum* received four times more legitimate visits than *A. napellus* and more robbery. (A) *Aconitum* flower with hood (galea) pulled back (solid arrow) revealing two nectaries on adapted petals (dashed arrow). (B) *Bombus terrestris* chewing the floral galea of *A. lycoctonum* to rob nectar. (C-D) Close-up view of bee visits captured by the Rana automated monitoring system showing C) *B. hortorum* anticipating foraging from *A. napellus* with its long proboscis extended, and D) *B. hortorum* foraging legitimately for nectar from *A. lycoctonum*. (E-F) Bumblebee visitation frequency and behavior (relative number of raceme visits per hour) to *A. lycoctonum* and *A. napellus* by E) *B. hortorum*; and, F) *B. terrestris* recorded by visual and automated (Rana) monitoring methods during peak flowering (June-July). *Note: the scales on the y axes differ*. Total numbers of visits are in parentheses. Sample numbers and observation periods: *A. lycoctonum* = 16 racemes from 11 individuals monitored for 177 hours on 20 days; *A. napellus* = 11 racemes from 9 individuals monitored for 116 hours on 17 days. Photographs: A) R. Fang; B-D) S. E. Barlow. *Related to Movie S1*.

Figure 2. Nectar and galea alkaloid concentrations and the effect on the rate of visits by nectar foraging bumblebees. A) The concentration of alkaloids (ppm) in the nectar and floral galeas of paired racemes is positively correlated in *Aconitum lycoctonum* ($n = 14$, $t = 3.885$, $P < 0.01$), but not in *A. napellus* ($n = 10$, $t = 0.466$, $P = 0.658$). *Related to Tables S1 and S3*. B) The rate of visits by *Bombus hortorum* to *Aconitum lycoctonum* racemes declines in response to 1) high nectar alkaloids (Linear regression: R-Sq(adj) = 0.27, $F = 5.8$, $P < 0.05$) and 2) three of the five most abundant alkaloids in nectar (Multiple linear regression: 8-*O*-methyllycoctonine, $t = -2.443$, $P < 0.001$; 8-*O*-methyllycaconitine, $t = -4.359$, $P < 0.01$; Alkaloid-1AL, $t = 2.70$, $P < 0.05$). *Related to Table S4*.

Figure 3. *Bombus terrestris* workers detect and avoid consuming solutions containing *Aconitum* spp. alkaloids. Individual bees were tested with 100 mM sucrose solutions containing 20 ppm of the most prevalent compound in the nectar of *A. lycoctonum* (8-*O*-methyllycoconitine, 8OM) or of *A. napellus* (aconitine, ACO) or 20 ppm of a mixture of alkaloids extracted from the galea of either species (AL = *A. lycoctonum,* AN = *A. napellus*). The y-axis indicates the relative response to a test solution compared to 100 mM sucrose (i.e. deterrence index). Bees found 8OM less repellent than ACO; the response to the solution containing ACO was not significantly different to the alkaloid mixtures for AL or AN (all $P > 0.05$). N_{suc} = 10/trt group, N_{8OM}= 8, N_{ACO} = 10, N_{AL} = 9, N_{AL} = 10. *Related to Figures S2 and S3.*

Table 1. Economics of nectar foraging on *Aconitum* spp. A) Mean abundance and energetic value of nectar in *A. lycoctonum* and *A. napellus*. B) Total ATP pay-off per population for a pollinator foraging on robbed flowers of *A. lycoctonum* and *A. napellus* in two years of observations. Values based on data in A. Mean vol/flower represents the amount accumulating over a 16 h period when bees were excluded and is a pooled measure from both nectar spurs per flower.

A)			%wt/vol (g/100ml)					
	Nectar present	Mean vol (μl)/flower	Total sugars	Gluc	Fruct	Suc	ATP/μl	ATP/flower
A.lyc	79%	1.97 ± 0.09	61.3	0	17.8	43.4	8.08*	15.9*
A. napel	48%	3.19 ± 0.49	63.0	0	11.4	51.6	8.35*	26.6*

B)	Year 1			Year 2		
	% larceny	Available to pollinator	Pay off (total ATP)	% larceny	Available to pollinator	Pay-off
A.lyc[#]	8	72.7%	1160*	41	46.6%	740*
A. napel	2	47.0%	1250*	25	36.0%	950*

The % of flowers available to pollinators was calculated by multiplying the number of flowers present with nectar by the % larceny. Pay-off values obtained by multiplying % flowers with nectar available to pollinators by the ATP per flower; * multiply by 10^{19}

STAR Methods

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Prof. Philip Stevenson (p.stevenson@kew.org).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study species

The study plant species were *Aconitum* aff. *napellus* L. and *A. lycoctonum* L. subsp. *neapolitanum* (Ten.) Nyman. Plants were established at Royal Botanic Gardens, Kew from European wild origin seed collections (RBG Kew references 1969-50099 and 1998-2073 SAUP respectively).

The wild bumblebee species observed visiting *Aconitum* flowers were *Bombus hortorum* (L.) and *Bombus terrestris* (L.) (nomenclature follows [35]). The former is a long-tongued species, and the latter is short-tongued and notable for its propensity to rob flowers with deep nectaries.

The bumblebee species used for bioassay in the laboratory were worker *B. terrestris audax* from colonies (Koppert NATUPOL, UK) kept at 25±2°C and 28±2% RH and fed *ad libitum* with honeybee collected pollen and sugar solution.

METHOD DETAILS

Bee-flower observations and raceme sampling

Bee visits to flowers were monitored manually by field observations and using a novel automated digital monitoring system, called Rana (Tumbling Dice Ltd, Newcastle upon Tyne, UK) (**Figure 1,** Movie S1) [36,37]. The Rana program uses motion and tuneable blob detection (computer vision) to detect small-scale movement, such as bees visiting flowers, while partly suppressing non-target extraneous events such as the effects of shadow and flower motion. Digital observations are combined into time-compressed, time-stamped movies (.avi) that are viewed and interpreted manually with video-editing

software (e.g. Virtualdub). Rana operates with a data logger (we used the Raspberry-Pi 1 Model A) and an autofocus camera (Logitech C525 720p HD webcams) and is tuned remotely via a standard web interface that, in this study, was accessed by USB tethering the Raspberry-Pi to a smartphone (Samsung Galaxy S5 smartphone). Throughout the flowering period, two Rana units were used to monitor a marked raceme from individuals of both *Aconitum* spp. for 1-3 days depending on flower senescence during clement weather between 07:00 and 20:00.

Manual observations were also performed for all racemes per individual and the numbers of insect visits per raceme were counted for 1-4 hours per observation day (typically 10:00-12:00 and 14:00-16:00) throughout the experimental period. Duplicate observations to marked racemes recorded manually and by Rana were identified by time record and accounted for within the final dataset.

For each insect flower visitor, behaviour was classified as: 1) legitimate nectar foraging i.e., insect entered the galea and accessed nectar; 2) attempted nectar robbery i.e., insect chewed the galea before abandoning flower; 3) nectar robbery i.e., insect chewed through the galea to reach nectaries; or 4) investigative visit only i.e., insect flew towards a flower and hovered but did not land or attempt to forage. To facilitate analysis, data were summarised as the relative number of visits per raceme per hour by the predominant legitimate pollinator (*B. hortorum*) and nectar robber (*B. terrestris*).

At the end of each 1-3 day monitoring period, marked racemes were bagged and collected the following morning. Nectar and galea samples were pooled from tens of flowers per raceme prior to chemical analysis. Final sample numbers reflect that, for some pooled samples, it was not possible to collect enough nectar for chemical analysis while some bee observation data was not available for corresponding analyses.

To calculate the incidence of nectar and the volume per flower in each year, we surveyed all flowers from up to 3 racemes from individuals of both species (Yr 1: AL n = 4, AN n = 11; Yr2: AL n= 7, AN n = 5). Racemes were bagged overnight and collected the following morning (16 h bagging period). Nectar was extracted from both nectary spurs using microcapillaries and pooled per flower.

Nectar and hood chemistry

Alkaloids were extracted from freeze dried corollas in 0.5M HCl for 24h (5% wt/volume) and partitioned into chloroform, evaporated to dryness and re-dissolved in methanol for isolation by HPLC using separation conditions described in [38]. Isolation was carried out on a Waters system (600E pump and996 PDA detector) using a Phenonmenex Luna column (150 mm 10 mm i.d., 10 µm lm particle size) with a gradient elution program based on A = MeOH and B = H_2O; A = 25% at t = 0 min, A = 100% at t = 20 min, and A = 100% at t = 40 min; column temperature 30°C and flow rate 4.7 ml/min. An extract from 19.5 g of *A. napellus* corollas yielded 14 mg aconitine and 70 g of *A. lycoctonum* corollas yielded 9.2 mg of lycaconitine and 4.2 mg 8-O-methyllycoctonine. Pure compounds were identified using Nuclear Magnetic Resonance spectroscopy (NMR). Spectra were acquired in $CDCl_3$ at 30˚C on a Bruker Avance 400 MHz instrument using instrument parameters described in [38]. Standard pulse sequences and parameters were used to obtain 1D ^{1}H, 1D ^{13}C, COSY (correlation spectroscopy), HSQC (heteronuclear single quantum coherence), and HMBC (heteronuclear multiple bond correlation) spectra. Chemical shift referencing was carried out with respect to internal TMS at 0.00 ppm. Aconitine and lycaconitine were determined by comparison with an authentic standard (JB Harborne) and published data [39,40] and quantified in nectar and corollas by LC-MS using calibration curves of pure compounds [41]. Nectar was recovered from nectaries using microcapillaries (10mu CamLab), weighed and diluted in 50mu methanol prior to analysis. HRESI-MS data were recorded using a Thermo LTQ-Orbitrap XL mass spectrometer (methods described in [38]). Sample introduction was via a Thermo Accela LC system performing chromatographic separation of 5µl injections on a Phenomenex Luna C18(2) column (150 mm X 3.0 mm i.d., 3 µm particle size) with a linear mobile phase gradient of 10–100% aqueous MeOH containing 0.1% formic acid over 20 min. Spectra were recorded in either positive or negative modes at 30,000 resolution. All other alkaloids were characterised by calculating molecular formulae from HR-MS and comparing with compounds

reported from *Aconitum* in the Combined Chemical Dictionary [14] (Table S1). Ionisation of the different alkaloids was not necessarily always equivalent in the ion source of the MS so non-metric multi-dimensional scaling (NMDS) was performed on absolute peak area integrations (Figure S1, Table S2). All other tests were carried out with alkaloid concentrations converted to ppm (=ug/g). e.g., total alkaloids in *A. napellus* and *A. lycoctonum* were determined using aconitine and lycaconitine respectively as representative compounds to estimate ecologically relevant concentrations for bioassay and in particular against mixtures of alkaloids for which molarity concentrations cannot be calculated accurately owing to the different molecular weights of the components (**Figure 2**, **Figure 3**, Figure S2, Figure S3, Table S3, Table S4).

Bee behavioural experiments

We used a gustatory sensitivity assay to measure consumption and behaviour of freely-moving bumblebees [15] to the nectar alkaloids of both *Aconitum* spp. (**Figure 3**, Figure S2, Figure S3). Briefly, individual worker bees (*B. terrestris audax*) were collected from colonies captured into a plastic vial with a perforated top. Prior to experiments, bees were placed in complete darkness at room temperature and deprived of food for 2 to 4 h, directly transferred to a holding tube (a modified 15 ml centrifuge tube with a 3 to 4 mm hole drilled at the tip and a metal mesh fixed inside the tube tip) and left for 3 min to acclimatize. A droplet (~3.5 µl) of 500 mM sucrose was presented at the tube tip via a female adapter connected to a syringe and the bee was allocated up to 5 mins to consume the droplet. Bees that did not consume this droplet were removed from the experiment. Once the bee extended its proboscis and consumed the sucrose drop, a 100 µl microcapillary tube that was preloaded with the test solution (approximately ¾ full) was presented to the bee. The 10 min observation period started when the bee contacted the test solution inside the microcapillary tube with its proboscis. The behaviour of the bee was recorded using a Dinolite AM4815ZT microscope camera attached to a laptop computer. Images of the microcapillary tube were taken before and after the test with the test solution using a computer scanner at 600 dpi to measure the amount of solution

consumed. The amount of solution consumed was measured using ImageJ (version 1.48). The behaviour of the bees recorded on video during the first 2 min of the test period was scored offline using the Noldus Observer for contacts with the solution and the duration of contact.

The test solutions were composed of 100 mM sucrose (Sigma-Aldrich, grade II, UK) and an extract of the total alkaloids from the galea of *A. napellus* or *A. lycoctonum* or the individual compounds, 8-*O*-methyllycoconitine, aconitine or lycaconitine, isolated from the original alkaloid mixture. The alkaloid extracts were diluted in 100 mM sucrose. For each round of experiments, a 100 mM sucrose control was included.

For all experiments, a 'deterrence index' was calculated for each subject from the volume of solution consumed during the test phase as follows: (average volume of 100 mM sucrose control per experiment – volume of test solution)/(average volume of 100 mM sucrose control per experiment + volume of test solution). This index was used to standardize the data to adjust for seasonal variation in the bees' relative response to the control solution.

QUANTIFICATION AND STATISTICAL ANALYSIS

The effect of species and plant part on total alkaloid concentrations (ppm) was analysed with two-way ANOVA using log transformed data undertaken in R [42]. A non-Metric Multidimensional Scaling analysis (nMDS) of alkaloid composition in the nectar and galeas were undertaken separately for each species (Figure S1, Table S2). Data were sqrt transformed prior to transformation using the Bray-Curtis similarity index (accepting a stress value < 0.2 [43]). For each species, plots of the first two nMDS axes were generated for exploratory purposes. PERMANOVA hypothesis tests using 9999 permutations were performed on these transformed datasets to test for significant differences in sample location in nMDS space (R, vegan package [44]). Correlations between alkaloid concentrations (ppm) in the

nectar and galeas of both species were analysed using Pearson's correlation coefficient tests in R [42] (**Figure 2**A, Table S3). Linear models and multiple linear regression models for the response of bee visits to nectar alkaloids were undertaken on log transformed data (**Figure 2**B1-2, Table S4). For multiple regression models, stepwise selection using forwards and backwards elimination of predictors resulted in the final models (R, MASS package [45]). Tests of bee responses to alkaloids in sucrose were performed using generalized linear models (GLM) with a linear or Poisson distribution in SPSS (**Figure 3**, Figure S2, Figure S3).

DATA AND SOFTWARE AVAILABILITY

The biological assay data supporting the results are deposited at: 10.6084/m9.figshare.5165350
Nectar and galea alkaloid data: 10.6084/m9.figshare.5165350

Bumblebee-alkaloid bioassay data: 10.6084/m9.figshare.5165350

SUPPLEMENTAL INFORMATION

Supplemental information includes three figures, four tables and one movie and can be found on-line at …..

Movie S1. Example of time-compressed video of bees visiting racemes of *A. napellus* and *A. lycoctonum* recorded using the Rana automated monitoring system. *Related to Figure 1.*