# Pyrrolizidine Alkaloids: occurrence in bee products and impact on honeybees (*Apis mellifera* L.)

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Degree of Doctor in Natural Sciences by

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Titre:

# "Pyrrolizidine Alkaloids : occurrence in bee products and impact on honeybees (*Apis mellifera* L.)"

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Neuchâtel, le 13 juillet 2017

Le Doyen, Prof. R. Bshary

*“The wing structure of the hornet,*

*in relation to its weight,*

*is not suitable for flight,*

*but he does not know this*

*and flies anyway.”*

Albert Einstein

1879 -1955

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# General summary

Pyrrolizidine alkaloids (PAs) are toxic plant secondary metabolites produced as defense against herbivores by a wide variety of plants, mainly of the Boraginaceae, Asteraceae and Fabaceae families. Consumption of products contaminated with PAs may lead to hepatotoxic, carcinogenic, genotoxic and teratogenic effects in animals and humans. PAs are mostly found in leaves, roots and seeds, and may directly enter the food chain via plant-derived food, such as teas or grains, or indirectly through the contamination of livestock fodder. Occasionally however, PAs are also found in honey or bee pollen when bees collect nectar and pollen from PA-producing plants. In plant pollen, the PA content is in some cases higher than the one in leaves, while little is known about the PA content of plant nectar. The presence of PAs in floral rewards raises two important questions. First, is nectar or pollen the main source responsible for PA contamination of honey? Second, what is the impact of these toxic secondary compounds on bee colonies? The aim of the present thesis is to examine these two questions using the European honeybee *Apis mellifera* L. and the viper bugloss *Echium vulgare* L. as a model system. *E. vulgare* is a common European plant producing copious pollen and nectar and it is an important resource for various bee species.

Research on the impact of PAs on honeybees and on bee products is a challenging field of research. PAs are a difficult class of toxins to investigate due to the large taxonomic diversity of PA-producing plants, the numerous known PA isomers, the lack of adequate reference material, and the absence of reliable standardized analytical methods. Therefore the extraction, isolation and purification of non-commercially available PAs from plants are research prerequisites.

Honeybees build large eusocial colonies relying on pollen and nectar, stored in the hive as food source. Workers collect large quantities of these floral resources, often mixing

numerous botanical sources, including *E. vulgare*. Pollen is stored in the hive as "bee bread", while honey is obtained from the concentration of nectar and contains only traces of pollen. Adult honeybees feed on these two products, which are the main sources of protein (bee bread) and carbohydrates (honey) for the entire colony. Unlike other bees however, honeybee larvae do not primarily feed on pollen and nectar: they are progressively fed by nursing bees, which consume large quantities of bee bread and produce protein-rich secretions with specific glands: the hypopharyngeal and mandibular glands. Indirectly, pollen is still the main source of protein for larvae and pollen secondary compounds, such as PAs, may potentially pose a risk for larvae when PA-producing plants are present near the hive. Therefore, examining the pathway of pollen and nectar secondary compounds in the hive is complex and implies analyses of pollen, nectar, bee bread, honey and, ideally, hypopharyngeal secretions.

The following doctoral thesis comprehensively investigates for the first time the route of the PA contamination from the plants to the honey, and the impact of pollen PAs on honeybees and honeybee larvae. It is divided into the following three chapters:

In chapter 1, we develop a protocol to facilitate the collection, extraction and LC-MS profiling of PAs from plant pollen from *E. vulgare* and *Eupatorium cannabinum*, two main PA-producing plants found in Switzerland. We also propose a method for extracting and isolating of large quantities of PAs from *E. vulgare* to obtain pure echimidine and echivulgarine for bioassays.

In chapter 2, we track the entry pathway of *Echium* PAs into honey. *Echium* species are suspected to underlie the high PA concentrations sometimes found in European honey, since these PA-containing honeys often contain trace amounts of *Echium* pollen. Since *Echium* pollen contains particularly high concentrations of PAs, it is currently assumed that *Echium* pollen is the main source of PA contamination in honey. Consequently, honey

filtration has been suggested as a measure to reduce PA levels in honeys derived from *Echium*. We compare the PA profiles of floral nectar and plant pollen of *E. vulgare* with the PA profile of honey produced at apiaries placed in the vicinity of *E. vulgare* plants. Our results strongly indicate that nectar is likely the main contributor to the PA content of honey, and not pollen as currently assumed. These findings have important implications for beekeeping practices and enable the formulation of guidelines to minimize PA levels in honeys.

In chapter 3, we comprehensively examine the effects of pollen PAs on the development of honeybee colonies by performing toxicological feeding assays with PAs on adult honeybees (*Apis mellifera*) and honeybee larvae. Our results show that *Echium* PAs are toxic to larvae at low concentrations, demonstrating for the first time that secondary compounds found in pollen have the potential to strongly impact bee development. In striking contrast, PAs at much higher, near realistic concentrations were tolerated by adult bees. However, since pollen is the exclusive source of protein for nursing bees, pollen PAs may still impact colony development if only a fraction of the PAs pass from the bee bread into the nursing secretions fed to larvae. To investigate this question we established a new method in which nursing honeybees were forced to feed on bee bread supplemented with known levels of PAs. Hypopharyngeal secretions produced by these bees were collected and analyzed. Surprisingly, only trace amounts of PAs were found in these secretions, demonstrating that nursing acts as a filter for some toxins in honeybee colonies. These results have two important implications. First, the honeybee is the most generalist bee species known. How this wide pollen spectrum is possible given the complex and diverse pollen chemistry has remained an unanswered question. Our results suggest that larval nursing relaxes the physiological constraints associated with pollen digestion. Second, various chemicals, such as pesticides, are likely to follow the same pathway from pollen and nectar into the hive.

Whether pesticides pass into the larval diet has not been investigated so far; our experimental system may be used to examine this important question.

In conclusion, this thesis brings two important answers. Firstly, despite its low PA content, plant nectar was found as the major vehicle of PAs into honeys, in contrast to the current hypothesis suggesting plant pollen as the major contributor of PA in honey. Secondly, we discovered a new feature deriving from the honeybee's eusocial behaviour. In fact, parental caring was found to play a key role for the protection of the future generations against plant secondary metabolites and therefore increase the chances of survival of the colony.

The thesis is the result of a collaboration between Unine and Agroscope. All bioassays were performed at the Agroscope Bee Research centre (Liebefeld), extraction and analytics of PAs were performed at Unine (Neuchâtel).

Keywords: Pyrrolizidine alkaloids, *Apis mellifera*, honey, nectar, pollen, *Echium vulgare*, collection, extraction, UHPLC-HRMS profiling, echimidine, hypopharyngeal secretions, honeybee larvae, ED50.

# Résumé

Les alcaloïdes pyrrolizidiniques (AP) sont des métabolites secondaires toxiques formés par une grande variété de plantes, notamment par celles de la famille des boraginacées, des astéracées et des fabacées, pour se défendre contre les herbivores. La consommation de produits contaminés par des AP peut avoir des effets tératogènes, génotoxiques, cancérigènes et hépatoxiques sur les animaux et les humains. Les AP se trouvent principalement dans les feuilles, les racines et les semences et sont susceptibles d'entrer directement dans la chaîne alimentaire par le biais des aliments d'origine végétale, comme les thés ou les céréales, ou indirectement par la contamination du fourrage destiné au bétail. Quelquefois, les AP peuvent également se trouver dans le miel ou le pollen lorsque les abeilles recueillent le nectar et le pollen des plantes produisant des AP. La teneur en AP est parfois plus élevée dans le pollen que dans les feuilles des plantes. Par contre, on sait encore peu de choses sur la teneur en AP du nectar des plantes. La présence d'AP dans les produits d'origine florale soulève deux questions majeures. Premièrement: le nectar ou le pollen sont-ils la principale source responsable de la contamination du miel par les AP? Deuxièmement: quel est l'impact de ces composés toxiques secondaires sur les colonies d'abeilles? La présente thèse a pour but d'examiner ces deux questions en utilisant l'abeille mellifère européenne *Apis mellifera* L. et la vipérine commune *Echium vulgare* L. comme système-modèle. *E. vulgare* est une plante européenne commune produisant une grosse quantité de pollen et de nectar et qui constitue une importante ressource pour différentes espèces d'abeilles.

Les recherches relatives à l'impact des AP sur les abeilles mellifères et les produits à base de miel représentent un domaine ambitieux. Les AP sont une catégorie de toxines difficile à étudier étant donné la grande diversité taxonomique des plantes produisant des AP, les nombreux isomères d'AP connus, le manque de matériel de référence approprié et

l’absence de méthodes d’analyse standard fiables. C’est pourquoi la recherche suppose au préalable l’extraction, l’isolement et la purification d’AP issus de plantes, non disponibles dans le commerce.

Les abeilles mellifères forment de larges colonies eusociales qui dépendent du pollen et du nectar stockés dans la ruche comme réserve alimentaire. Les ouvrières recueillent de grandes quantités de ces ressources florales, mélangeant souvent de nombreuses sources botaniques, parmi elles *E. vulgare*. Le pollen est stocké dans la ruche sous la forme d’un pain d’abeille, tandis que le miel est obtenu à partir de la concentration du nectar et ne contient que quelques traces de pollen. Les abeilles mellifères adultes se nourrissent de ces deux produits qui sont les principales sources de protéines (pain d’abeille) et d’hydrates de carbone (miel) de l’ensemble de la colonie. Contrairement aux autres abeilles, les larves de l’abeille mellifère ne se nourrissent pas de pollen et de nectar au début de leur développement: elles sont nourries progressivement par les abeilles nourricières, qui consomment de larges quantités de pain d’abeille et produisent des sécrétions riches en protéines à l’aide de glandes spécifiques: les glandes hypopharyngiennes et mandibulaires. Le pollen est la principale source de protéines et les composés secondaires du pollen, tels que les AP, peuvent représenter un risque potentiel pour les larves lorsque des plantes produisant des AP sont présentes à proximité de la ruche. C’est pourquoi l’étude du parcours suivi par les composés secondaires du pollen et du nectar jusque dans la ruche est complexe et suppose des analyses de pollen, de nectar, de pain d’abeille, de miel et idéalement des sécrétions hypopharyngiennes.

La thèse de doctorat présentée ici étudie pour la première fois de manière approfondie la voie de contamination par les AP depuis les plantes jusqu’au miel de même que l’impact des AP contenus dans le pollen sur les abeilles mellifères et leurs larves. La thèse est divisée en trois chapitres:

Dans le chapitre 1, nous avons développé un protocole pour faciliter la collecte, l'extraction et le profilage LC-MS des AP contenus dans le pollen d'*E. vulgare* et d'*Eupatorium cannabinum*, deux plantes majeures productrices d'AP en Suisse. Nous proposons également une méthode permettant d'extraire et d'isoler de grandes quantités d'AP provenant d'*E. vulgare* afin d'obtenir de l'échimidine et de l'échivulgarine pures pour les tests biologiques.

Dans le chapitre 2, nous examinons la voie d'entrée des AP issus d'*Echium* dans le miel. Les espèces d'*Echium* sont suspectées d'être à la base des fortes concentrations d'AP que l'on trouve parfois dans le miel européen, puisque les miels contenant des AP présentent souvent des traces de pollen d'*Echium*. Sachant que le pollen d'*Echium* contient des concentrations particulièrement élevées d'AP, l'hypothèse actuelle est qu'il est la principale source de contamination du miel par ce métabolite. Par conséquent, il a été suggéré de filtrer le miel pour réduire les teneurs en AP dans les miels provenant d'*Echium*. Nous avons comparé les profils AP du nectar de fleurs et du pollen des plantes d'*E. vulgare* aux profils AP du miel produit dans des ruchers situés à proximité de plantes d'*E. vulgare*. Nos résultats indiquent que le nectar est probablement le contribuant principal de la teneur du miel en AP et non pas le pollen, comme supposé jusqu'à aujourd'hui. Ces découvertes ont des implications importantes pour les pratiques apicoles et permettent d'établir des directives afin de minimiser la concentration en AP des miels.

Dans le chapitre 3, nous étudions de manière approfondie les effets des AP contenus dans le pollen sur le développement des colonies d'abeilles mellifères en effectuant des tests toxicologiques alimentaires avec des AP sur les abeilles mellifères adultes (*Apis mellifera*) et sur leurs larves. Selon les résultats obtenus, les AP d'*Echium* sont toxiques pour les larves en faibles concentrations. Il est ainsi démontré pour la première fois que les composés secondaires trouvés dans le pollen sont susceptibles d'influencer

considérablement le développement des abeilles. Le contraste est d'autant plus frappant que les AP en concentrations nettement plus élevées, proches des concentrations réelles, étaient tolérées par les abeilles adultes. Toutefois, sachant que le pollen est la seule source de protéines des abeilles nourricières, il suffit qu'une fraction d'AP passe du pain d'abeille dans les sécrétions nourricières destinées à l'alimentation des larves pour que les AP du pollen aient un impact sur le développement de la colonie. Afin d'étudier cette question, nous avons développé une nouvelle méthode dans laquelle les abeilles mellifères nourricières étaient obligées de se nourrir de pain d'abeille dont la teneur en AP était connue. Les sécrétions hypopharyngiennes produites par ces abeilles étaient ensuite recueillies et analysées. Seules des traces d'AP ont été trouvées dans ces sécrétions, ce qui est surprenant et prouve que l'acte nourricier agit comme un filtre pour certaines toxines dans les colonies d'abeilles mellifères. Ces résultats ont des implications majeures. Premièrement, l'abeille mellifère est l'espèce d'abeille le plus généraliste que l'on connaisse. Comment ce large spectre de pollen est-il possible étant donné la complexité et la diversité chimiques du pollen ? C'est une question qui reste en suspens. Nos résultats suggèrent que l'alimentation des larves réduit les contraintes physiologiques associées à la digestion du pollen. Deuxièmement, différents produits chimiques, tels que les pesticides sont susceptibles de suivre le même chemin en passant du pollen et du nectar à la ruche. Actuellement, aucune étude n'a été faite pour savoir si les pesticides passaient dans le régime alimentaire des larves; notre système expérimental pourrait servir à étudier cette question importante.

En conclusion, cette thèse apporte des réponses cruciales. Premièrement, en dépit de sa faible teneur en AP, il s'est avéré que le nectar des plantes était le vecteur majeur des AP dans le miel, contrairement à l'hypothèse actuelle qui veut que le pollen des plantes soit le contribuant essentiel des AP dans le miel. Deuxièmement, nous avons trouvé une nouvelle

caractéristique dérivée du comportement eusocial des abeilles mellifères. En effet, nous avons découvert que les soins parentaux jouaient un rôle clé dans la protection des générations futures contre les métabolites secondaires des plantes et que par conséquent, ils augmentaient les chances de survie de la colonie.

La thèse est le résultat de la collaboration entre l'Unine et Agroscope. Tous les essais biologiques ont été réalisés au centre de recherche apicole d'Agroscope (Liebefeld), l'extraction et l'analyse des AP ont été effectuées à l'Unine (Neuchâtel).

Keywords: Alcaloïdes pyrrolizidiniques, *Apis mellifera*, miel, nectar, pollen, *Echium vulgare*, collecte, extraction, profilage UHPLC-HRMS, echimidine, sécrétions hypopharyngiennes, larves d'abeille, ED50.

# Introduction

Plants and animals often evolve defense strategies to increase their survival and reproduction. The production of toxic secondary metabolites, such as pyrrolizidine alkaloids (PAs), represents a widespread and efficient mechanism evolved by plants to reduce herbivory.[1,2,3] PAs have been identified in about 3% of the world's flowering plants.[4] They mainly belong to three plant families: Boraginaceae (all genera, e.g. *Echium* spp.), Asteraceae (tribes Senecioneae and Eupatorieae) and Fabaceae (mainly the genus *Crotalaria*).[7] More than 95% of the PA-producing plants investigated thus far belonged to these families[1] and more than 660 different PAs are currently described in the literature[5].

## Structure of PAs

PAs are ester alkaloids with polar basic characteristics. They consist of a necine base and one or more necinic acids (Figure 1). The necine base is composed of two heterocyclic rings carrying a nitrogen group, that can either be 1,2-unsaturated or saturated. Four main types of necine bases occur naturally in PAs: platynecine (usually non-toxic), retronecine, heliotridine and otonecine (Figure 2A).[6] Necinic acids are short or long carbon branched chains bearing various substituents (hydroxy, methoxy, epoxy, carboxy, acetoxy etc.), creating numerous structural stereo- and diastereoisomers.[7,8] One or more necinic acids are connected through ester bound to the necine base in position 1- and/or 7- to form monoesters, open-chain diesters or macrocyclic diesters (Figure 2B). Through chemical modifications of both necine base and necinic acids, a wide variety of PA structures can be produced by plants. PAs are classified in six main groups: senecionine type, triangularine type, lycopsamine type, monocrotaline type, phalaenopsine type and miscellaneous PAs.[7] The lycopsamine type represents the largest group of PAs known with more than one hundred different structures, and it is composed of mono- or diesters containing at least one

hydroxylated 2-isopropylbutyric acid (e.g. lycopsamine, Figure 2B). Senecionine type PAs are macrocyclic diesters that are derived from, or structurally similar to senecionine (Figure 2B). The essential structural features for toxic PAs are: the 1-2 double bond in the necine base, the presence of one or two hydroxyl groups in C-7 and/or C-9 position, the esterification of at least one of the hydroxyl groups in the necine base, and the esterification of the hydroxyl group(s) with branched mono- or dicarboxylic acid(s). PAs occur in nature as free bases (tertiary PAs) or N-oxides (Figure 4).[9] N-oxides are considered as pre-toxic forms of PAs because they are more soluble and can be easily excreted. However, upon ingestion, PA-N-oxides can be converted in the digestive tract to the corresponding tertiary forms which are toxic.[7],[10,11]

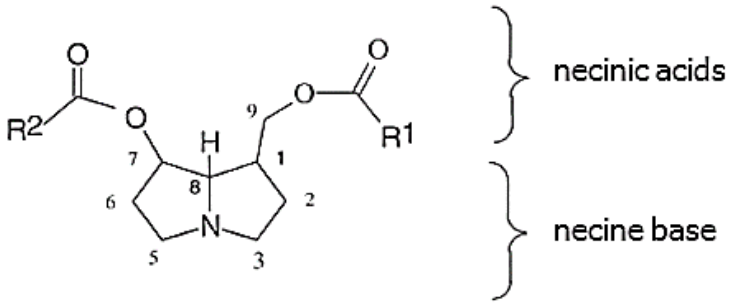


**Figure 1** Structure of a pyrrolizidine alkaloid (reproduced from Roeder *et al*. 2000).[12]

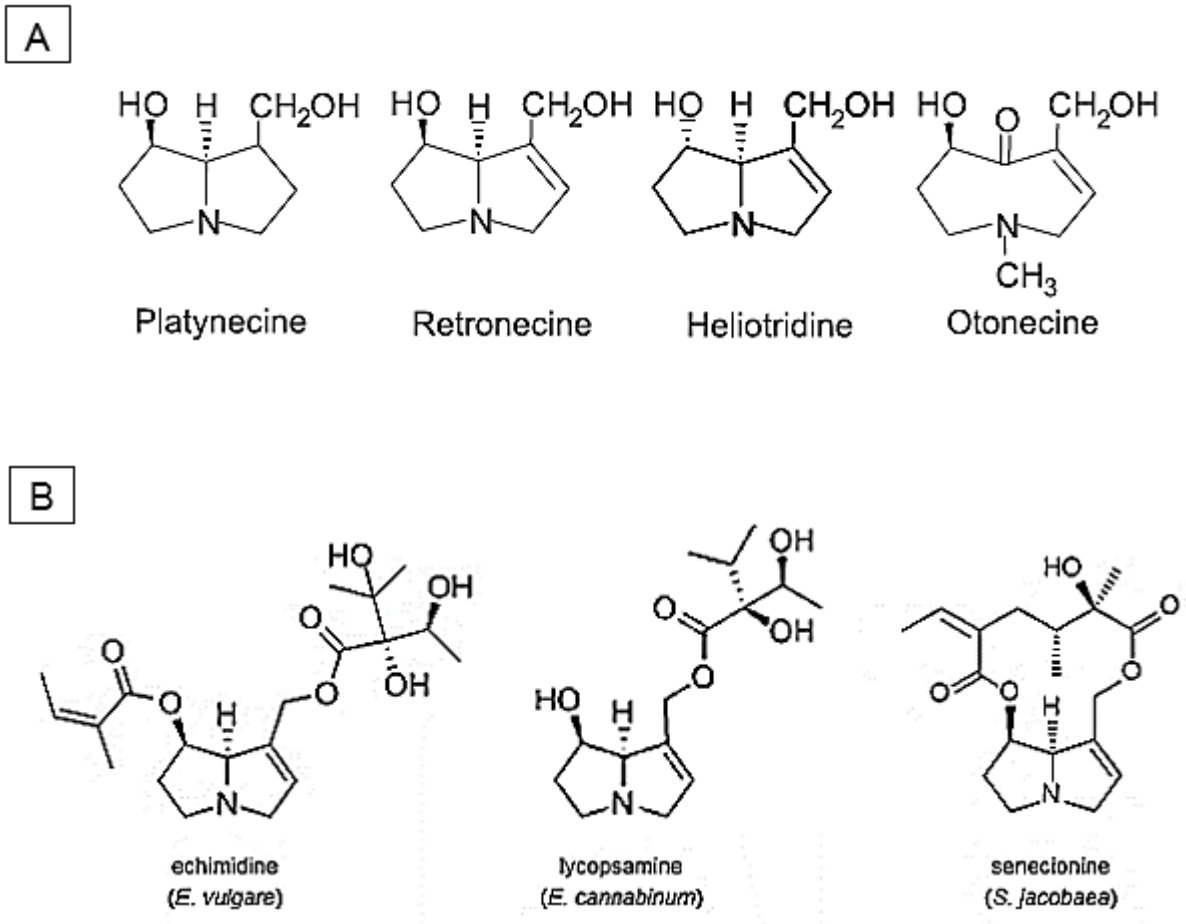


**Figure 2 A**) Necine bases: Platynecine (e.g. platyphylline, senecionine type PA). Retronecine: majority of the senecionine, triangularine, lycopsamine and monocrotaline type PAs, including PAs identified for example in *E. vulgare* (echimidine, echivulgarine, both lycopsamine type PAs), *Eupatorium cannabinum* (lycopsamine, intermedine, both lycopsamine type PAs) or *Senecio jacobaea* (senecionine, senecionine type PAs). Heliotridine (e.g. rinderine, lycopsamine type PA). Otonecine (e.g. senkirkine, senecionine type PAs). Structures of the necine bases are reproduced from Fu *et al*. 2004.[13] **B**) Examples of PAs from *Echium vulgare*: echimidine (open-chain diester alkaloid); *Eupatorium cannabinum*: lycopsamine (monoester alkaloid); *Senecio jacobaea*: senecionine (macrocyclic diester alkaloid). Echimidine structure is reproduced from www.chemfaces.com, lycopsamine and senecionine structures from Stegelmeier *et al*. 2016).[14]

## Biosynthesis of PAs

PAs are produced by the secondary metabolism of plants. They are synthesized starting from amino acids and a single PA-producing plant can produce different types of PAs. Early studies on the biosynthesis of PAs were made using carbon isotope labeling of candidate precursors.[15-18] Putrescine was found as a common precursor of the necine base of the majority of PAs, but its origin varies between plant families. In fact, putrescine can derive either from L-ornithine (e.g. genus *Crotalaria*) or L-arginine (e.g. genus *Senecio*). Two putrescines are usually fused for the synthesis of one molecule of homospermidine which is then cyclized in various steps to form the necine base (Figure 3). Necinic acids are mainly derived from L-valine, L-leucine, L-isoleucine and L-threonine, and synthesized from different pathways.[19-22] In nature, PAs are synthesized as soluble N-oxides in the roots (ex. *Symphytum*) or in the shots (ex. *Heliotropium*) of the plant, and they are translocated to other compartments and generally stored in the vacuoles.[23]

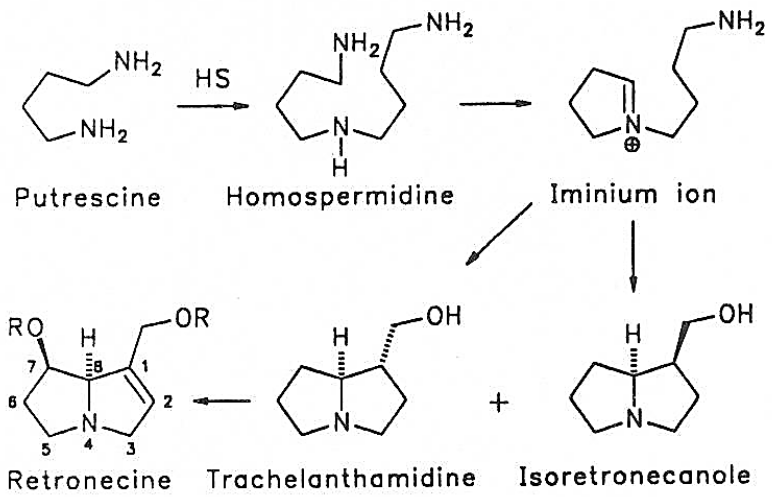


**Figure 3** Synthesis of the PA necine base (reproduced from Roeder *et al.* 2000).[12]

## Toxicity and metabolism of PAs

Several food products from various geographical origins were found to contain PAs in different amounts. Exposure to PAs through food may either be caused by the intentional consumption of the plant (e.g. *Petasites* and *Symphytum* consumed as vegetables) or by accidental consumption of food containing parts of PA-producing plants, such as roots, aerial parts or seeds.[24] The first recorded instance of PA poisoning in humans was occurred in 1920 in South Africa when many people in the Western Cape suffered from liver cirrhosis after eating bread made with wheat that was probably contaminated with seeds of *Senecio burchellii.*[25] To date, the largest reported outbreak of human intoxication by PAs was in Afghanistan in 1974 when an estimated 35'000 people were affected after grains were contaminated with *Heliotropium* seeds. Among the 7'200 cases examined, 1'600 were affected and many died 3-9 months after the onset of clinical signs.[26] Other intoxication events caused by PA-containing seeds and plant parts mixed with grains have been reported from Asian countries.[27,28] Consumption of contaminated food containing high concentrations of PAs can lead to toxic acute effects mainly affecting the liver, and in worst cases lead to death.[29] Additional damage can occur to the lungs, blood vessels, kidney, pancreas, guts, bone marrow and brain.[30] The lowest known dose causing acute toxicity in humans was 3 mg PA/kg b.w. per day (exposure of a boy for 4 day-period, lethal outcome) and 0.8 -1.7 mg PA/kg b.w. per day (exposure of a girl for a 2 week-period, HVOD)[31] In Europe, episodes of acute intoxications are nowadays rare thanks to the implementation of agricultural technologies avoiding contamination of flour with PA-containing seeds.[31]

While acute toxicity is relatively rare, the risk of sub-acute and chronic intoxications is still of considerable concern for the European population and worldwide. The risk is mainly correlated to the consumption of herbal remedies obtained from PA-producing plants, teas and bee products, such as honey and bee pollen.[32] These products usually contain low

levels of PAs; however, long-term consumption of various contaminated products can lead to chronic exposure.[31] Potential genotoxic and tumorigenic effects of PAs were observed from *in vitro* and *in vivo* studies on eukaryotic model systems.[33,34]

The action mechanism of PAs in mammals is well known. PAs are absorbed from the gastrointestinal tract and transported to the liver where they get metabolized into toxins. 1,2-unsaturated PAs are oxidized by hepatic cytochrome P450 enzymes into dihydropyrrolizine esters, and react as nucleophiles with nucleotides and proteins, forming adducts that cause structural modifications and damages (e.g. DNA modifications) (Figure 4).[35,36] Only a small fraction of the ingested PAs can be detoxified by the enzyme esterase through hydrolysis of the ester bound connecting the necinic acids to the necine base, while the majority undergo bio-activation. N-oxides, because of their higher water-solubility, may be easily excreted, however, since they are reduced in the gut by the bacterial flora into tertiary PAs after ingestion they are considered as pre-toxic forms.[35,36]

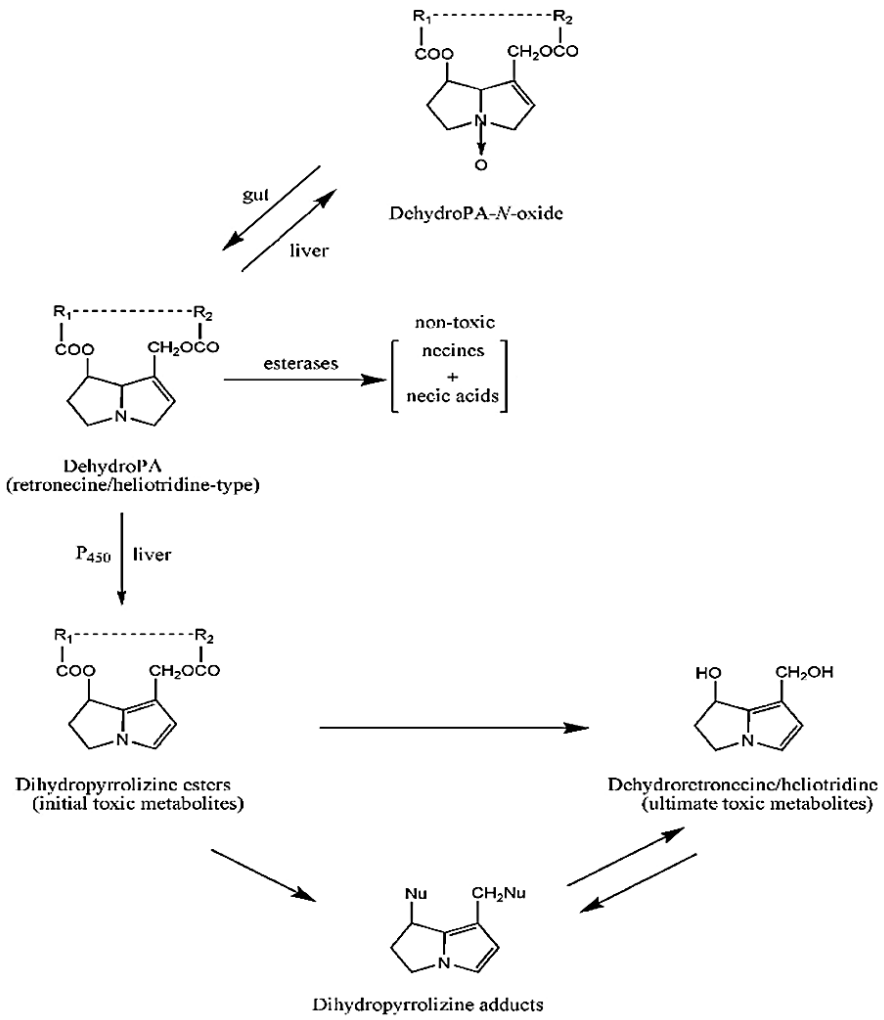


**Figure 4** Mechanism of bioactivation and detoxification of PAs in vertebrates (reproduced from Edgar *et al*. 2011).[37]

## PA-producing plants in Europe

PA producing plants occur worldwide in almost every habitat and exhibit different traits (annual, perennial, invasive etc.). To date, more than 660 PAs and their respective N-oxides have been identified from approximately 6'000 angiosperm plant species.[38] *Echium* spp., *Senecio* spp. and *Eupatorium* spp. are important PA-producing plant species in the European agro-ecosystem as they represent common PA sources of food and feed contamination, potentially posing a risk for animal's and human's health. Previous studies

have shown that PAs of *Echium* spp. are the main source of contamination in European honey, followed by PAs of *Eupatorium* spp. and *Senecio* spp..[39-41]

## *Echium* spp. (Boraginaceae)

**Figure 5** Flowering plants of *Echium vulgare* (Photo: Verena Kilchenmann)

*Echium* is a genus of 60 species belonging to the Boraginaceae family. They usually grow as wild plants of various suitable climate zones throughout the world. A multitude of *Echium* species produce toxic PAs (*E. angustifolium, italicum, plantagineum, pininana, sericeum, vulgare* etc.).[7] *E. vulgare* (Viper Bugloss, Figure 5) is the main *Echium* species in Switzerland and, together with *E. plantagineum* (Patterson's Curse), in European ecosystems. Both species produce lycopsamine type PAs, mainly echimidine and echimidine-derived isomers (Figure 2B). PAs found in *E. vulgare* have been well characterized before.[42,43] Typical PAs of *E. vulgare* are echimidine, acetylechimidine, echivulgarine, vulgarine and acetylvulgarine. PAs from *E. vulgare* are frequently detected in

European honeys.[39-41] In fact, this plant is known to produce copious amounts of nectar and pollen that are harvested from bees between May and September when the plant is flowering.

## *Eupatorium* spp. (Asteraceae)

**Figure 6** Flowering plants of *Eupatorium cannabinum* (Photo: Ruedi Ritter).

*Eupatorium* is a common genus of the Asteraceae family. This genus contains up to 60 species depending on the classification system. Although many *Eupatorium* species are poisonous to humans and grazing livestock due to the production of PAs (such as *E. perfoliatum*, *E. cannabinum* and *E. fortunei*), they have been frequently used in folk medicine.[44] Within this genus, *E. cannabinum* (Figure 6), also known as Hemp Agrimony, is a native plant species in European ecosystems. This species is perennial and blooms from July till early September. *E. cannabinum* produces lycopsamine-type PAs; however, little is known about the pattern of PAs produced by this plant. Various sources report lycopsamine

(Figure 2B), intermedine, supinine, echinatine, their beta-angelyl/tiglyl esters and their isomers as main PAs produced by this plant, together with other minor PAs.[8,45-47]

## *Senecio* spp. (Asteraceae)

**Figure 7** Flowering plants of *Senecio jacobaea* (Photo: Ruedi Ritter).

Within the Asteraceae family, 1'200 species of *Senecio* are currently recognized worldwide. In the past years, an increase of some *Senecio* species has been observed in Central Europe.[29] *Senecio inaequidens*, an invasive plant, is rapidly spreading in Europe and posing a health concern.[48,49] Another plant, *S. jacobaea* (Figure 7), also known as *Jacobaea vulgaris* or tansy ragwort, was often found as a contaminant of foods and feeds,[29] and its geographical distribution is extending due to climate change.[50] *S. jacobaea* blooms from June to November, and its pollen is morphologically similar to the majority of the other Asteraceae, such as *E. cannabinum*. PAs produced by this plant generally derive from senecionine-N-oxide (senecionine type PAs), synthesized in the roots.[51] Once it is

transported through the phloem, senecionine-N-oxide is converted into several related PAs. The pattern of PAs produced from senecionine-N-oxide differs between *Senecio* species.[52,53] Generally, PAs from *Senecio* are macrocyclic diesters, such as senecionine (Fig. 2 C). From the WHO report EHC 80,[54] the PAs which have been confirmed in *S. jacobaea* are senecionine (Figure 2B), jacobine, jaconine, jacozine and seneciphylline. However, other PAs were claimed to be present, such as erucifoline, integerrimine, riddelline, senecivernine, together with other minor PAs.[8,55]

## PAs and honeybees

Honeybees are insects of great economic importance thanks to their fundamental role as pollinators of flowering plants, and additionally, for the production of honey, pollen, wax, propolis and other bee products. Honeybees rely on nectar as source of carbohydrate and plant pollen as protein source. Due to their foraging activities, bees are exposed to secondary metabolites when they collect these products from PA-producing plants. Moreover, some of these plants, such as *E. vulgare*, are frequently visited by honeybees. Only a few toxicological studies of PAs on adult honeybees have previously been conducted.[56,57] In 1993, Detzel and Wink for the first time tested heliotrine on honeybees.[56] Different test series with different concentrations of heliotrine were tested, from 0.005% to 1%. A 48 hours ED50 for heliotrine was calculated as 0.1%, highlighting for the first time the potential adverse effects of PAs on honeybee's health. Later in 2009, Reinhard and colleagues tested a mixture of PA tertiary bases and a mixture of PA-N-oxides from *Senecio vernalis* on honeybees, separately.[57] N-oxides mixture showed no relevant toxic effects on bees, while after 48 hours the mixture of tertiary bases killed more than 50% of the bees at concentration of 2%. It was demonstrated once more the toxicity of PAs on adult bees at relatively high concentrations. Particularly, N-oxides showed a lower toxicity than tertiary bases. The same research group also tested the toxic difference between monocrotaline

and 1,2-dihydromonocrotaline.[57] Monocrotaline provided at 2% in sucrose solution was highly toxic for bees while 1,2-dihydromonocrotaline showed non-toxic effects, demonstrating that the double bound in position 1,2 of the necine base is a key element for the toxicity of PAs. As opposed to some species of solitary bees being able to cope with PAs,[58] generalist honeybees do not seem to have developed any strategy to front PAs in floral rewards.[59] Nevertheless, PAs may pose a risk to honeybees, when PA-producing plants are the only available source of pollen and nectar in the environment. Pollen particularly may pose a risk for honeybees' health due its high PA content.[38,43] Moreover, pollen is an important protein source used by honeybees to produce hypopharyngeal secretions as nourishment for larvae. Consequently, pollen containing PAs may also pose a risk for larvae's health, although indirectly. No studies testing this eventuality and no toxicological studies of PAs on honeybee larvae are reported in the literature. In conclusion, more studies are required to understand the impact that PAs have on a honeybee colony development and to understand if honeybees are capable to cope with PAs in natural condition and how.

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# Aim of the study

## Chapter 1 - Isolation of PAs from *E. vulgare* and PA profiling in plant pollens

As tool for plant and food research, we provide guidelines to facilitate the collection, extraction and LC-MS profiling of PAs from plant pollen from *E. vulgare* and *E. cannabinum*, two common European PA-producing plants visited by honeybees. Moreover, to compensate the lack of standards, we extracted various PAs from plants to be used as analytical standards and as test compounds in the toxicological bioassays presented in chapter 3 (See Appendix 1).

## Chapter 2 - Origin of PAs in honey

In order to determine the pathway of PAs from floral rewards into honey, we compared the PA content and the PA concentration and profile in floral nectar and plant pollen of *E. vulgare* to the concentration and profile found in honeys harvested from apiaries placed in the vicinity of blooming plants of *E. vulgare*. Results from this chapter enable the formulation of guidelines to allow beekeepers to minimize PA levels in their honey production.

## Chapter 3 - Toxicological studies of PAs on honeybee adults and larvae

In the last chapter we performed feeding assays with PAs extracted from *E. vulgare* (see Appendix 1), on honeybee adults and larvae (*Apis mellifera*). We also evaluated the transfer of PAs into the secretions used by nurse honeybees to feed the larvae in a newly developed experimental set-up. Taken together, the data obtained are important to understand the risk of pollen PAs to bee colonies and to identify possible mechanisms evolved by bees to overcome toxicity.

# CHAPTER 1

# Pyrrolizidine alkaloid profiling in plant pollen by UHPLC-HRMS

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**Abstract**

Many plant species produce pollen-containing PAs that honeybees collect and bring as a protein source into the beehive. Methods for collection of PA containing pollen, together with the extraction and analysis of PAs, are important tools for plant and food research. Several procedures are described in the literature for the analysis of PAs. Mainly two approaches are commonly used: In the first approach, the total PAs (N-oxides and free-bases) are analyzed as the sum of the necine bases by gas chromatography coupled with mass spectrometry (GC-MS). The second approach is based on liquid chromatography coupled with mass spectrometric detection (LC-MS). This method can detect both the free alkaloids

and their N-oxides simultaneously and has become the method of choice in recent years. However, due to the lack of standards on the market and the multitude of PAs discovered so far, the detection, identification and quantification of PAs represents a continuous challenge. In the following chapter, methods for the collection, extraction and profiling of PA plant pollen from *Echium vulgare* and *Eupatorium cannabinum* are presented.

**Keywords:** Pyrrolizidine alkaloids, *Echium vulgare*, *Eupatorium cannabinum*, pollen, collection, extraction, UHPLC-HRMS profiling, echimidine, lycopsamine, intermedine.

## Introduction

Pyrrolizidine alkaloids (PAs) are secondary metabolites produced by some plants as a defense mechanism against herbivores and phytophagous insects. They can occur as N-oxides or free-bases/tertiary PAs (Figure 1). In plants, the N-oxides are often found in higher concentrations as compared to the corresponding free-bases/tertiary PAs.[1,2] PA containing plant species mainly belong to the families of Boraginaceae (all genera), Asteraceae (mainly genera of Senecioneae and Eupatorieae), Fabaceae (mainly genus of *Crotalaria*) and Apocynaceae.[2] PAs can get into bee products, such as honey and pollen, when bees collect nectar or pollen of PA containing plants. PAs have been detected in plant pollen.[3,4] High concentrations of PAs have also previously been reported in bee collected pollen[4,5] presenting a food safety concern for the consumers of pollen as a nutritional supplement. PAs are classified as esters of hydroxylated methyl pyrrolizidines, consisting of a necine base (1,2-saturated or unsaturated) and one or more necinic acids.[2] Esters of 1,2-unsaturated retronecine- and otonecine-type PAs are toxic for humans and animals.[6,7] The structural diversity of more than 400 known PAs[8,9] represents an analytical challenge, and no standardized method has been established so far to determine the PA content in bee products. Many PAs are difficult to quantify, due to the lack of standard materials. Two main analytical approaches are commonly used: a sum parameter GC-MS method[4,8,10] and a

targeted LC-MS method allowing the individual identification of PAs and PA-*N*-oxides.[3,8,11] The GC-MS method covers most PAs, except the otonecine-type PAs, but derivatization is required and structural information of the original PAs is lost. On the other hand, the targeted LC-MS method identifies individual PAs, but unknown PAs are not detected and the chromatographic separation of many PA isomers is also not easily obtained. To at least partially overcome these limitations, ultra-high pressure liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS) as a way to profile both known and unknown PAs can be used.[12] Compared to conventional HPLC, UHPLC enables a more rapid separation with higher resolution and is more efficient at separating closely related molecules, e.g. positional isomers.[13] UHPLC relies on the use of smaller particles (sub-2µm) and instrumentations able to resist higher pressure, typically up to 1000 or 1500 bars. HRMS systems, such as time-of-flights (TOF) or electrostatic trap (Orbitrap™) represent an attractive way to record data from UHPLC separation at high frequency and in a non-targeted manner. HRMS measures ions with high mass and spectral accuracies and allows for the determination of elemental compositions, which in turn may assist the identification of unknowns. Furthermore, recent instruments are fully compatible with quantitative or semi-quantitave analysis.[14] Here we describe a UHPLC-HRMS-based method for the profiling of PAs in plant pollen from *Echium* and *Eupatorium* species, which may be applied to other plant genera with minor adaptation.

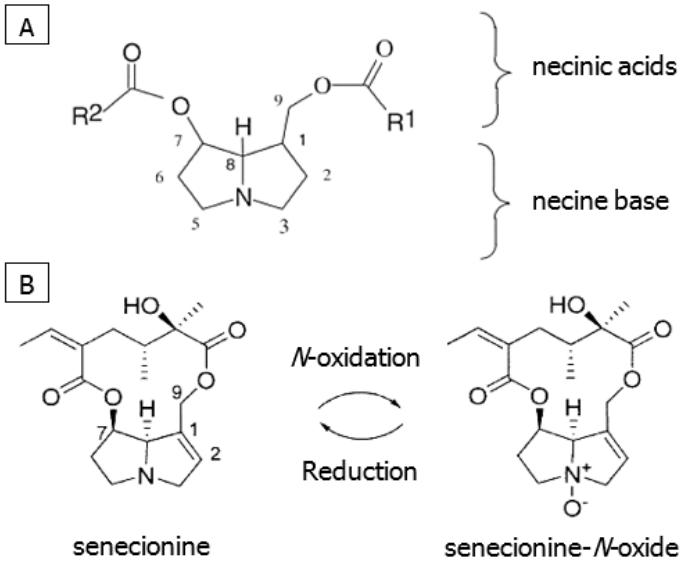


**Figure 1 A)** Structure of PAs: Necinic acids are connected to the necine base by ester bond in position 1 and/or 7. Necinic acids can be bound to form a macrocyclic ester.[15] **B)** Example of PA free base and *N*-oxide macrocyclic esters.[16]

## Materials and Methods

### Equipment and Chemicals

Steel-made forceps were purchased from A. Dumont (#0208-55-PO), 1.5 mL and 2 mL tubes from Eppendorf (#0030120086, #0030120094, respectively), 2 mL glass LC-MS tubes (screw top) from Kinesis (#KVP6112), polystyrene Petri dishes (Ø 10 cm) from Greiner (#663102), glass beads (Ø 2 mm) from Sigma-Aldrich, nitrile gloves (powder free) from Kimberly-Clark (#52003M), cyclohexane SupraSolv (#102817), methanol SupraSolv (#106011) and formic acid for Analysis Emsure (#100264) from Merck, ultra pure water was obtained from a Millipore system, Bedford, MA, USA. Echimidine (CAS #520-68-3) and lycopsamine (CAS #10285-07-1) were purchased from Phytolab (#89553 and #89726, respectively). Acetonitrile ULC/MS (#012041) and Water ULC/MS (#232141) were purchased from Biosolve.

## Collection of plant pollen from PA-containing plants

It is necessary to adapt the collection technique for pollen to the type and shape of the flower. We present two examples: collection of pollen from *E. vulgare* (Boraginaceae) and from *E. cannabinum* (Asteraceae).

## Pollen collection from *E. vulgare*

Plant pollen from *E. vulgare* (Figure 2), located as blue/violet dust on the anthers of the flower can be collected using steel-made surgery forceps or using pure cyclohexane. Rainy days are not recommended for collection, since pollen can be washed out from the anthers.

1. Verify that most of the flowers are well developed and fully open.
2. Tightly bag the plants on the field with fine mesh nets to avoid the pollen harvest from insects (at least one day before the collection) (Figure 3).
3. Spread a layer of paraffin around the lower part of the stem to avoid non-flying insects from climbing the plant to collect pollen.
4. Plant pollen can be collected two days after bagging the plant and is stored at -20°C until extraction. It is suggested to bring a cooler box container filled with dry ice onto the field in order to place the freshly collected samples immediately on dry ice.

## Collection with forceps

1. Wear Latex gloves during the collection.
2. Using forceps, scratch the pollen from the surface of the anthers of the flowers, carefully avoiding PA contamination by the stamen tissues.
3. Collect the pollen using pre-weighted 1.5 mL tubes. Collect 1 mg of pollen in each tube.
4. Insert the forceps carefully inside the tube and release the pollen on the walls of the vial. Try to avoid any electrostatic charge of the tube that could compromise the collection.

**Collection with cyclohexane**

1. Remove the stamens from the flower.
2. Immerge the stamens in a pre-weighted LC-MS glass tube containing cyclohexane.
3. Shake the stamens delicately into the cyclohexane to release the pollen.
4. If possible bring the tubes back to the laboratory to evaporate the cyclohexane under laminar-flow hood. Determine the weight of the dry pollen.
5. Store the dry pollen at -80°C until extraction.

Pros: This technique allows for a faster collection of pollen when compared to the method by forceps. Both collection methods give similar concentrations of PAs as we have determined in our laboratory.

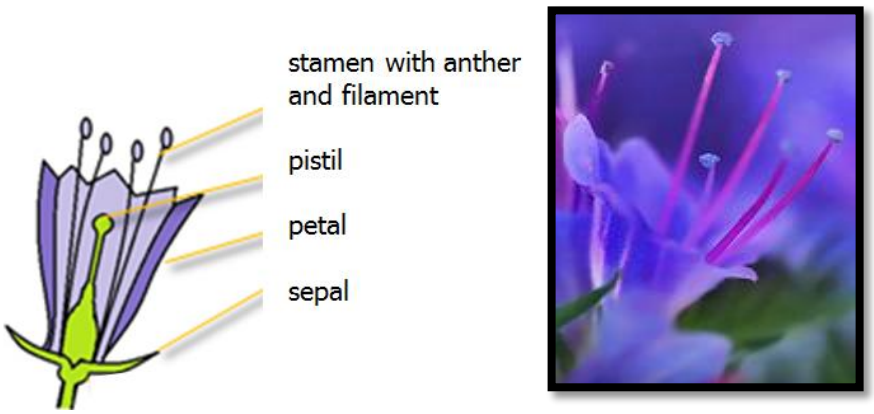


**Figure 2** Structure of a flower of *Echium vulgare*.

**Figure 3** A plant of *E. vulgare* bagged with a net supported by a metal structure.

### Pollen collection from *E. cannabinum*

Plant pollen from *E. cannabinum* (Figure 4), located along the stamens, cannot be collected using forceps due to the small dimensions of the tubular floral units (approximately 7 mm).

### Collection on Petri dishes

1. Bag the plant and apply the paraffin layer on the lower part of *E. cannabinum* (Figure 5).
2. After two days, shake the entire floral head of *E. cannabinum* over a Petri dish in order to collect the pollen that is released by this procedure.
3. Remove the impurities from the dish using forceps.
4. Collect the pollen into a pre-weighted 1.5 mL tube
5. Store the tube at -80°C until extraction.

Pros: This method allows for a rapid collection of a high amount of pollen from *E. cannabinum*.

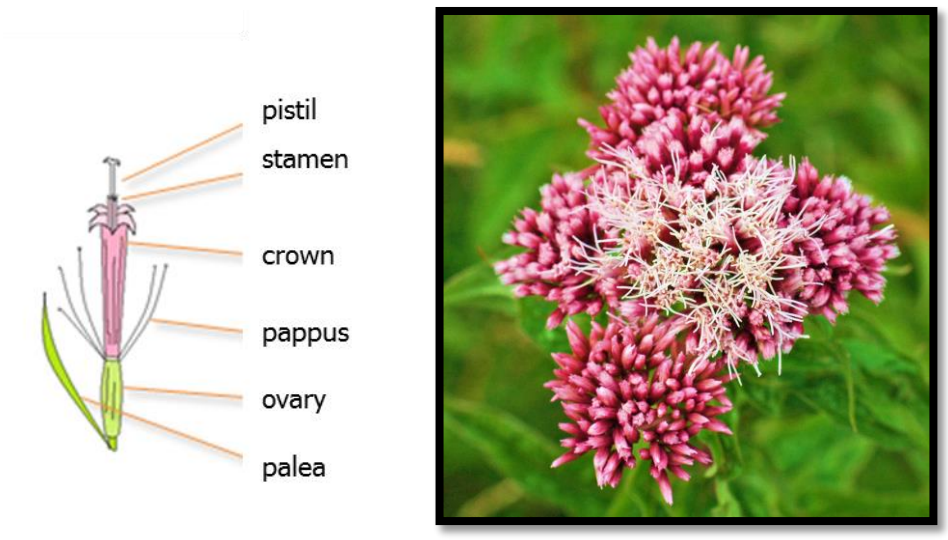


**Figure 4** Structure of a floret and a floral head of *Eupatorium cannabinum.*

**Figure 5** An *E. cannabinum* plant bagged with a net.

**Extraction of PAs from plant pollen**

PAs are polar organic compounds with basic characteristics. They are soluble in polar organic solvents or in mixtures of solvents and acidified water.

1. Transfer 1 mg of plant pollen in a pre-weighted 2 mL microcentrifuge tube using a metal spatula.

2. Dissolve the pollen in 100 µL of extraction solvent consisting of 70% methanol, 29.5% ultrapure water and 0.5% formic acid.
3. Add 4-8 glass beads to the tube.
4. Shake the tube at 30.0 Hz for 4 minutes.
5. Centrifuge the tube at 18407 *g* for 4 minutes.
6. Collect the supernatant (extract) in a new tube.
7. Transfer with a pipette 5 µL of the extract and dilute 5-20 times with the extraction solvent into a glass LC-MS vial containing a conical glass insert.

Pros: This technique can be used for extracting plant pollen from many plant genera.

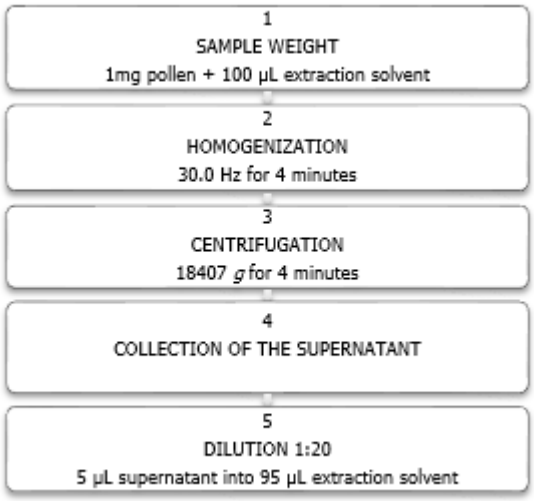


**Figure 6** Scheme illustrating the steps of PA extraction from plant pollen.

**Profiling of PAs in plant pollen with UHPLC-HRMS**

Non-targeted analysis using LC-HR-MS system allows the detection of alkaloids found in *Echium vulgare* (echimidine/-N-oxide, vulgarine/-N-oxide, acetylechimidine/-N-oxide, acetylvulgarine/-N-oxide and echivulgarine/-N-oxide) and alkaloids found in *Eupatorium cannabinum* (intermedine/-N-oxide, lycopsamine/-N-oxide). Separation of the alkaloids is performed using an Acquity UPLC (Waters). The UPLC system is coupled to a Synapt G2

QTOF mass spectrometer (Waters). An Acquity BEH C18 column (50x2.1 mm i.d., 1.7 um particle size, Waters) fitted with guard column (5x2.1 mm) of identical phase is utilized. The column is maintained at 30°C and a binary gradient of separation is performed at a flow rate of 0.4 mL $min^{-1}$. The mobile phase consists of 0.05% formic acid in water (solvent A) and 0.05% formic acid in acetonitrile (solvent B). The gradient program used is: 0-4 min 5-40% B, 4-6 min 40-100% B, 6-9 min 100% B, 9.1-10.5 min 5% B. Injection volume is 1 µL. The autosampler needle is washed with methanol/acetonitrile/isopropanol (1:1:1), followed by 0.05% formic acid in water between injections to eliminate carryover. The QTOF operates in electrospray positive mode over a mass range of 50-600 Da. MS conditions are: Capillary voltage +2800 V, cone voltage +30 V, source temperature 120°C, desolvation gas temperature and flow 350°C and 800 L/h, respectively, scan time 0.4 s. A leucine-enkephaline solution at 400 ng/mL is infused throughout the analysis to ensure high mass accuracy (<2 ppm). Fragmentation spectra are recorded in separate analyses in MSe mode using a collision energy ramp of 10-30 eV. Data is recorded using Masslynx 4.1 and PAs are identified based on their retention times, exact mass fragmentation characteristics and comparison with the existing literature and databases containing information on PAs known in *Echium* and *Eupatorium* genus. External calibration for the quantification of the PAs is made using echimidine, intermedine and lycopsamine as standards. Five calibration points are made: 0.02, 0.1, 0.5, 2, and 10 µg/mL. Linear responses are obtained from 0.02 to 2 µg/mL (Table 1). The limit of detection (LOD) and limit of quantitation (LOQ) for echimidine, lycopsamine and intermedine are 1.5 ng/mL and 2 ng/mL, respectively. As only a limited number of reference standards are available, a number of other PAs and PA-*N*-oxides commonly found in *Echium* (echimidine-N-oxide, vulgarine/-N-oxide, acetyl-echimidine/-N-oxide, acetylvulgarine/-N-oxide, echivulgarine/-N-oxide) are indirectly quantified as echimidine equivalents. PAs and their PA-*N*-oxides and isomers in pollen of *E. vulgare* and

*E. cannabinum* determined with LC-MS analysis are reported in Table 2. Two examples of extracted ion chromatograms of PAs are shown in Figure 7.

**Table 1** calibration curves obtained for echimidine, lycopsamine and intermedine.

| Echimidine or Lycopsamine or Intermedine (µg/mL) | Echimidine (intensity) | Intermedine (intensity) | Lycopsamine (intensity) |
|---|---|---|---|
| 0.02 | 2950 | 3130 | 3670 |
| 0.1 | 13660 | 15540 | 16200 |
| 0.5 | 63940 | 62050 | 71200 |
| 2 | 243800 | 200400 | 277000 |
| Calibration curve* | y = 122755x + 686 | y = 104410x + 1900 | y = 138740x + 1143 |

* weighed by 1/x.

**Table 2** Retention and mass characteristics of known *Echium*-type and *Eupatorium*-type PAs (free bases/-*N*-oxides and isomers).

| PA peak n° | Compound Name | RT (min) | Chemical formula | $[M + H]^+$ experimental | $[M + H]^+$ calculated | Error (mDa) |
|---|---|---|---|---|---|---|
| | • *Echium vulgare* | | | | | |
| | Echimidine | 2.28 | C20H31NO7 | 398.2184 | 398.2179 | 0.5 |
| 1 | Echimidine-N-oxide | 2.27 | C20H31NO8 | 414.2126 | 414.2128 | -0.2 |
| | Acetylechimidine | 2.74 | C22H33NO8 | 440.2282 | 440.2284 | -0.2 |
| 3 | Acetylechimidine-N-oxide | 2.71 | C22H33NO9 | 456.2234 | 456.2234 | 0.0 |
| | Echivulgarine | 3.81 | C25H37NO8 | 480.2575 | 480.2575 | 0.0 |
| 5 | Echivulgarine-N-oxide | 3.83 | C25H37NO9 | 496.2552 | 496.2547 | 0.5 |
| | Vulgarine | 2.33 | C20H31NO7 | 398.2174 | 398.2179 | -0.5 |
| 2 | Vulgarine-N-oxide | 2.43 | C20H31NO8 | 414.2133 | 414.2128 | 0.5 |
| | Acetylvulgarine | 2.88 | C22H33NO9 | 440.2285 | 440.2284 | 0.1 |
| 4 | Acetylvulgarine-N-oxide | 2.87 | C22H33NO9 | 456.2243 | 456.2234 | 0.9 |
| | • *Eupatorium cannabinum* | | | | | |
| 6 | Lycopsamine | 1.12 | C15H25NO5 | 300.1807 | 300.1811 | -0.4 |
| 7 | Intermedine | 1.16 | C15H25NO5 | 300.1810 | 300.1811 | -0.1 |
| 8 | Lycopsamine-N-oxide or Intermedine-N-oxide* | 1.27 | C15H25NO6 | 316.1756 | 316.1760 | 0.4 |
| 9 | Lycopsamine-N-oxide or Intermedine-N-oxide* | 1.35 | C15H25NO6 | 316.1758 | 316.1760 | 0.2 |

* Distinction between the N-oxides of intermedine and lycopsamine not possible due to the lack of standards.

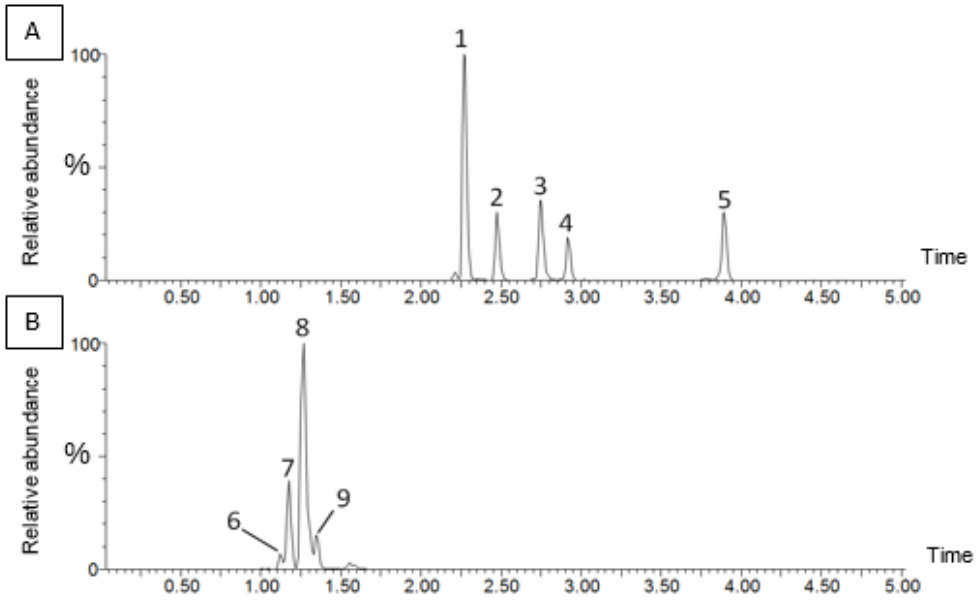


**Figure 7** Extracted ion chromatograms of PAs identified in plant pollen of *E. vulgare* (A) and *E. cannabinum* (B). Peak numbers correspond to the PAs described in Table 2 (1: echimidine-N-oxide; 2: vulgarine-N-oxide; 3: acetylechimidine-N-oxide; 4: acetylvulgarine-N-oxide; 5: echivulgarine-*N*-oxide; 6: lycopsamine; 7: intermedine; 8-9: lycopsamine-N-oxide or intermedine-N-oxide).

Pollen samples of *E. vulgare* and *E. cannabinum* analysed contain mainly PA-N-oxides while free bases were only present as traces. Table 3 shows the MS/MS fragments of PA-N-oxides detected in pollen samples. In addition, PAs not previously reported to be present in pollen of *E. vulgare* and *E. cannabinum* can also be detected by UHPLC-HRMS. They are tentatively identified through the determination of their molecular formula based on high mass and spectral accuracy measurements by the high resolution QTOF mass spectrometer (Table 4).

**Table 3** MS/MS fragment ions for PA-N-oxides from *E. vulgare* or *E. cannabinum*.

| Pyrrolizidine alkaloid | $[M + H]^+$ | MS/MS fragment ions |
|---|---|---|
| • *Echium vulgare* | | |
| Echimidine-N-oxide | 414.2126 | 396.2029, 352.1764, 338.1608, 254.1403, 220.1350, 120.0817 |
| Acetylechimidine-N-oxide | 456.2234 | 438.2131, 396.2025, 338.1610, 254.1393, 220.1339 |
| Echivulgarine-N-oxide | 496.3402 | 478.2448, 396.2030, 338.1613, 254.1397, 220.1341, 120.0816 |
| Vulgarine-N-oxide | 414.2133 | 396.2021, 314.1612, 256.1189, 172.0976, 138.0923, 136.0764 |
| Acetylvulgarine-N-oxide | 456.2243 | 438.2132, 356.1714, 298.1295, 214.1081, 180.1030, 120.0818 |
| • *Eupatorium cannabinum* | | |
| Lycopsamine/Intermedine-N-oxide | 316.1756 | 172.0973, 155.0947, 138.0917, 111.0684 |

**Table 4** PAs that have not been previously reported from *E. vulgare* and *E. cannabinum*.

| Compound name | RT (min) | Chemical formula | $[M + H]^+$ experimental | $[M + H]^+$ calculated | Error (mDa) |
|---|---|---|---|---|---|
| • *Echium vulgare* | | | | | |
| Curassavine-N-oxide* | 1.54 | C16H29NO5 | 316.2125 | 316.2124 | 0.1 |
| • *Eupatorium cannabinum* | | | | | |
| Leptantine* | 1.41 | C15H27NO6 | 318.1922 | 318.1917 | 0.5 |
| Amabiline* | 1.48 | C15H25NO4 | 284.1865 | 284.1862 | 0.3 |
| Uplandicine* | 1.71 | C17H27NO7 | 358.1868 | 358.1866 | 0.2 |

*Tentative identification

## Data processing

The following procedure is used for the detection and quantification of PAs. Provided that the identity of the PA is known, this procedure may also be applied to other types of alkaloids from other plant species.

1. To identify the different alkaloids, generate an extracted ion chromatogram (EIC) using a mass window of ± 0.01 Da around the values provided in Table 2.
2. Verify the mass accuracy of the ions compared to the theoretical values.
3. In the EICs integrate the peaks and calculate concentrations in ng/g or µg/g of pollen using your own calibration curves. Example of equations are provided in Table 1.

In this study we obtained a total PA concentration of 7.43 and 5.32 mg/g in pollen of *E. vulgare* and *E. cannabinum*, respectively (Table 5).

**Table 5** Total PA content found in pollen from *E. vulgare* and *E. cannabinum*.

| Plant pollen origin | PA Concentration (mg/g) | Standard deviation (mg/g) |
|---|---|---|
| • *Echium vulgare** | 7.43 | ± 2.45 |
| • *Eupatorium cannabinum** | 5.32 | ± 1.98 |

*Samples collection performed in 2013 from two different location in Switzerland.

## Conclusion

Liquid chromatography based approaches are frequently chosen for the identification and quantification of PAs in plants and for food analysis, allowing the determination of individual PAs and PA-N-oxides. This is particularly important for determining the exact type of PAs which are present in a given plant species and also in food analysis, where the spectrum of PAs may help deduce the plant species that contribute to PA contamination. Additionally, the sample preparation is faster and does not need derivatization as required for gas chromatography based approaches. However, many types of PAs are still not available on the market as reference substances. Therefore, a number of PAs are quantified using a reference substance that is closely related but not identical to the analysed PAs. Hence, quantification may not be entirely accurate, since the response factor of the detector may differ between PAs, even when they are closely related to each other. Furthermore, positional isomers, such as lycopsamine and intermedine, are also not easy to separate. Finally, while some plant species have been relatively well characterized regarding their PA content (e.g. *E. vulgare*), little is known of many other PA-producing plant species. Recently, ultra-high performance liquid chromatography coupled with high resolution mass spectrometry has been described as a selective and sensitive method for the analysis of PAs in plants.[12,17-19] This method provides an accurate mass information based on which unknown PAs may also be detected in an untargeted manner without the need for reference standards for each PA, as has been shown in the present study. In the future we anticipate

that UHPLC-HRMS will become the method of choice for the profiling of both known and unknown PAs in plants.

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# CHAPTER 2

# Pyrrolizidine alkaloids from *Echium vulgare* in honey originate primarily from floral nectar

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**Abstract**

Pyrrolizidine alkaloids (PAs) in honey can be a potential human health risk. So far, it has remained unclear whether PAs in honey originate from pollen or floral nectar. We obtained honey, nectar and plant pollen from two observation sites where *Echium vulgare* L. was naturally abundant. The PA concentration of honey was determined by targeted analysis using a high pressure liquid chromatography-mass spectrometry system (HPLC-MS/MS),

allowing the quantification of six different PAs and PA-N-oxides present in *E. vulgare.* *Echium*-type PAs were detected up to 0.153 µg/g in honey. Nectar and plant pollen were analyzed by non-targeted analysis using ultra-high pressure liquid chromatography-high resolution-mass spectrometry (UHPLC-HR-MS), allowing the detection of 10 alkaloids in small size samples. *Echium*-type PAs were detected between 0.3 - 95.1 µg/g in nectar and 500 - 35000 µg/g in plant pollen. The PA composition in nectar and plant pollen was compared to the composition in honey. Echimidine (+N-oxide) was the main alkaloid detected in honey and nectar samples, while echivulgarine (+N-oxide) was the main PA found in plant pollen. These results suggest that nectar contributes more significantly to PA contamination in honey than plant pollen.

**Keywords:** Pyrrolizidine alkaloids, *Echium vulgare*, honey, nectar, plant pollen, high pressure liquid chromatography-mass spectrometry (HPLC-MS/MS), ultrahigh pressure liquid chromatography-high resolution mass spectrometry (UHPLC-HR-MS).

## Introduction

Pyrrolizidine Alkaloids (PAs) are toxic compounds produced by plants as a chemical defense against herbivores.[1,2] Many unrelated plant species produce PAs, and some of them are particularly abundant in European agro-ecosystems. These PA-containing plants mainly belong to the Asteraceae (Senecioneae and Eupatorieae tribes), Boraginaceae (all genera), and Fabaceae (genus *Crotalaria*) families.[3,4] PAs may get into the food chain when food products are either contaminated with or derived from PA-containing plant tissues.[5-7] PAs can occur as N-oxides or as free-base/tertiary forms. These two forms are both hepatotoxic and genotoxic.[8,9] In plants, the N-oxides are found in higher concentrations than the corresponding free-bases (tertiary PAs).[1,10] Acute poisoning or chronic exposure to PAs mostly affects liver function, since PAs are activated by nucleophilic compounds through the liver's detoxification enzymes.[6,11-13] The chronic intake of low PA-levels in food can lead to

liver cirrhosis and cancer.[14] Legal limits for PAs in food have not been established yet in the European Union or in Switzerland. However, the German Federal Institute for Risk Assessment (BfR) recommends an intake of not more than 0.007 µg of 1.2-unsaturated PAs per day per kg bodyweight.[15]

Honey is one of the best studied food products with respect to PA contamination. When honeybees collect nectar and plant pollen from PA-containing plants, PAs are transferred into bee products such as honey or bee-collected pollen.[16,17] PAs have been detected in honey samples from various geographical and botanical origins.[4,18-27] PA concentrations of up to two orders of magnitudes over the limits recommended by the BfR have been reported in monofloral honeys from *Echium vulgare* or *E. plantagineum* [28-32] and from *Senecio jacobaea*.[31,33,34]

Honey is mostly composed of concentrated nectar and contains only traces of pollen. Therefore, PAs contained in nectar constitute an important potential source of PAs in honey. However, the concentration of secondary compounds may be considerably higher in plant pollen than in nectar,[35,36,37] and some pollen types contain particularly high amounts of PAs.[17] Consequently, it remains unclear whether the PA content in nectar is high enough to substantially contaminate honey,[38] and more generally, whether the PAs in honey predominantly originate from pollen or nectar.[39,40]

Prior research has suggested pollen as the major source of PA contamination in honey.[14,17] Contamination of honey could be caused by the liberation of PAs from pollen.[38] Lastly, experiments with plant pollen from *Senecio vernalis* added to PA-free honey have suggested that contamination of honey may occur through diffusion of PAs from pollen into honey.[40]

Unraveling the entry mechanism by which PAs contaminate honey is important for reducing risks associated with PA-containing bee products.

Two approaches can be used to examine the pathway from the different plant tissues into

bee products. First, quantitative analyses of nectar, pollen and honey may help determine which plant part is the main contributor to the total PAs found in honey. Second, differences in the PA composition (relative abundance of different PAs) found in nectar can be compared to that found in pollen. This information can be used to determine whether the PA composition in honey more closely matches that of nectar or pollen. In the present study, we performed qualitative and quantitative analysis of the different alkaloids found in *Echium* nectar and pollen.

We selected *Echium vulgare* as a model to study the pathway by which PAs are transferred into honey. This plant species is the only species of the genus *Echium vulgare* regularly found in Switzerland. It is widely distributed in Europe and has been previously described as a major source of PA contamination of European honeys.[19,21] We chose two observation sites where *E. vulgare* was blooming during the bee season. These sites were located in two different climatic regions, one located to the north and the other to the south of the Alps.

## Material and Methods

### Chemical reagents

The echimidine used in this study was obtained from Phytolab (Vestenbergsgreuth, Germany), while the heliotrine was from Latoxan (Valence, France). For plant extraction, milli-Q water was used. Formic acid and glass beads (Ø 2 mm) were purchased from Sigma-Aldrich (Buchs, Switzerland). HPLC grade methanol was purchased from Merck (Dietikon, Switzerland). Cyclohexane (> 98% purity), sulphuric acid, and ammonia were purchased from Merck Chemicals (Darmstadt, Germany). The solvents and additives used for LC-MS were water, acetonitrile, and ULC-MS grade formic acid acquired from Biosolve (Valkenswaard, Netherlands).

## Observation sites

We selected two observation sites where *Echium* plants were abundant around bee colonies. The first observation site was located north of the Alps near Basel, close to the border between Switzerland and France (hereafter Basel). The other observation site was located close to Italy, along the southern flank of the Alps in the Verzasca valley (hereafter Verzasca). At the Verzasca site, two beekeepers participated in the project (hereafter Verzasca 1 and Verzasca 2). The aerial distance between these two apiaries was approximately 400 meters.

## Honey and plant sample collection

**Honeys**: In total, four samples of honey were included from Basel and six from Verzasca. In Basel, the honeys were harvested on 8 June and 27 July 2012, as well as on 29 June and 8 August 2013. In Verzasca 1, the honeys were harvested on 1 August 2012, 2 August 2013, and 1 August 2014, while in Verzasca 2 they were harvested on 30 July 2012, 2 August 2013, and 1 August 2014. Eight additional honey samples were obtained from apiaries in diverse regions of Switzerland. All of the additional honey samples were produced between 2009 and 2011. **Plant material***:* In Basel, *E. vulgare* was in blossom during June and July, while in the Verzasca valley *E. vulgare* was in blossom from June until August. Samples of nectar and plant pollen from *E. vulgare* were collected at the two observation sites. Samples were only collected under dry weather conditions to avoid wash-out of plant pollen from the anthers and dilution of nectar by the rain. In Basel, the samples were collected on 18 June and 4 July 2013 and on 19 June and 27 June 2014. In Verzasca, the samples were collected on 29 June and 17 July 2013 and on 6 July and 18 July 2014. In total, 20 nectar samples from Basel (n=10 in 2013 and n=10 in 2014), 16 nectar samples from Verzasca (n=7 in 2013 and n=9 in 2014), 14 plant pollen samples from Basel (n=5 in 2013 and n=9 in 2014) and 13 plant pollen samples from Verzasca (n=4 in 2013 and n=9 in

2014) were collected. On the day before any given sample collection, plants were tightly bagged with a fine mesh, and a layer of insect glue was spread around the lower part of the stem in order to prevent insect visits. Nectar was collected using a Pasteur pipette previously elongated to a capillary on a flame. The pipette was directly placed into the corolla, carefully avoiding any disruption of floral tissues. In 2013, pollen from anthers of *Echium* flowers (plant pollen) was collected with metal forceps. Plant pollen was carefully removed from the surface of the anthers in order to prevent contamination of the pollen with other flower parts, especially the anthers. This procedure yielded low amounts of plant pollen. In order to facilitate collection and avoid contamination with other plant parts, plant pollen was collected in 2014 by immersing the stamens into cyclohexane.[38] The cyclohexane was subsequently evaporated. For comparison, two plant pollen samples were collected from the same plant using both methods (forceps and cyclohexane). Since the two samples gave comparable results (data not shown), we concluded that the cyclohexane did not wash out PAs from the pollen, and hence both collection methods would yield similar results. All samples were kept on dry ice during collection and subsequently stored at -80°C until extraction.

**Sample preparation of honey for quantification of PAs with LC-MS/MS**

Honey samples were prepared as described in Dübecke *et al*.[19] Since PA-N-oxides are polar organic compounds with basic characteristics, they are soluble in polar organic solvents or in mixtures of solvents and acidified water. 10 g of honey, together with 100 ng heliotrine as internal standard and 30 mL of 0.05M sulphuric acid were vigorously shaken for 20 min. Samples were then filtered overnight using a 2 mm mesh to remove particles that could block the solid-phase extraction. Clean-up was conducted using SPE-SCX Cartridges (Varian) washed previously with methanol and conditioned with 9 mL of 0.05M sulphuric acid. Samples were loaded onto the column, washed with 9 mL of deionized water, eluted into a glass vial using ammoniated methanol,[18] and dried at 40°C in an ambient air stream.

Samples were then reconstituted in 1 mL deionized water, shaken vigorously, and filtered into a 2 mL glass vial using a 0.45 µm syringe filter.

The PA concentration was determined by targeted analysis using a HPLC-MS/MS-system as described in Dübecke *et al*.,[19] allowing the detection of six different PAs or PA-N-oxides (echimidine, echimidine-N-oxide, acetylechimidine, acetylechimidine-N-oxide, echivulgarine and echivulgarine-N-oxide) commonly found in *E. vulgare.*[17] The total PA concentration was calculated as the sum of the six different PAs. LC-MS/MS analysis was performed using an HTC PAL autosampler of CTC Analytics AG, a Shimadzu LC-system with a Thermo Hypersil Gold C18 column (50 x 2.1 mm, 1.9 µm particle size) and an Applied Biosystems API4000 QTRAP triple quadrupole mass spectrometer. Concentrations were corrected against the recovery of the internal standard. For quantification, external calibration was performed using echimidine as the standard. The limit of quantitation (LOQ) for echimidine in honey was 1 ng/g. As no further reference standards were available, echimidine-N-oxide, acetylechimidine, acetylechimidine-N-oxide, echivulgarine, and echivulgarine-N-oxide were indirectly quantified using the calibration of echimidine, assuming the same response factor and thus the same LOQ. A linear range was achieved from 0.5 to 100 ng/mL for the echimidine standard. Recovery of echimidine near the LOQ was 97%. Repeatability was 5.4% as determined with six independent sample preparations measured by the same person on the same day.

**Extraction of PAs from nectar and plant pollen, and UHPLC-HRMS analysis**

**Nectar**: 5 µL of nectar was directly transferred into a glass vial containing a 200 µL insert and diluted 10 times with the extraction solvent (70% methanol, 29.5% ultra-pure water and 0.5% formic acid, v/v). **Plant pollen**: 1 mg of plant pollen was accurately weighed using a microbalance scale (Mettler Toledo), mixed with 100 µL of extraction solvent as described above and transferred into a 2 mL Eppendorf tube. Five glass beads were added and the

tube was vigorously shaken at 30 Hz for 4 min to disrupt the pollen structure and to extract the PAs. Following centrifugation (18400 *g*, 4 min), 5 µL of the supernatant was transferred into a glass vial containing a 200 µL insert and diluted 10 times with the extraction solvent. Non-targeted analysis using the UHPLC-HR-MS system was carried out for the detection and quantification of various alkaloids found in *E. vulgare* (echimidine, echimidine-N-oxide, vulgarine, vulgarine-N-oxide, acetyl-echimidine, acetyl-echimidine-N-oxide, acetylvulgarine, acetylvulgarine-N-oxide, echivulgarine and echivulgarine-N-oxide[17]). In brief, separation of the alkaloids was performed on an Acquity BEH C18 column (50 x 2.1 mm i.d., 1.7 µm particle size, Waters), using an Acquity UHPLC™ system (Waters) coupled to a Synapt G2 QTOF mass spectrometer (Waters). A binary gradient was performed at a flow rate of 0.4 mL $min^{-1}$. The mobile phase consisted of 0.05% formic acid in water (solvent A) and 0.05% formic acid in acetonitrile (solvent B). The gradient program was as follows: 0-4 min 5-40% B, 4-6 min 40-100% B, 6-9 min 100% B, 9.1-10.5 min 5% B. The temperature of the column was maintained at 30°C and that of the autosampler at 25°C. Injection volume was 1 µL. The QTOF operated in electrospray positive mode over a mass range of 50-600 Da. MS conditions were: Capillary voltage +2'800 V, cone voltage +30 V, source temperature 120°C, desolvation gas temperature and flow 350°C and 800 L/h respectively, and scan time 0.4 sec. A leucine-enkephaline solution at 400 ng/mL was infused throughout the analysis to ensure high mass accuracy (<2 ppm). PAs were identified on the basis of their retention times, exact mass fragmentation characteristics, and by comparing with the existing literature and databases containing information on known PAs in the *Echium* genus. External calibration was made using echimidine as a standard for the quantification of the PAs. Linear responses were obtained from 5 to 4'000 ng/mL (supporting information). The limit of quantitation (LOQ) for echimidine was 2 ng/mL. Since no reference standards other than echimidine were available, additional PAs and PA-N-oxides (echimidine-N-oxide, vulgarine, vulgarine-N-oxide, acetyl-echimidine, acetyl-echimidine-N-oxide, acetylvulgarine,

acetylvulgarine-N-oxide, echivulgarine, and echivulgarine-N-oxide) were indirectly quantified as echimidine equivalents assuming the same response factor. Retention and mass characteristics are shown in “supporting information”.

**Water content in nectar and honey**

**Nectar**: 20 µL of nectar was frozen with liquid nitrogen in a previously weighed vial and lyophilized (LabConco, USA). The water content was calculated as the difference in weight before and after lyophilization. In total, ten samples of nectar from Basel (n=3 from 2013, n=7 from 2014) and ten samples of nectar from Verzasca (n=3 from 2013, n=7 from 2014) were analyzed. **Honey**: 5 g of honey was liquefied at 55°C and cooled to room temperature. The content of water was determined according to the harmonized methods of the European Honey Commission[41] using a refractometer (Mettler Toledo RE40). In total, ten honey samples, four samples of honey from Basel (n=2 from 2012, n=2 from 2013) and six from Verzasca (n=1 from 2010, n=1 from 2012, n=2 from 2013, n=2 from 2014) were analyzed for their water content.

**Sediment of honey**

In total, nine honey samples, four samples from Basel (n=2 from 2012, n=2 from 2013) and five samples from Verzasca (n=1 from 2010, n=1 from 2012, n=1 from 2013, n=2 from 2014), were analyzed for their honey sediment, including the pollen fraction.[42] 5 g of honey diluted with 10 mL of water was homogenized until the honey was completely dissolved. The tube was subsequently centrifuged at 1’000 *g* for 4 min and the water discarded. This procedure was repeated three times for a total amount of 15 g of honey. The final pellet was washed twice with 10 mL of water to clean it from the sugar residues, subsequently dried at 35°C for 24 h, and then weighed.

## Results

### PA concentrations in honey

*Echium*-type PAs were found in most of the honeys collected at both observation sites. The total PA concentrations of the honey samples produced in Basel in July and August during 2012 and 2013 were very low, 0.003 µg/g and 0.002 µg/g respectively (Figure 1), and near the LOQ, while no PAs were measurable in the samples harvested in June 2012 and 2013 (data not shown). Higher concentrations of PAs were detected in the samples produced in Verzasca. Levels ranged from 0.002 µg/g to 0.153 µg/g, and varied substantially between the collection years and the two apiaries (Figure 1). With regards to the type of alkaloids, echimidine (sum of tertiary base and the corresponding N-oxide) was present in the highest concentrations, followed by echivulgarine (+N-oxide) and acetylechimidine (+N-oxide) respectively. Echimidine (+N-oxide) accounted on average for 72% of the total PAs found in honey. Its concentration was four to six times higher than that of echivulgarine (+N-oxide). Similar results were obtained for honey samples containing *Echium*-type PAs from various other locations within Switzerland, where echimidine (+N-oxide) was also found to be the dominant alkaloid (Table 1).

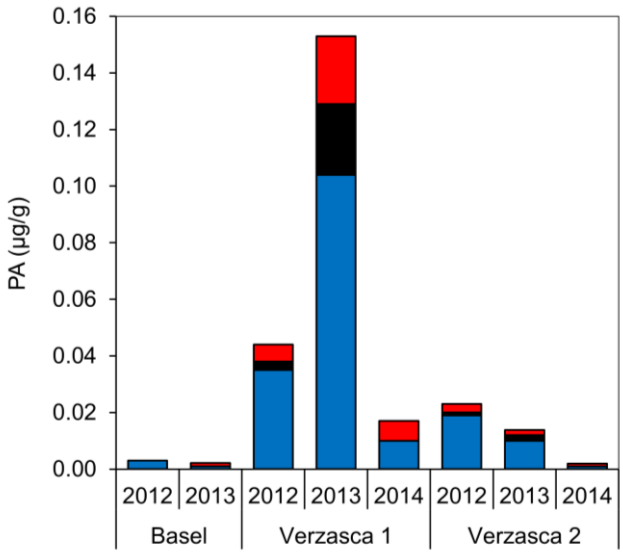

**Figure 1** Concentrations of the three main *Echium*-type PAs in honeys from the two observation sites. ■ echivulgarine (+N-oxide); ■ acetylechimidine (+N-oxide); ■ echimidine (+N-oxide).

**Table 1** PA content of positive honey samples. Echimidine (+N-oxide) was the main PA contaminant detected. n.d = Not detected; N = Northern; S = Southern.

| Honey type | Origin | echimidine (+N-oxide) (µg/g) | acetylechimidine (+N-oxide) (µg/g) | echivulgarine (+N-oxide) (µg/g) |
|---|---|---|---|---|
| Polyfloral / honeydew | Swiss plateau | 0.014 | 0.001 | 0.002 |
| Polyfloral | Swiss plateau | 0.009 | 0.001 | 0.004 |
| Polyfloral | N flank of Alps | 0.006 | n.d | 0.001 |
| Alpine mountain flower | Central Alps | 0.006 | 0.006 | n.d |
| Alpine mountain flower | Central Alps | 0.008 | 0.001 | 0.001 |
| Fir honeydew | Central Alps | 0.006 | n.d | 0.002 |
| Polyfloral / chestnut | S flank of Alps | 0.034 | 0.001 | 0.004 |
| Alpine mountain flower | S flank of Alps | 0.009 | 0.001 | 0.001 |

## PA concentrations in nectar and plant pollen

Plant pollen contained high concentrations of PAs, while much lower concentrations of PAs were found in nectar (Figure 2). The PA concentrations of plant pollen collected in Basel ranged from 1’600 to 35’000 µg/g and were on average 7’428 µg/g (in 2013) and 24’453 µg/g (in 2014) respectively. Plant pollen from Verzasca collected in 2013 and 2014 contained PAs ranging in concentration from 500 to 12’900 µg/g. Average PA concentrations of 5’427 µg/g and 9’661 µg/g were measured in pollen collected in 2013 and 2014. In contrast, the PA content of nectar was on average more than 500 times lower than the PA concentration in pollen. The PA concentration in nectar samples collected in Basel ranged from 4.8 to 95.1 µg/g. PA concentrations were on average 21.3 µg/g (in 2013) and 40.1 µg/g (in 2014). Nectar from Verzasca contained PAs from 0.3 to 51.5 µg/g, on average 15.7 µg/g (in 2013) and 18.0 µg/g (in 2014). The PAs in nectar and plant pollen were mainly N-oxides,

and the contribution of PAs as free bases to the total amount of each type of PA was very low (data not shown).

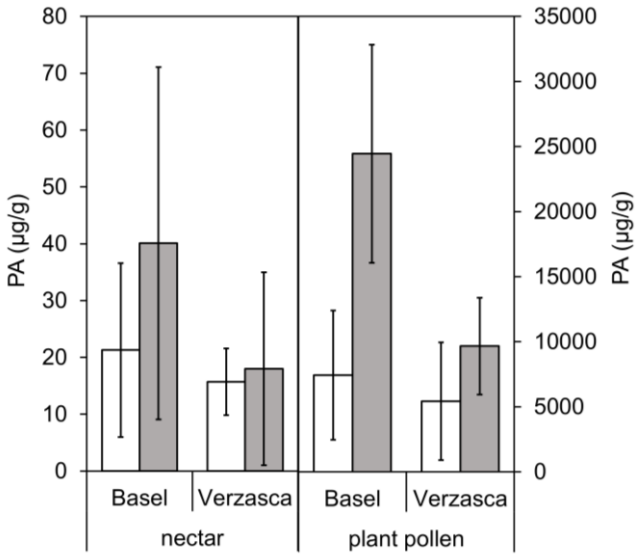


**Figure 2** Sum of *Echium*-type PAs (echimidine-N-oxide, vulgarine-N-oxide, acetylechimidine-N-oxide, acetylvulgarine-N-oxide, echivulgarine-N-oxide and their corresponding tertiary bases) in nectar (Basel: n=10 in 2013 and n=10 in 2014; Verzasca: n=7 in 2013 and n=9 in 2014), plant pollen (Basel: n=5 in 2013 and n=9 in 2014; Verzasca: n=4 in 2013 and n=9 in 2014) of *E. vulgare*, collected at the two observation sites. ☐ year 2013; ■ year 2014.

**Proportions of various *Echium*-type PAs in nectar and plant pollen**

In this study, we compared the proportions in which the various *Echium*-type PAs were present in honey with the proportions of these alkaloids present in nectar and pollen. The distribution of the alkaloids in samples collected in 2014 is shown in Figure 3. We detected the same types of PAs in nectar and plant pollen. However, the percentage of several PA-types varied between the investigated plant matrices. In nectar, approximately half of the PA content was echimidine (+N-oxide), while the other half consisted of acetylechimidine (+N-oxide) and low amounts of vulgarine (+N-oxide), echivulgarine (+N-oxide), and

acetylvulgarine (+N-oxide). In contrast, echivulgarine (+N-oxide) was the main alkaloid-type in plant pollen (63% of the total PA content). The four other alkaloid-types were present at substantially lower concentrations. Similar PA profiles were obtained with samples collected in 2013 (data not shown). In summary, the echimidine-type was the dominant alkaloid in honey as well as in nectar, while pollen mainly contained the echivulgarine-type.

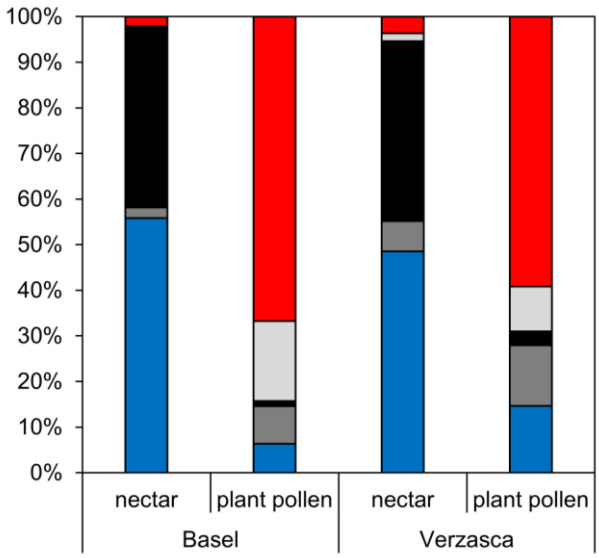


**Figure 3** Proportions of PAs in nectar (n=10 from Basel; n=9 from Verzasca) and plant pollen (n=9 from Basel; n=9 from Verzasca) from *Echium vulgare* collected in 2014 at two observation sites. ■ echivulgarine (+N-oxide); ■ acetylvulgarine (+N-oxide); ■ acetylechimidine (+N-oxide); ■ vulgarine (+N-oxide); ■ echimidine (+N-oxide).

## Discussion

### Nectar of *E. vulgare* as a primary source for PAs in honey

The proportion of the different types of PAs found in honey was similar to that found in nectar, but strikingly different from that found in pollen. Echimidine (+N-oxide) was the dominant alkaloid found in honey and nectar. In contrast, plant pollen mostly contained echivulgarine (+N-oxide). This unequal quantitative distribution of alkaloids strongly suggests nectar as the primary source of PAs in honey.

Prior research has found concentrations of PAs in plant pollen of up to 14'000 µg/g.[17] We measured similar average PA concentrations in plant pollen from Basel in 2013 (7'428 µg/g), from Verzasca in 2013 (5'427 µg/g) and in 2014 (9'661 µg/g), but pollen collected in 2014 in Basel contained a higher amount of PAs (24'453 µg/g). Climatic and genetic variations may affect the amount of PA produced by the plants and thus the PA content in pollen. Boppré *et al*.[17] found echivulgarine-N-oxide as the major alkaloid in *Echium* pollen, followed by vulgarine-N-oxide, echimidine-N-oxide, and acetylechimidine-N-oxide. Similar proportions of alkaloids were found in our study, thus supporting their results. Since the total PA content of plant pollen was found in concentrations that were 1'000 times higher than in honey, the authors concluded that plant pollen has the potential to be a significant source for PAs in honey.[17] However, they did not investigate nectar as a potential source of alkaloids in honey, and did not report the unequal proportions of the alkaloids in honey compared to pollen. Since we integrated analyses of *Echium* nectar and of honeys harvested at the same locations, we obtained a more complete picture of the contamination pathway for this plant species, suggesting that floral nectar contributes more significantly to honey contamination than pollen. Our results suggest that pollen may play a small role in the PA contamination of honey and that a small proportion of PAs may be released from pollen into honey as previously suggested by Kempf.[38,40] For example, the proportion of echivulgarine (ca. 15% of the total PAs) found in the honey from Verzasca 1 (2013; Fig. 1) was higher than what would be expected from pure nectar, which contains proportionally less echivulgarine (less than 5% of the total PAs).

**Estimation of concentration and dilution factors for nectar and pollen during honey ripening**

In order to estimate an approximate factor by which the components of nectar, such as sugars and PAs, are concentrated during the process of honey ripening, we measured the

water content in nectar and honey. Nectar samples contained water in the range of 30% to 95%, depending on climatic conditions, with an average of 66% (standard deviation = 18.3%). Honeys contained on average 17.3% (standard deviation = 1.6%) water. Therefore, PAs are concentrated during the processing of nectar to honey by a factor of approximately four (Table 2).

Honey contains traces of pollen that depend on several factors. When bees forage from flowers, they come into contact with the anthers, so that pollen may fall into nectar that is later collected. Pollen grains can also stick to the bee's body hair. Furthermore, apicultural practices can influence the amount of pollen in honey.[43] For these reasons, the pollen content of honey is variable. We therefore assessed the weight of the honey sediment, which mainly consists of pollen. Some honey also contains other components, e.g. “honeydew indicators,” such as algal cells and mold spores.[43] Thus the weight of pollen may be overestimated by this procedure. On average, our honey samples from Basel and Verzasca contained a sediment of 0.048 mg/g (standard deviation = 0.032). This value is close to the range of 0.14 to 0.2 mg/g found by Maurizio in honey from Germany and Switzerland.[43] In other words, pollen in honey is usually diluted at least 5’000 fold in honey (Table 2).

We measured an average total PA concentration of 25 µg/g (standard deviation = 22 µg/g) in nectar and an average total concentration of 13’551 µg/g (standard deviation = 9787 µg/g) in plant pollen. Thus, the PA concentration in nectar is approximately 500 times lower than it is in plant pollen. However, during honey ripening, the concentration of nectar increases about four fold, while the pollen content is diluted to at least 1:5000. Therefore, despite its initial lower PA concentration, the nectar of *E. vulgare* plays a substantially greater role in the PA contamination of honey than pollen (Table 2). This is in agreement with our observation of similar PA proportions of nectar and honey.

**Table 2** Estimation of the contribution of PAs from nectar and pollen to the PA content in honey.

| | nectar | pollen |
|---|---|---|
| **average PA conc. (µg/g)** | 25 | 13551 |
| **concentration/dilution** | 4:1 | >1:5000 |
| **PA contribution to honey** | ++++ | + |

**PA concentration in monofloral honeys**

Previous studies have found PA concentrations of up to 2.85 µg/g in monofloral honey of *E. vulgare* or *plantagineum*,[28-32,39] and up to 3.9 µg/g in monofloral honey of *S. jacobaea*.[33,34,39] The highest concentrations of PAs ever measured (up to 13 µg/g) were found in honey collected from hives in a field of *Senecio jacobaea.*[31] In the present study, we measured an average alkaloid content of 25 µg/g in nectar from *E. vulgare* plants (Fig. 2; Table 2). This value is on the same order of magnitude as the maximum PA concentrations ever measured. Therefore, the concentration of PAs in nectar is indeed high enough to explain the observed PA concentrations in honey.

The highest total PA concentration found in our honey samples was 0.153 µg/g, measured in a sample from Verzasca (Fig. 1). In fact, this honey is a polyfloral honey characteristic of the area of production. In such honeys, nectar from *E. vulgare* can be diluted more than 150 times with nectar from other plant species, explaining the final PA observed in this sample. We analyzed the pollen grains of this honey by microscopy. The majority of the pollen (88%) originated from *Castanea sativa*, while only 2.8% originated from *E. vulgare*. Sensorial and melissopalynological analyses revealed *Castanea sativa*, *Rubus sp.*, *Tilia sp.*, *Rhododendron sp.*, and *E. vulgare* as other significant components of the honey. Thus it stands to reason that a substantially higher amount of alkaloids could be expected in a monofloral honey of *E. vulgare*.

In conclusion, we found that most of the PAs in our honey samples were attributable to nectar, contrary to previous assumptions that proposed pollen as the primary source.[17,38,40] If pollen were the main source of PAs in honey, PA contamination of honey could be reduced by passing the honey through a filtration system designed to remove pollen. However, since nectar is the main contributor of PAs, such filtration would not substantially decrease the concentration of PAs, and would thus be an ineffective technical solution to the problem. A more suitable approach of reducing PA contamination in honey would be to avoid PA producing forage plants, such as those belonging to the Boraginaceae, in large numbers near apiaries. Some PA producing plants are an important foraging source for several solitary bee species. In small numbers, these plants will not usually pose a serious contamination threat.

**Acknowledgement**

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**Supporting information**

**Table S1** UHPLC-HRMS retention and mass characteristics (MS/MS ions) of *Echium*-type PAs.

| Peak n° | Compound Name | RT (min) | Chemical formula | $[M + H]^+$ experimental | $[M + H]^+$ calculated | Error (mDa) |
|---|---|---|---|---|---|---|
| 1 | Echimidine | 2.28 | C20H31NO7 | 398.2184 | 398.2179 | 0.5 |
| 2 | Echimidine-N-oxide | 2.27 | C20H31NO8 | 414.2126 | 414.2128 | -0.2 |
| | Vulgarine | 2.33 | C20H31NO7 | 398.2174 | 398.2179 | -0.5 |
| 3 | Vulgarine-N-oxide | 2.43 | C20H31NO8 | 414.2133 | 414.2128 | 0.5 |
| | Acetylechimidine | 2.74 | C22H33NO8 | 440.2282 | 440.2284 | -0.2 |
| 4 | Acetylechimidine-N-oxide | 2.71 | C22H33NO9 | 456.2234 | 456.2234 | 0.0 |
| | Acetylvulgarine | 2.88 | C22H33NO8 | 440.2285 | 440.2284 | 0.1 |
| 5 | Acetylvulgarine-N-oxide | 2.87 | C22H33NO9 | 456.2243 | 456.2234 | 0.9 |
| | Echivulgarine | 3.81 | C25H37NO8 | 480.2575 | 480.2575 | 0.0 |
| 6 | Echivulgarine-N-oxide | 3.83 | C25H37NO9 | 496.2552 | 496.2547 | 0.5 |

**Figure S1** UHPLC-HRMS chromatograms of echimidine standard (1), PAs in pollen and nectar from *E. vulgare*: echimidine-N-oxide (2), vulgarine-N-oxide (3), acetylechimidine-N-oxide (4), acetylvulgarine-N-oxide (5), echivulgarine-N-oxide (6). Peak numbers also refer to Table S1.

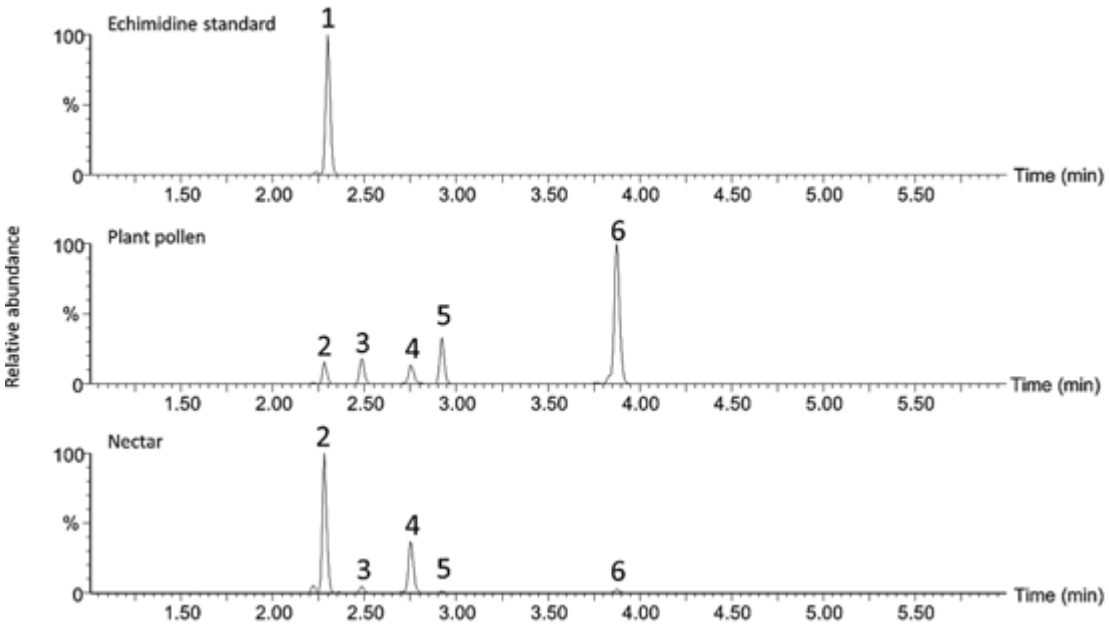
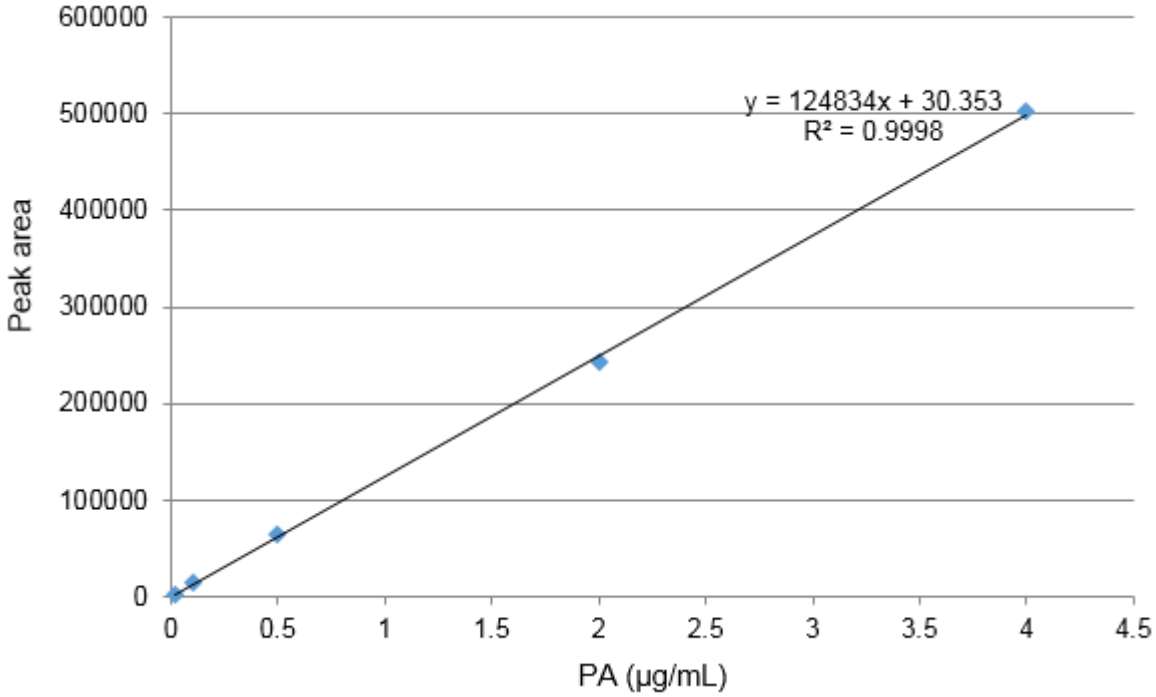



**Figure S2** Linear range of echimidine standard in UHPLC-HRMS. Linearity was achieved from 0.005 to 4 µg/mL. This material is available free of charge via the Internet at http://pubs.acs.org.

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# CHAPTER 3

# Larval nursing protects honeybee larvae from pollen secondary metabolites

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**Abstract**

Pollen of various plants contains toxic secondary compounds, sometimes at concentrations higher than those in flowers or leaves. The ecological significance of these compounds remains unclear and their impact on bees largely unexplored. Here, we studied the impact of pyrrolizidine alkaloids (PAs) found in pollen of *Echium vulgare* on honeybee adults and larvae. Echimidine and echivulgarine were isolated and added to diets in order to perform toxicity bioassays. While adult bees showed a relatively high tolerance to PAs, larvae were much more sensitive. In contrast to other bees, a honeybee larval diet typically only contains traces of pollen and predominantly consists of hypopharyngeal and mandibular secretions

produced by nurse bees, which feed on large quantities of pollen. We thus ascertained the transfer of PAs to nursing secretions produced by bees consuming pollen supplemented with realistic amounts of PAs. Maximum PA concentrations in these secretions were two orders of magnitude smaller than those in the nurse diets and well below the toxicity threshold for larvae. Our results indicate that pollen secondary compounds have the potential to impact bee development, but suggest that honeybee nursing behavior may protect larvae against pollen toxins.

**Keywords:** *Apis mellifera*, pollen secondary compounds, pyrrolizidine alkaloids, *Echium vulgare*, hypopharyngeal secretions, honeybee larvae.

## Introduction

Plants have evolved a wide array of chemical defenses against herbivores,[1,2] involving an impressive diversity of secondary metabolites. In turn, herbivores have responded with numerous adaptations including enzymatic metabolism or sequestration of toxins.[3] Bees are a special case among insect herbivores, as they do not consume foliar tissues, but feed exclusively on pollen and nectar.[4] For bees, pollen is the main source of amino acids while nectar is the predominant source of carbohydrates. The impact of plant secondary metabolites on bees has long remained a neglected field of research and largely restricted to the effect of nectar secondary compounds.[5] Yet several studies have recently suggested that secondary metabolites can reach particularly high concentrations in plant pollen.[6-10] Mechanisms to cope with toxic compounds are likely to differ widely among solitary bees, bumblebees and honeybees, given their different life histories. Larvae of solitary bees and bumblebees feed directly on a mix of pollen and nectar. In striking contrast, eusocial honeybees nurse their larvae with protein-rich hypopharyngeal and mandibular secretions throughout their larval development.[11-13] In honeybees, the composition of this secreted jelly depends on whether the larva becomes a queen, a worker bee or a drone, but for the first

three to four days all larvae receive jelly that is free or almost free of pollen.[14,15] After this period, worker larvae receive a modified jelly that is less rich in protein, but contains more sugar and small amounts of pollen,[14,16-18] up to 5% of the total protein consumed during larval development.[15,19] Yet pollen is still the primary source of protein for honeybee colonies. Workers store pollen pellets mixed with honey, nectar and glandular secretions as bee bread in the hive[20,21] and newly emerged bees consume large quantities of bee bread during the first days of life, as it is central to the growth of their hypopharyngeal glands and the production of nursing secretions.[22,23] It has also been shown that pollen consumption is the most important factor influencing the longevity of newly emerged bees.[23-26] Throughout the season, honeybees have a wide host-plant spectrum due to the large number of workers and the long foraging distances. They are therefore potentially exposed to a wide array of secondary metabolites, maybe influencing the fitness of honeybee colonies. In the present study, we examine the effect of plant secondary compounds on honeybee colonies using *Echium vulgare* as a model. This widespread plant in Europe produces copious amounts of floral nectar and pollen and is extensively visited by honeybees. *E. vulgare* contains high levels of pyrrolizidine alkaloids (PAs) in many tissues, including leaves and pollen.[8,10,27] Typical PAs found in pollen of *E. vulgare* are echimidine-N-oxide and echivulgarine-N-oxide. However, PA N-oxides are at least partially converted in the digestive tract of honeybees into the more toxic tertiary PAs [28]. Moreover, honeybees have no detoxification mechanism by *N*-oxidation to cope with PAs.[28] Differently to nectar that contains PAs in low concentrations, the high PA content in *E. vulgare* pollen,[8,29,30] on average 13 mg/g,[10] constitutes a potential risk for newly emerged honeybees and honeybee larvae. Here we examined the impact of PAs isolated from *E. vulgare* on adults and honeybee larvae (Figure 1). We firstly fed newly emerged honeybee workers with pollen supplemented with field realistic levels of echimidine, and examined whether the PAs had an impact on worker survival. Secondly, we examined the effect of both echimidine and echivulgarine across a

range of concentrations on the development of honeybee larvae. Finally, we quantified the levels of PAs found in the hypopharyngeal and mandibular secretions (royal jelly) produced by nurses fed with bee bread that was supplemented with realistic levels of echimidine. For this, we developed a modified hive system that enabled us to trace plant secondary metabolites from pollen into larval jelly. Taken together, our results show that the production of hypopharyngeal and mandibular secretions in honeybees protects larvae against direct exposure to plant pollen secondary metabolites.

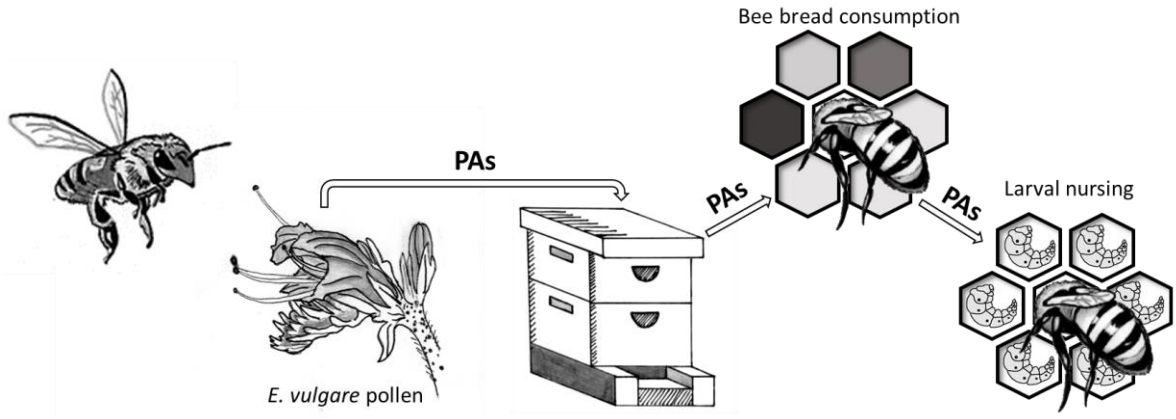


**Figure 1** Pathway of PAs from pollen of *E. vulgare* into bee bread and larval diets. Pollen of *E. vulgare* containing PAs is harvested by forager bees and stored in the hive as bee bread together with other pollen types. Newly emerged honeybees consume bee bread as a protein source for the development of their hypopharyngeal glands. Mature nursing bees consume bee bread to produce hypopharyngeal and mandibular secretions to feed larvae.

## Material and methods

### Chemicals and equipment

The reference standard echimidine (purity 94%, w/w) was obtained from Phytolab (Vestenbergsgreuth, Germany). For the extraction of PAs, methanol (HPLC grade), formic acid (purity 98%, w/w) and glass beads (Ø 2 mm) were purchased from Sigma-Aldrich

(Steinheim, Germany), while ammonia was purchased from Merck Chemicals (Darmstadt, Germany). MilliQ water was obtained from a Millipore system. BondElut SCX SPE cartridges (1 mL) were from Agilent Technologies (USA). For chromatographic analysis, water, acetonitrile, and formic acid, all of ULC-MS grade, were from Biosolve (Valkenswaard, the Netherlands). For toxicity assays, SupraSolv acetone, glycerol (85% for analysis), sodium chloride and potassium sulfate (Emsure), D(+)-glucose anhydrous for biochemistry and D(-)-fructose for biochemistry were all purchased from Merck (Darmstadt, Germany). Methyl benzethonium chloride (MBC) was obtained from Sigma-Aldrich (Steinheim, Germany) and yeast extract for the preparation of microbiological culture media was from Becton, Dickinson and Company (Allschwil, Switzerland). Polystyrene grafting cells (code CNE/3) were obtained from Nicoplast Society (Maisod, France), sterile 48-well suspension culture plates from Greiner Bio-One (Frickenhausen, Germany) and Plexiglas desiccators from Thermo Scientific (Nalgene 5314-0120). Cotton dental rolls (8 mm) were purchased from Hartmann (Neuhausen, Switzerland), brushes for grafting were from Leonhardy (code 19645, Nürnberg, Germany) and sterile 0.2 µm cellulose acetate syringe filters were from Hahnemuehle (Dassel, Germany).

**Extraction and purification of PAs from *Echium vulgare***

Echimidine and echivulgarine used in the bioassays were extracted from *E. vulgare* (leaves and inflorescences) collected at different locations in Switzerland (for details see Appendix 1). Briefly, plant material was lyophilized, extracted in methanol, and N-oxides were reduced with zinc dust to tertiary bases. After acid-base liquid-liquid extraction, tertiary PAs were separated using a semi-preparative system, evaporated and lyophilized. Echimidine and echivulgarine were adjusted according to their purity level (see Appendix 1).

**Honeybee colonies (*Apis mellifera*)**

Colonies were located at the Swiss Bee Research Centre at Agroscope, Bern, Switzerland (GPS coordinates: 46°55'49''N, 7°25'9''E). All colonies were treated for *Varroa* infestation and tested negative for European foul brood. Newly emerged honeybees: For each test series, frames hosting emerging broods were selected from three different bee colonies and incubated at 35°C in frame cages. After 24 h, newly emerged honeybees were collected in a glass recipient, delicately mingled to obtain a homogeneous population sample, and distributed equally in Liebefeld hoarding cages[24] made of stainless steel (13 x 6 x 10 cm). Colonies for royal jelly production: 600 g of bees (corresponding to 5'000-6'000 workers; no queen) were carefully brushed into small Miniplus® hives, an experimental unit that expresses all normal behaviors of a full sized colony.[31] The new colonies were kept in the dark at 13°C for 3 h. Then they were fed and kept at ambient temperature. In total, six colonies were created: three experimental colonies and three controls. The Miniplus systems were modified in that an external cage (30 x 20 x 30 cm) with wooden sides and covered with a fine metal net was screwed over the entrance hole. This external cage allowed cleaning activities of the bees but prevented foraging and forced the nursing bees to feed exclusively on the bee bread and honey placed inside the hive system. Honeybee larvae: First instar larvae were obtained from three different bee colonies in 2015 and in 2016. In each colony, a comb with empty cells or emerging brood was placed in an excluder cage. Three days later, the queen of each colony was confined in the excluder cage for 24 h. The oviposition was confirmed by visual inspection after the queen was released. After three days, the first instar larvae were collected with a disinfected, fine paintbrush.

**Bee-collected pollen, honey and bee bread**

Pollen: External pollen traps were positioned at the entrance of four bee colonies in Liebefeld in early April 2015. Pollen loads were collected daily and immediately stored at -25°C. In

total, 5 kg of pollen was harvested and combined. Melissopalinological analysis revealed *Acer* sp. (73.9%), *Cornus* sp. (9.7%), *Brassica* sp. (6.4%), *Aesculus* sp. (2%), and *Cotinus* sp. (1.6%) as major pollen types. Minor pollen types were present at lower percentages, but no pollen from *E. vulgare*. No PAs above the limit of detection (LOD) were measured by the chemical analyses. Honey: A polyfloral honey harvested at the end of May 2015 (before the flowering of *E. vulgare*) was used for the preparation of the supplemented pollen provisions to mask the repellent effect of PAs. Bee bread: 400 g of bee bread was harvested in 2016 from various bee colonies. Bee bread was removed from the combs with a metal spatula, carefully avoiding any wax particles, frozen at -20°C and homogenized with an electric mill. Bee bread contained no PAs above the LOD.

**Toxicity of echimidine on newly emerged honeybees**

In total, four artificial provisions were prepared by mixing 2.25 g of bee-collected pollen with 1.00 g of honey. To this pollen/honey mixture 0.5 mg, 5 mg or 25 mg of echimidine was added in 62.5 µL of acetone, resulting in concentrations of echimidine in the provision of 150 µg/g, 1530 µg/g and 7690 µg/g, w/w respectively. These concentrations correspond to an exposure of 2, 20 and 100 µg/bee respectively, assuming that all 50 bees in a cage consume the same amount of provision. The highest experimental echimidine concentration in pollen provisions (7’690 µg/g) was the maximal non-repellent concentration as tested in preliminary experiments. The control provision contained only 62.5 µL of acetone. The amount of pollen consumed by 50 bees within the first six days after emergence was evaluated with feeding experiments prior to our toxicity study. 50 bees consumed approximately 0.65 g of pollen, resulting in 13 mg pollen per bee. Aliquots of 0.65 g provision were offered to 50 bees per cage at day 0 (D0). After one hour, sucrose solution (50:50, w/w) was provided *ad libitum* and replaced every three days. Cages were placed in an incubator at 30°C and 75% relative humidity. Dead bees were removed and counted every

day. The experiment was stopped after all bees were dead. Experiments and controls were conducted in triplicates, for a total of three independent test series (approximately 450 bees per data point).

**Toxicity of PAs on honeybee larvae**

Chronic exposure test series on larvae were performed according to Aupinel *et al*.,[32] with minor modifications. Artificial diets were prepared by dissolving yeast and sugars in MilliQ water. The solution was then filtered (0.22 µm mesh) and combined with royal jelly (see details in Table 1) previously produced at the Swiss Bee Research Centre. Echimidine and echivulgarine were dissolved in acetone and supplemented at equal concentrations in diets A, B and C. The densities of the larval diets increased from diet A to C. Since diets were offered as volumes, PAs were adjusted according to volumes instead of weights (for details see Table 2). For negative controls, 10 µL of acetone was added to the diet. In total, six concentrations of echimidine and five concentrations of echivulgarine were tested. The cumulative PA dose consumed per larva is listed in Table 2, assuming that each larva would consume the entire diet. The plastic cells for hosting the larvae were disinfected with 70% v/v ethanol and dried at 50°C. Cells were then transferred into 48-well tissue culture plates previously filled with cotton dental roll pieces soaked with 500 µL of a 15.5% v/v glycerol in 0.4% v/v MBC solution. At D1, larvae grafted at the first instar stage were placed into the grafting cells containing 10 µL of diet A without alkaloid, before an additional 10 µL of diet A containing the double concentration of the alkaloids was added. Plates were placed into a hermetic Plexiglas desiccator containing a saturated solution of potassium sulfate (96% RH). The desiccator was closed and placed into an incubator at 34.5°C. At D3, larvae were fed with 20 µL of diet B, while at D4, D5 and D6, larvae were fed with 30, 40 and 50 µL of diet C, respectively. Larval growth was observed every day under a binocular magnifier. Dead larvae were discarded and not replaced. Diets that were not entirely consumed by

larvae at D7 were removed with cotton dental rolls. Cells were transferred into a new sterile culture plate and placed into a desiccator containing a saturated solution of sodium chloride (70% RH). The desiccator was closed and placed into an incubator at 34.5°C. At D15, culture plates were individually placed into plastic boxes, together with a piece of honeycomb, until the bees emerged.

**Table 1** Composition of the diets used in the larval tests.

| | DAY 1 | DAY 3 | DAY 4 | DAY 5 | DAY 6 |
|---|---|---|---|---|---|
| **Diet** | **A** | **B** | **C** | **C** | **C** |
| **Volume per larva (µL)** | **20** | **20** | **30** | **40** | **50** |
| **Royal jelly (g)** | 47.6 | 47.0 | 46.6 | 46.6 | 46.6 |
| **Yeast extract (g)** | 1.0 | 1.4 | 1.9 | 1.9 | 1.9 |
| **D(+)-Glucose (g)** | 5.7 | 7.0 | 8.4 | 8.4 | 8.4 |
| **D(-)-Fructose (g)** | 5.7 | 7.0 | 8.4 | 8.4 | 8.4 |
| **MilliQ $H_2O$ (g)** | 40.0 | 37.6 | 34.7 | 34.7 | 34.7 |
| **Total (g)** | 100 | 100 | 100 | 100 | 100 |

**Table 2** Concentrations of echimidine and echivulgarine in the different diets offered to honeybee larvae until D6.

| | PA conc. in the diets (µg/g) | PA in Diet A (µg/larva) | PA in Diet B (µg/larva) | PA in Diet C (µg/larva) | PA in Diet C (µg/larva) | PA in Diet C (µg/larva) | cumulative PA in 7 days (µg/larva) |
|---|---|---|---|---|---|---|---|
| **Volume per larva (µL)** | | **20** | **20** | **30** | **40** | **50** | **160** |
| **Echimidine** | 10 | 0.21 | 0.22 | 0.34 | 0.45 | 0.56 | 1.8 |
| | 15 | 0.31 | 0.33 | 0.50 | 0.67 | 0.84 | 2.6 |
| | 20 | 0.42 | 0.44 | 0.67 | 0.89 | 1.12 | 3.5 |
| | 30 | 0.62 | 0.65 | 1.00 | 1.34 | 1.67 | 5.3 |
| | 40 | 0.83 | 0.87 | 1.34 | 1.79 | 2.23 | 7.1 |
| | 80 | 1.66 | 1.74 | 2.68 | 3.57 | 4.46 | 14.1 |
| **Echivulgarine** | 10 | 0.21 | 0.22 | 0.34 | 0.45 | 0.56 | 1.8 |
| | 20 | 0.42 | 0.44 | 0.67 | 0.89 | 1.12 | 3.5 |
| | 40 | 0.83 | 0.87 | 1.34 | 1.79 | 2.23 | 7.1 |
| | 80 | 1.66 | 1.74 | 2.68 | 3.57 | 4.46 | 14.1 |
| | 160 | 3.33 | 3.49 | 5.36 | 7.14 | 8.93 | 28.2 |

**Transfer of PAs from bee bread into royal jelly**

In total, three independent test series were conducted. Each test series was composed of an experimental and a control colony. Preliminary trials suggested that 60 g of bee bread was sufficient for the needs of the colony. Experimental colonies received bee bread supplemented with echimidine, while the control colonies received bee bread without echimidine. Echimidine was supplemented at a concentration that was not repellent but still realistic for natural conditions, so that bees would feed on large amounts of pollen to produce royal jelly. 120 mg of echimidine was dissolved into 1 mL of acetone and mixed with 60 g of bee bread, resulting in a final echimidine concentration of 2'000 µg/g. As a control, 60 g of bee bread was mixed with 1 mL of acetone. For each colony, 60 g of bee bread was pasted into the wax cells of an empty comb. Larvae at the first instar stage were obtained from three bee colonies and grafted into plastic cells fixed to queen rearing frames. The frame containing the larvae, the comb hosting the bee bread, together with a comb filled with 500 g of a polyfloral spring honey and a comb filled with water, were placed into queen-less colonies in a modified Miniplus system as described above. The colonies were kept at ambient temperature in a room with natural light. After three days, they were transferred to 13°C for 3 h prior to collection of the cells containing royal jelly. Wax caps and larvae were removed from cells containing royal jelly. Cells with royal jelly were detached from the queen rearing frame and stored at -20°C. New cells hosting newly-grafted larvae were glued to the queen rearing frame, and placed back into the colony for the production of a new batch of royal jelly. The procedure was repeated every three days for a total of three harvests per colony. The bee bread remaining after the three harvests was weighed in order to calculate the amount of bee bread consumed per colony.

**Quantification of PAs in royal jelly, bee bread and bee-collected pollen, using UPLC-HRMS analysis**

Royal jelly: 100 mg of royal jelly was weighed using a microbalance scale (Mettler Toledo), mixed with 1'000 µL of extraction solvent A (98% ultrapure water and 2% formic acid, v/v) and transferred into a 2 mL Eppendorf tube. Five glass beads were added, and the tube was shaken at 30 Hz for 4 min. Following centrifugation (18'400 *g* for 4 min), the supernatant was collected and purified on a BondElute SCX SPE cartridge. Cartridges were washed with 1 mL of methanol and conditioned with 1 mL of the extraction solvent A. Samples were loaded onto the column and washed with the extraction solvent A. After drying, samples were eluted into a glass vial using ammoniated methanol,[33,34] and dried at 40°C for 2 h using a centrifugal evaporator (CentriVap, Labconco). Samples were then re-dissolved in 500 µL of a 70% methanolic solution using an ultrasonic bath. 100 µL of the supernatant was transferred into a glass vial containing a 200 µL insert. Bee bread: 10 mg of bee bread was accurately weighed, mixed with 1'000 µL of extraction solvent B (70% methanol, 29.5% ultrapure water and 0.5% formic acid, v/v) and transferred into a 2 mL Eppendorf tube. Five glass beads were added, and the tube was shaken at 30 Hz for 4 min. Following centrifugation (18'400 *g* for 4 min), 10 µL of the supernatant was transferred into a glass vial containing a 200 µL insert and diluted 20 times with the extraction solvent. Bee-collected pollen: 1 mg was accurately weighed, mixed with 100 µL of extraction solvent B (70% methanol, 29.5% ultrapure water and 0.5% formic acid, v/v) and transferred into a 2 mL Eppendorf tube. Five glass beads were added, and the tube was shaken at 30 Hz for 4 min. Following centrifugation (18400 *g*, 4 min), 5 µL of the supernatant was transferred into a glass vial containing a 200 µL insert and diluted 10 times with the extraction solvent. UPLC-HRMS: the detection and quantification of PAs in royal jelly, bee bread and bee-collected pollen was performed according to Lucchetti *et al*. (2016). In brief, the PA analysis was

performed on an Acquity BEH C18 column (50 × 2.1 mm i.d., 1.7 µm particle size, Waters), using an Acquity UPLC$^{TM}$ system (Waters) coupled to a Synapt G2 QTOF mass spectrometer (Waters). The injection volume was 1 µL. The QTOF operated in electrospray positive mode over a mass range of 50−600 Da. A leucine-enkephalin solution at 400 ng/mL was infused throughout the analysis to ensure high mass accuracy (<2 ppm). PAs were identified on the basis of their retention times, exact mass fragmentation and characteristics, and comparison with existing literature and databases containing information on known PAs in *Echium* spp. Quantification was achieved by external calibration using echimidine from Phytolab as standard. Linear responses were obtained from 5 to 4'000 ng/mL. For echimidine, the limit of quantitation (LOQ) was 2 ng/mL (s/n 10) and the limit of detection (LOD) 0.7 ng/mL (s/n 3).

**Statistical analyses**

For statistical analyses, we used the statistical package SPSS 11 (SPSS 2005) for Macintosh OS X. Differences in survival rates of adult bees were compared with Kaplan Meier[35] using a pairwise log-rank test without censored observations, since all bees were dead at the end of the experiment. Every death was considered as a single event independent of each other. Differences in larval survival rates were also compared with Kaplan Meier using a pairwise log-rank test. The larvae that completed the development and emerged as adults were considered as censored observations. Differences between treatment groups in all the feeding experiments were corrected with Bonferroni ($p<0.001$), using the option 'pairwise for each stratum'. Using R statistical package, the effect dose 50 ($ED_{50}$) recorded on D21 was calculated using a three parameter log-logistic function with a lower limit at 0.

## Results

### Toxicity of echimidine on adult bees

We tested the effect of echimidine in the pollen diet by offering provisions supplemented with echimidine to newly emerged adults. Bees consumed control provisions or provisions at 2 µg, 20 µg or 100 µg echimidine per bee within six days. The maximal lifespan of the bees in our assays was 63 days. No relevant mortality was observed within the first 15 days for any of the tested echimidine concentrations and controls. Thus, no acute echimidine toxicity was observed. However, the lifespan of adults fed with echimidine provisions at 100 µg/bee was significantly shortened compared to the lifespan of bees fed with control provisions or provisions at 2 or 20 µg/bee (pairwise log-rank test with Bonferroni corrections, $p<0.0016$) (Figure 2).

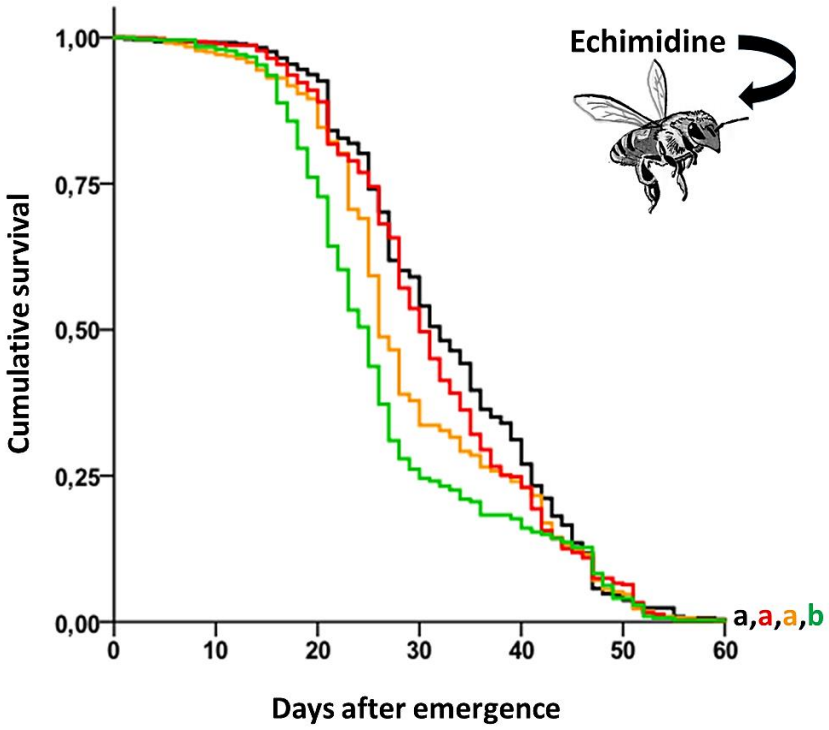


**Figure 2** Toxicity of echimidine on adult bees. Survival of the control group is represented in black (n=459), survival of the bees fed with echimidine 2 µg/bee in yellow (n=451), with

20 µg/bee in red (n=455) and with 100 µg/bee in green (n=448). Results for each concentration are reported as a sum of three test series performed in triplicates. Letters at the end of the curves designate significant difference between the treatment groups (pairwise log-rank tests, corrected with Bonferroni, $p<0.0016$).

**Toxicity of PAs on honeybee larvae**

Chronic exposure test series using diets supplemented with six different concentrations of echimidine were performed on honeybees. No relevant mortality was observed from D1 to D3 for any of the tested echimidine concentrations. When larvae were exposed to a cumulative dose of 14.1 µg echimidine per larva, all the larvae died within nine days (Figure 3). Echimidine at 5.3 µg/larva induced a mortality of 97% until the imago stage, while 50% of the larvae fed with a cumulative dose of 3.5 µg echimidine completed metamorphosis and subsequently emerged as adults (Figure 3). Diets with a total of 3.5 µg echimidine per larva resulted in slightly lower, although statistically significant (pairwise log-rank tests with 99% confidence interval, Bonferroni-corrected, $p<0.0016$) larval survival compared to control diets. However, the emergence rate (74%) was similar to the control (73%). No significant differences ($p<0.0016$) in survival were observed between controls and a diet with a cumulative dose of 1.8 µg/larva. Emergence rates were 73% (control) and 76% (1.8 µg/larva). The $ED_{50}$ recorded on D21 (adult emergence) was 3.81 µg for echimidine.[36] In conclusion, total echimidine doses from 3.5 to 14.1 µg/larva showed a significant dose-related toxicity on honeybee larvae, while 1.8 µg/larva, corresponding to an echimidine concentration of 10 µg per gram of diet, was non-toxic. Chronic exposure tests were repeated with commercially available echimidine from Phytolab and gave comparable results (data not shown).

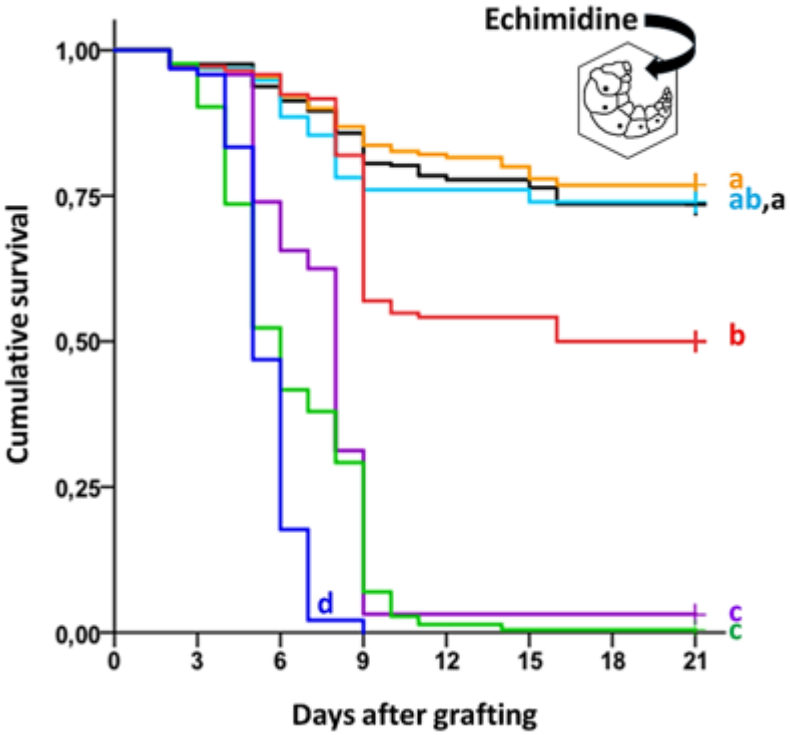


**Figure 3** Toxicity of echimidine on larvae. The survival rate of the control larvae is represented in black (n=288). The survival rate of larvae fed with echimidine 1.8 µg/larva is represented in yellow (n=190), with 2.6 µg/larva in pale blue (n=96), with 3.5 µg/larva in red (n=144), with 5.3 µg/larva in violet (n=96), with 7.1 µg/larva in green (n=216) and with 14.1 µg/larva in blue (n=96). Bioassays were terminated at D21, after bees emerged as adults. Letters at the end of the curves designate significant difference between treatments (pairwise log-rank tests, corrected with Bonferroni, $p<0.0016$). Survival for each concentration is averaged over at least two independent test series.

Chronic exposure test series on honeybee larvae were also performed with echivulgarine. Larval diets were supplemented with five different concentrations of echivulgarine. No relevant mortality was observed from D1 to D3 for all tested echivulgarine concentrations. The majority of the larvae died within nine days when exposed to a cumulative dose of 28.2 µg echivulgarine per larva (Figure 4). This dose induced a mortality of 98% until the imago stage. Mortality was 75% for cumulative doses of 14.1 µg/larva. However, 25% of the larvae

fed a diet supplemented with a total of 14.1 µg echivulgarine per larva went through metamorphosis and subsequently emerged as adults (Figure 4). No statistically significant differences ($p<0.0016$) in survival until the imago stage were observed between controls and diets supplemented with echivulgarine doses of 7.1 µg/larva or lower. Emergence rates were 74% (control), 77% (1.8 µg/larva), 73% (3.5 µg/larva) and 74% (7.1 µg/larva). Hence, echivulgarine doses of 14.1 and 28.2 µg/larva showed a significant dose-related toxicity on honeybee larvae. The $ED_{50}$ recorded on D21 (adult emergence) was 12.53 µg echivulgarine.[36]

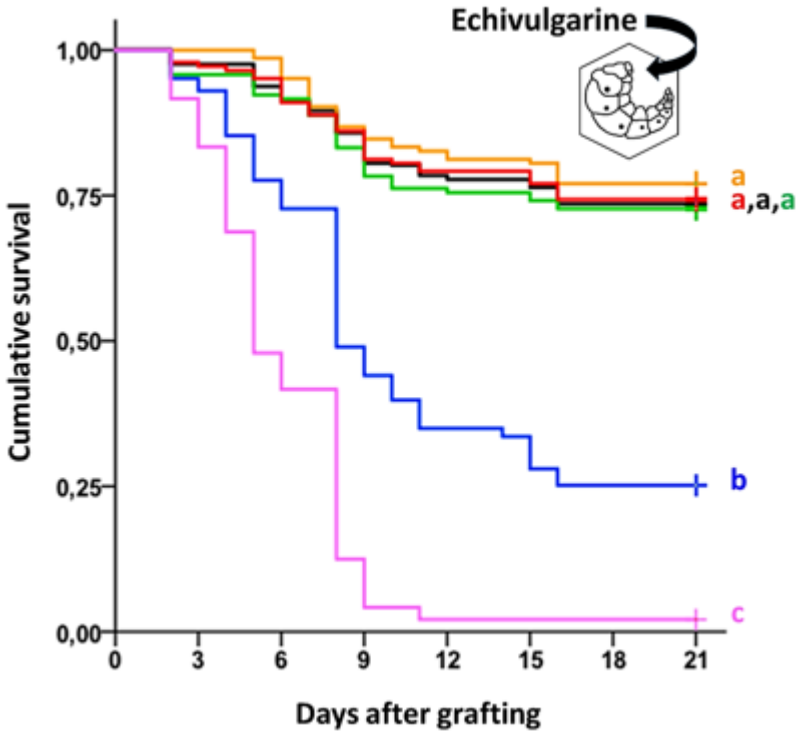


**Figure 4** Toxicity of echivulgarine on larvae. Survival of the control group is represented in black (n=288). Survival of the group fed with 1.8 µg/larva is represented in yellow (n=144), with 3.5 µg/larva in red (n=143), with 7.1 µg/larva in green (n=144), with 14.1 µg/larva in blue (n=143) and with 28.2 µg/larva in pink (n=48). Bioassays were terminated at D21, after bees emerged as adults. Letters at the end of the curves designate significant difference

between treatments (pairwise log-rank tests, corrected with Bonferroni, $p<0.0016$). Survival for each concentration is averaged over at least two independent test series.

**Transfer of echimidine from bee bread into royal jelly**

Modified Miniplus systems were used to study the transfer of echimidine from bee bread into royal jelly. Nursing bees fed on bee bread supplemented with 2'000 µg echimidine per gram. On average, 35.4 g of bee bread was consumed in the experimental colonies and 41.8 g in the controls. Thus, experimental and control colonies consumed similar amounts of bee bread. Nursing bees produced similar amounts of royal jelly, on average 298 mg royal jelly per cell in experimental colonies and 260 mg royal jelly in controls. Echimidine concentrations in royal jelly were on average 3.8, 2.0 and 0.6 µg/g per harvest respectively (Table 3), while echimidine concentrations in royal jelly of the control colonies were below the LOD. The highest measured echimidine concentration was 6.9 µg/g.

**Table 3** Echimidine concentration in royal jelly produced by nursing bees while consuming echimidine (2000 µg/g) supplemented bee bread. Average echimidine concentrations are reported for the first (n=10), second (n=3) and third (n=9) harvest.

| Harvest n° | Echimidine in royal jelly (µg/g) | Range (µg/g) |
|---|---|---|
| **1** | 3.8 (± 1.3) | 2.3 – 6.9 |
| **2** | 2.0 (± 0.2) | 1.9 – 2.3 |
| **3** | 0.6 (± 0.3) | 0.3 – 1.0 |

## Discussion

The impact of pollen secondary compounds on bees remains a largely unexplored field of research.[5,37,38] Two central questions need to be addressed to determine the ecological and evolutionary significance of pollen secondary compounds and to evaluate how pollen secondary metabolites impact pollen foraging behavior in bees. Firstly, do pollen secondary compounds, at realistic doses, impact bee development and fitness at both larval and adult stages? Secondly, can adult bees detect pollen secondary compounds and are they deterred by them? Our study comprehensively addresses the first of these two questions using the honeybee and *E. vulgare* as a model system.

Our most salient result is that at field realistic doses, pyrrolizidine alkaloids (PAs) found in high concentrations in pollen of *E. vulgare* have the potential to strongly impact larval development. Bioassays performed with honeybee larvae reproduced a scenario of chronic exposure to PAs, a situation that would mimic the case of a larva developing on provisions of PAs-containing pollen. Honeybee larvae were particularly sensitive to PAs and toxic effects were observed at an $ED_{50}$ of 3.81 µg per larva for echimidine or 12.53 µg per larva for echivulgarine. Given that pollen of *E. vulgare* contains PAs at concentrations of 0.5-35 µg/mg[10] and that each bee larva requires an amount of protein that would correspond to 125-187.5 mg of pollen,[25] these results indicate that at field realistic doses, pollen secondary compounds of *E. vulgare* would have the potential to impact bee development. Provisions of *Echium* pollen have been previously shown to be toxic to solitary bee larvae,[39,40] although conclusive evidence that the PAs were underlying the mortality is so far lacking. However, honeybees strongly differ from solitary bees in that only about 5% of the protein in the larval diet is directly derived from pollen.[15] The majority of the protein in honeybee larval diets, irrespective of their caste, comes from worker hypopharyngeal and mandibular secretions. Remarkably, the diet of honeybee larvae contains no pollen or only trace amounts of pollen

during the first three days of their development,[14,15] when larvae are more sensitive to PAs than at later developmental stages (See Supporting Information).

Yet the nearly exclusive source of protein for honeybee workers is pollen.[19] Consequently in complex eusocial bees the effects of pollen secondary compounds are potentially important for newly emerged workers, which feed on large quantities of bee bread for the development of their hypopharyngeal glands and for the production of nursing jelly. Our study indicates that adult bees show a substantially higher tolerance to pollen PAs than larvae. Concentrations of echimidine up to 1'530 µg/g pollen had no significant effect on adult survival, corresponding to more than 100 times the lowest concentration in the larval diet (20 µg/g) having a significant effect on larval survival. An echimidine concentration of 7'690 µg/g provision (100 µg/bee), thus comparable to the PA content in pure *E. vulgare* pollen (13'000 µg/g[10]), significantly shortened the lifespan of newly emerged honeybees. Given that numerous pollen types[19] are typically mixed in honeybee colonies to produce bee bread, this effect is probably not relevant in a natural environment. Higher echimidine concentrations could not be tested because such diets had a deterrent effect on honeybees. The high tolerance of honeybee workers to pollen secondary compounds is in agreement with a previous study, where adult bees fed with sucrose solutions containing monocrotaline and a mixture of PAs isolated from *Senecio vernalis*, showed a relatively high tolerance to PAs, and no acute toxicity within 48 h was observed at realistic doses.[28] In agreement with our study, deterrent effects of high PA concentrations have been reported previously,[28,41] although in these studies, the alkaloids were given in sucrose solution and not in pollen.

Given that adult bees tolerated 100 times higher PA concentrations than larvae, the feeding of workers on PA-containing pollen may still have important implications for colony development if the PAs were transmitted to the larvae through hypopharyngeal and mandibular gland secretions. In our third experiment, concentrations up to 6.9 µg/g of echimidine were detected in royal jelly produced by workers fed with bee bread containing

echimidine at 2’000 µg/g. Hence, the echimidine concentration in royal jelly was reduced by at least two orders of magnitude and these low levels indicate that only a fraction of the pollen PAs in the consumed bee bread passes into the larval jelly. Maximal concentrations were below 10 µg/g, a dietary concentration that was non-toxic to larvae (Figure 3, Table 2). Taken together, our results show that even if the levels of PAs in pollen of *E. vulgare* have the potential to negatively impact larval development, honeybee colonies in a natural environment are most likely little affected by pollen secondary compounds of this plant for the following reasons: first, unlike other bees, honeybee larvae feed on diets containing remarkably little pollen, especially during the first few days of larval development; second, adults are much less sensitive to PAs than larvae; third, only a very small fraction of PAs are transmitted into nursing secretions; fourth, adult honeybees were deterred by high concentrations of PAs in pollen. Future research should determine whether adult bees have the ability to perceive pollen PAs, or whether the deterrence is solely the result of the detrimental effect of PAs on their metabolism; fifth, different pollen types are mixed in bee bread and even if one or several pollen types contain secondary compounds, these toxic compounds will be diluted. In solitary bees, pollen dilution has been suggested as a mechanism to reduce pollen toxicity.[42,43]

Our results have important implications for our understanding of pollen utilization by bees. They demonstrate that pollen secondary compounds have the potential to influence bee fitness by impacting larval survival, in agreement with a recent study on the effect of *Lupinus* pollen alkaloids on bumblebee colony development.[38] However, in contrast to honeybees, larvae of solitary bees and bumblebees directly consume pollen and nectar provisions and are thus directly exposed to pollen secondary compounds. A growing number of studies on solitary bees suggest that many pollen types exhibit properties that hamper larval development on non-host pollen,[39,43-46] in striking contrast to studies on honeybees that suggest toxic pollen types are rare.[19] Our results show that honeybees have a unique way

of reducing larval exposure to pollen secondary compounds through larval nursing. Consequently, larval nursing may bypass protective properties of the pollen and allow the broad pollen spectrum that is typical of complex eusocial honeybees.

Finally, while our study focused on plant secondary metabolites, it is worth mentioning that pesticides found in pollen may follow a similar route from flowers to bee bread and from there into hypopharyngeal and mandibular secretions. The current OECD guidelines for testing pesticides on honeybees *prior* to legislation focus on worker and larval toxicity, without considering whether these compounds are transmitted into larval jelly.[36] Hence, our modified, experimental Miniplus hives may serve as a model system to evaluate which types of chemicals pass into the larval diet and hence to which chemicals larvae are exposed.

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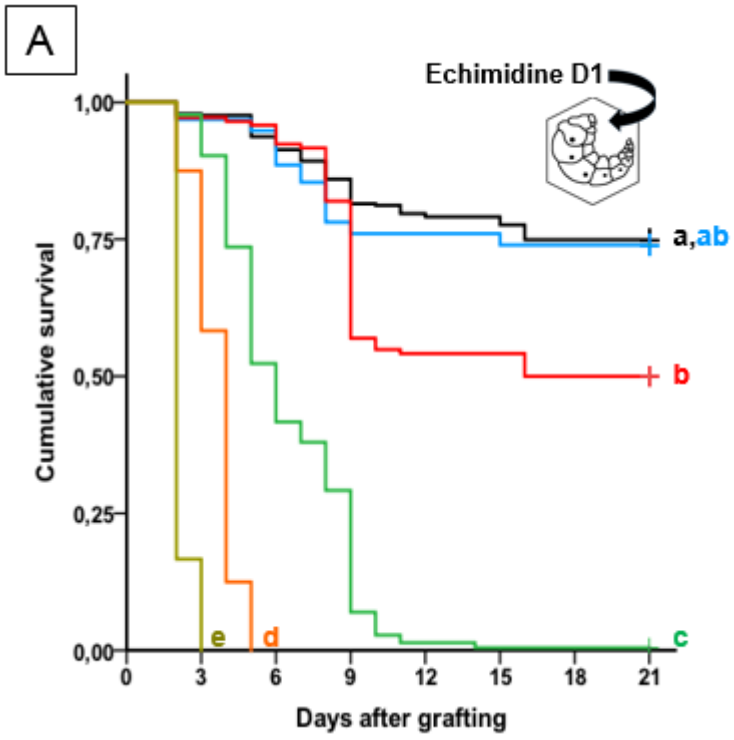
**Supporting Information**

**Figure S1 A)** Toxicity of echimidine on larvae, when echimidine is provided at D1. **B)** Toxicity of echimidine on larvae, when echimidine is provided at D3. The survival rate of the control larvae is represented in black (D1 n=335; D3 n=335). The survival rate of larvae fed with echimidine 2.6 µg/larva is represented in pale blue (D1 n=96), with 3.5 µg/larva in red (D1 n=144), with 7.1 µg/larva in green (D1 n=216; D3 n=24), with 71 µg/larva in orange (D1 n=24; D3 n=24) and with 710 µg/larva in olive green (D1 n=24; D3 n=24). Bioassays were terminated at D21, after bees emerged as adults. Letters at the end of the curves designate significant difference between treatments (pairwise log-rank tests, corrected with Bonferroni, $p<0.0016$). Survival for each concentration is averaged over at least two independent test series.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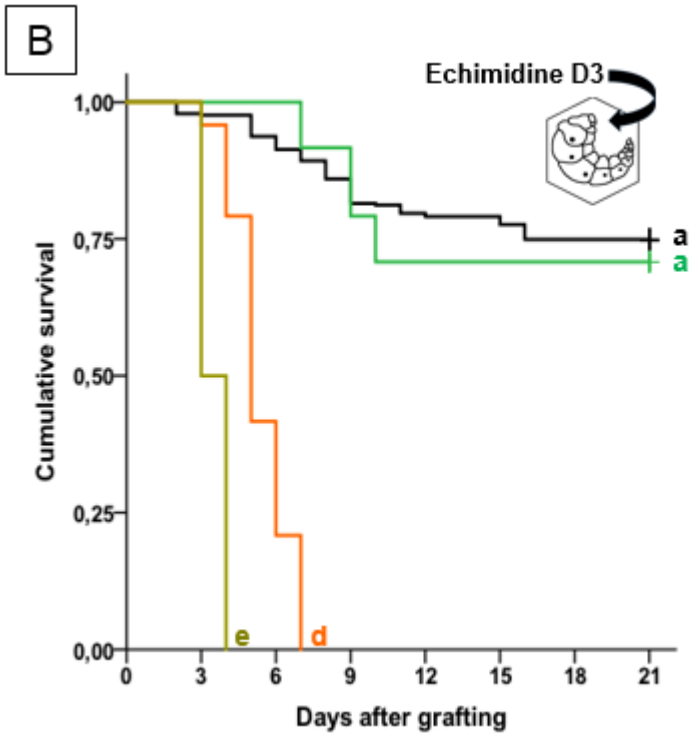
Days after grafting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Days after grafting

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# General discussion

## Analytical challenges of PAs

A prerequisite for the analysis of pyrrolizidine alkaloids is the high sensitivity and selectivity of the method, and due to this, several analytical methods previously employed to detect and monitor PAs are no longer used.[1] LC-MS-based approaches are nowadays considered the most useful. These approaches can reach very low limits of detection and be more informative about PA types than GC-MS-based approaches, which detect the total amount of PAs in a sample, but lose the identity of each single PA. The UHPLC-HRMS system used in this study resulted in an excellent analytical method to identify individual PAs, also because of a non-targeted approach. Contrary to targeted analysis that allows the identification of a specific molecule based on the fragmentation profile and retention time compared to a standard, in non-targeted analysis we have an overview of almost all the molecules in the matrix, allowing the detection of all PAs, whether known or unknown. This information can help to identify the plant species contaminating samples based on the PA profile.

Precise quantification of the PAs is only possible with reference substances that can be used as calibration standard. Of the 660 different PA structures known up to date, only 35 reference substances are commercially available.[2] Since there are more PAs than commercial available standards, some PAs can only be indirectly quantified. Thanks to the extraction and purification of echimidine and echivulgarine performed in our study, we could precisely identify and quantify these two PAs in various matrixes. However, minor PAs and unknown PAs were tentatively identified based on their predicted molecular formula, mass, accuracy, and quantified as echimidine (e.g. *Echium* PAs) or lycopsamine (e.g. *Eupatorium*

PAs) equivalents due to the lack of their corresponding standards.

Furthermore, the analysis of PAs can be problematic due to the complexity of the PA-containing matrix. For example, analysis of PAs in honey, royal jelly and other food products can be difficult to analyze. A clean-up step is necessary to remove the main interfering factors, such as sugars (e.g. in honey), or lipids (e.g. in royal jelly).

Other problems, such as the lack of inter-laboratory consistency and the wide variety of PA isomers, sometimes difficult to separate, may cause misinterpretation of the total PA content of a sample, highlighting the urgent need for reliable systems for food and feed controls. However, UHPLC-HRMS appears to be the most reliable method for the future PA research. Future developments will hopefully see a greater availability of standards and validation of methods.

## PAs in herbal medicine, food, feed, and regulations

Validated methods *ad hoc* for every product that can potentially contain PAs, such as herbal medicines, food (e.g. honey and teas) and feed, are important tools to control the risk of exposure. Nevertheless, limits for PAs in these products must be set by strict regulations. The commerce of PA-producing plants for medicinal purpose in Europe has been regulated. PA-producing plants are often used as medicinal plants in developing countries, where herbs in folk medicine play a more important role than in highly industrialized countries. However, due to the ‘‘Green wave’’, the use of herbal remedies in Europe has increased exponentially, together with the risk of pyrrolizidine exposure. In 1992, the Germany authorities set the limit of 1,2-unsaturated PA intake from herbal medicinal products to 1 µg/day for a maximum of six-weeks period of application. If this period is surpassed, the concentration must be decreased to 0.1 µg per day, while the use by toddlers, pregnant and lactating women is forbidden.[3] This limit was afterwards reduced, in 2016, to 0.35 µg/day by

the Committee on Herbal Medicinal Products (HMPC)[4], and, in Switzerland, will be adopted after a transitional period of three years.[5]

Regarding food, in 2011, both EFSA and the German Federal Institute for Risk Assessment published their data on the presence of PAs in various food products, focusing mainly on honey.[6,7] It was concluded by the EFSA that there is a possible health concern, especially for those toddlers and children consuming high amounts of honey. The BfR recommended not exceeding a daily intake of 0.007 µg/kg of body weight,[7] highlighting a concern for children's exposure. Considering a person who weighs 60 kg consuming 20 g of honey, the maximum PA concentration of that honey should not exceed 21 µg/kg. Even if only a recommendation, this value is often used as reference for European honeys.

In a recent comprehensive study supported by EFSA,[8] various samples of animal- and plant-derived products, including dairy products, eggs, meat, teas and herbal food supplements, all commercialized in Europe, were analyzed for the presence of PAs. The majority of the animal-derived products analyzed were found to be contaminated with low concentrations of PAs, while teas, especially black, rooibos and chamomile, were frequently found to be contaminated with PAs at high concentrations. From all of the evidence presented, teas represent the major source of PAs in the European population's diet. However, honey also represents a potential source of PAs, and both require data and safety monitoring.[9]

Tea and honey have the potential to pose a health risk when consumed over longer periods (chronic toxicity). Previous studies conducted on honey from various geographical and botanical origins have shown concentrations of PAs up to 3'900 µg/kg.[10-13] Concentrations from 2'850 to 13'000 µg/kg of a single PA-type were found in monofloral honeys from *E. vulgare* or *plantagineum* or *S. jacobaea*, which were surpassing more than 600 times the limit of PA concentrations recommended for PAs by the BfR. Moreover, the PA content can

vary between different years at the same locations of harvest. From our study conducted in Verzasca, the PA content of honey produced at the same location was ranging from 2 µg/kg to 153 µg/kg in different years. Consequently, in some years, the PA concentration in these honeys was surpassing the maximal concentration recommended by the BfR, while in other years, the PA concentration was below the recommendation. In 2013 in particular, the PA concentration detected in honey was 7 times higher than the recommendation, even if the honey was a polyfloral honey. High variations of the PA content were also noticed between different apiaries at the same location. In general, honeys produced in Switzerland do not pose a risk for human health. General recommendations currently followed by European countries try to minimize the PA intake and consequently reduce the risk of long-term adverse effects. The recommendation also involves those food products with a low PA content (e.g. eggs, milk and meat) that may still pose a health risk when consumed frequently.[14-18] Since PAs have been found in a wide range of food products, and due to the wide applications of honeys as a sweetener and high volumes of tea consumed in Europe, the risk of a substantial PA exposure may considerably increase in case of cross-consumption of various products containing PAs. It is recommended to minimize the exposure to PAs to the lowest level achievable,[19] due to their long-term carcinogenic potential. A careful selection of honeys based on production time, geographical origin, together with melissopalynological and chemical analyses, is necessary to exclude those honeys with a high PA content that can potentially result in long-term adverse effects on humans.

However, the detection and quantification of all the PAs potentially contained in honeys is complicated due to the lack of reference substances and the existence of PAs not yet described. Surveys of the landscape surrounding the apiaries may help avoiding PAs in honey. PA-plants in the vicinity of the apiaries usually do not pose a big risk if they are not

abundant or if other attractive plants are blooming simultaneously. In Switzerland, the flowering period for relevant PA-plants, such as *Senecio* spp., *Echium* spp. and *Eupatorium* spp. starts in late spring or early summer, based on field observations. Consequently, honeys harvested in spring are most likely free from PAs. Techniques such as filtration of honey to remove PA-containing pollen may be inefficient, as according to our study where floral nectar, rather than pollen, was found to be the major source for PAs in honeys. This important result was obtained through the comparison of the PA spectrum of the two sources with the PA spectrum of honey, and the estimation of the concentration or dilution of these two sources for the production of honey. New effective systems to reduce the risk of PAs in food and feed are required in order to protect consumers' safety.

One way to avoid PAs in food other than honey, such as meat, eggs and milk, would involve measures to avoid PAs in feed causing human's indirect exposure. In a study performed in Mexico,[20] common storing methods such as drying to produce hay, pelleting, ensilaging and composting were tested for their efficacy in reducing the content of PAs when present as contaminants in the starting material. Methods involving only physical processes were found to be the least effective in PA reduction, while ensilaging and composting, involving chemical and biological activities, resulted in a drastic reduction of the PA-content. The reduction found by these methods might be enough to prevent acute intoxications mainly in feed, but do not guarantee the safety of the product for long-term consumption. One year later, the FAO (Food and Agriculture Organization) released a Code of Practice containing various mechanical-based, chemical-based and biological-based approaches to help farmers to prevent and reduce the PA contamination in weed-derived food and feed.[21]

## Evolution and spread of PA-producing plants in Europe

Current regulations, together with scrupulous analysis of PAs in food and feed, play a key

role in protecting human's safety. However, an increasing presence and adaptation of PA plants has been recently observed in Europe, raising up the alert levels for the humans' and ecosystems' health. Many PA plants are pioneers, and are thus able to settle in alien environments and potentially become invasive.[22] *Senecio inaequidens*, an invasive PA plant, nowadays represents a serious concern in various European countries due to its high rates of spreading.[23,24] Moreover, hybridization events have occurred between PA-producing plant species. Hybrid plants can produce a new pattern of PAs or increase the PA content in their tissues.[25,26] The spread due to climate change and the introduction of new plant species influencing the ecosystem should be monitored. As challenge for future production programs, new methods allowing strict controls on the PA content in food and feed should be established.

## Secondary metabolites and plant-insect interactions

Despite the spread and adaptation of PA-producing plant in Europe pose an increasing concern, some herbivores may will be advantaged from this scenario. Cases of plant-herbivores interactions and mutualistic associations involving plant secondary metabolites are the demonstration of the coevolution occurring between plants and animals. Beside their role as feeding deterrents for most herbivores, secondary metabolites represent a source that some animals, especially insects, require for their survival. In fact, a wide variety of insects have evolved strategies or adaptations not only to overcome the defense systems of these plants, but also to utilize them for their own advantage. Among Lepidoptera, many species utilize PAs as their acquired defense mechanisms after their sequestration, usually in their N-oxide forms, from plants into the body of the insect.[27-29] Others utilize PAs for egg protection[30,31] or to develop sexual organs (e.g. *Coremata*).[32] The chemical transformation of PAs into pheromones for reproductive purposes is another strategy that other Lepidoptera have developed (e.g. Arctiidae and Danainae).[27,28,33] Other insects, such as the moth *Tyria*

*jacobaeae*, have developed a multisubstrate flavine monoxygenase that efficiently convert the PAs into their N-oxide forms.[34,35]

Although the role of plant secondary metabolites in leaves and roots as defense against herbivory, the role of secondary compounds in plant pollen and plant nectar, considered as floral rewards for pollinators, remains unclear.[36] Plants may use chemical compounds to protect accessible floral rewards against excessive harvests that can impede their reproduction.[37,38] It has also been postulated that some secondary compounds in pollen play a role in attracting specialized bees.[39] From the pollinator's prospective, floral rewards are a fundamental source of protein and carbohydrates, and the presence of secondary compounds in them may have positive or negative effects on pollinators' fitness. In a recent study it was demonstrated that nectar secondary metabolites could reduce parasite infections.[40] Negative effects on the fitness and larval development of various pollinators were also recorded.[36,37,41-43] For example, larvae of several bee species not specialized to the plant genus *Ranunculus* failed to develop on *Ranunculus* pollen.[37,38] The pollen of *Ranunculus* contains high levels of the toxin ranunculine, and thus it has been hypothesized that the high mortality was due to pollen secondary compounds. Pollen diets supplemented with high concentrations of ranunculin were toxic to non-specialist solitary bee larvae. However, at concentrations mirroring the natural concentrations in *Ranunculus* pollen, ranunculin had no toxic effect on the larvae, suggesting another, ranunculin-independent mechanism responsible for the observed toxicity,[43] such as the lack of essential nutrients, the poor protein content, difficulty in the pollen digestion or the presence of another unknown toxic compound.[37] These studies have shown that pollen is not a universally exploitable resource for all pollinators. In both cases, chemical composition of floral rewards appears to impact pollinators' performances. Pollinators may cope with secondary compound thanks to specific biochemical-physiological mechanisms (detoxification, conjugation, sequestration,

target-site insensitivity, rapid excretion, and endosymbionts), or thanks to behavioral mechanisms (deterrence or avoidance, diet mixing, storage).[44] Biochemical-physiological mechanisms are typical for specialized pollinators foraging nearly exclusively on some toxic plants;[45] these mechanisms provide in specialists a greater tolerance to defense compounds than in generalists. The advantage of such specialization is likely the access to competitor-free resources; however, these specialized pollinators are dependent on a single plant species. Diet mixing[46,47] as well as the production of nursing secretions (chapter 3) are possible strategies that eusocial and generalist honeybees use to reduce the concentration of secondary compounds in their diet. Generalist behavior, in contrast to specialization, allows the pollinators to exploit different plant species, thereby compensating unfavorable properties of floral rewards,[45,46] but at the same time increases the risk of exposure to a wide range of plant secondary metabolites. However, generalist pollinators may also have physiological adaptations to cope with unfavorable chemical compositions in plant rewards,[38] demonstrating how little is known about pollen chemistry and its impact on pollinator fitness. Future research should examine the chemical composition of nectar, pollen and the physiological adaptations that pollinators develop to overcome the unfavorable properties of plant rewards, in order to better understand the mechanisms of coevolution occurring between plants and insects.

## Final considerations

Natural products are often considered healthy. However, efforts to limit PAs in food and feed are necessary to reduce the contamination risk and to guarantee the consumers' safety. On the other hand, PA-producing plants are a natural element of the European environment; such plants play an important role for the maintenance of biodiversity since various insects are specialized or rely on them for their development. A strict legislation, together with the monitoring and the education of stakeholders (consumers, farmers, apiarists) are necessary

key elements to provide consumers' safety, while various levels of transdisciplinary research are essential requirements to understand more deeply the importance of PAs in nature, and to learn how to deal with them.

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# Appendix 1

## Extraction and purification of PAs from *E. vulgare*

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**Abstract**

*E. vulgare* is a common PA plant of European agro-ecosystems and was therefore chosen as model system in this doctoral study. It is a major contributor for the PA contamination of bee products in Europe.[1,2] Main alkaloids produced at different concentrations in its tissues and found in bee products are echimidine and echivulgarine, together with their N-oxides. *E. vulgare*'s alkaloids were extracted and purified from plant material (leaves and inflorescences) collected at different locations in Switzerland and in different years. After lyophilization of plants, extraction in methanol, reduction of the N-oxides with zinc dust to tertiary bases, and acid-base liquid-liquid extraction, tertiary PAs were separated using a semi-preparative system, evaporated and lyophilized once more. For analysis and bioassays, PAs were adjusted according to their purity level.

**Keywords:** *E. vulgare*, extraction, purification, echimidine, echivulgarine, lyophilization, acid-base liquid-liquid extraction, pyrrolizidine alkaloids.

## Chemicals used to extract and purify PAs from plants

Methanol (technical grade) was purchased from Thommen-Furler (Rüti bei Büren, Switzerland). Methanol (HPLC grade), formic acid (purity 98%, w/w), zinc dust (purity ≥ 98%, w/w), sodium chloride (purity ≥ 99%), acetonitrile (HPLC grade), ammonium hydroxide solution (≥ 28% in water, purity ≥ 99%, w/w), celite filter aid treated with sodium carbonate and flux calcined were all from Sigma-Aldrich (Steinheim, Germany). MilliQ water was obtained from a Millipore system. Sodium sulfate anhydrous was purchased from Fluka Chemie (Buchs, Switzerland), and dichloromethane from Honeywell (Seelze, Germany). 270 mm filters were obtained from J.C. Binzer (Hatzfeld, Germany).

## Extraction and purification of echimidine, acetylechimidine and echivulgarine from *Echium vulgare*

Plant specimens of *E. vulgare* were collected at various locations in Switzerland (Basel, Bern, Thun and Verzasca valley), at various points of time during the blooming season of *E. vulgare* from June until August 2013 and 2014. Specimens were collected by grasping the base of the plant with a gloved hand and pulling the closed hand up the stem to collect the majority of the inflorescences (stamens, pistils, petals and sepals) and leaves of the plant. Specimens were preserved with dry ice after collection, subsequently stored at -80°C and later lyophilized for 48 h. The dry stock (0.2 kg) was milled (Frittsch pulverizette), and extracted with 6 L technical methanol under continuous stirring for 24 h at 25°C. The supernatant was collected and filtered on a 270 mm paper filter, while the pellet was re-suspended in 3 L methanol and extracted one more time. Supernatants were combined and the solvents evaporated (Büchi rotavapor). To separate the chlorophylls, the dry extract (139

g) was reconstituted in 600 mL of a solution of 39.5% methanol, 60% water and 0.5% formic acid (v/v), stirred in an ultrasonic bath for 60 min and filtered. PA N-oxides were then reduced to tertiary bases with zinc dust.[3] After conversion, zinc dust was filtered out with celite powder on a membrane. An acid-base liquid-liquid extraction was performed on the PA-containing solution. For this, the pH was adjusted to 9.5 with ammonium hydroxide and dichloromethane (approximately one third of the volume of the PA-containing solution) was added together with the PA-containing solution in a separator funnel. Saturated sodium chloride was added to increase the separation efficiency. The funnel was shaken vigorously multiple times and the dichloromethane phase collected. The procedure was repeated three more times with additional dichloromethane. Sodium sulfate was added to dry the organic solution and the solvent of the combined extracts was evaporated, which yielded an oily residue. The residue was reconstituted in acetonitrile:water (70:30, v/v), divided into aliquots of 500 µL, and loaded onto a semi-preparative system composed of a 1525 EF pump (Waters, Milford, MA, USA) and a dual wavelength UV detector (2487, Waters) paired with a semi-preparative UV cell (path length 3 mm). The separation was performed on an XTerra MS C18 column (19 x 150 mm, 5 µm, Waters) using the following gradient conditions in water + formic acid 0.05% (solvent A) and acetonitrile + formic acid 0.05% (solvent B): 5% B for 3.15 min, 5-40% B from 3.15-52.27 min, 40-100% B from 52.27-59.00 min, holding at 100% B from 59.00-70.00 min, re-equilibrating at 5% B from 70.50-84.50 min. The flow rate was set as follows: 0.00-59.00 min at 8.0 mL/min, 59.50-84.00 min at 13.0 mL/min, and back to 8.0 mL/min at 84.50 min. UV detection was performed at 195 nm. The fractions were collected every minute in 13 x 100 mm glass tubes using a Gilson FC203B fraction collector. An aliquot of the fractions was re-injected in UHPLC-QTOFMS for confirmatory analysis. HPLC fractions containing exclusively either echimidine or acetylechimidine or echivulgarine were combined, evaporated and lyophilized. In total, from approximately 4 kg of fresh plant material, 1.08 g of echimidine, 57.5 mg of acetylechimidine and 53.2 mg of echivulgarine

were isolated. The purity level of echimidine (94%), acetylechimidine (92%) and echivulgarine (62%) was determined by UPLC-HRMS using echimidine from Phytolab as standard (Figure 1). No other PA peak was detected in the purified echimidine, acetylechimidine and echivulgarine. For feeding experiments, concentrations of the isolated PAs were corrected for their purity level.

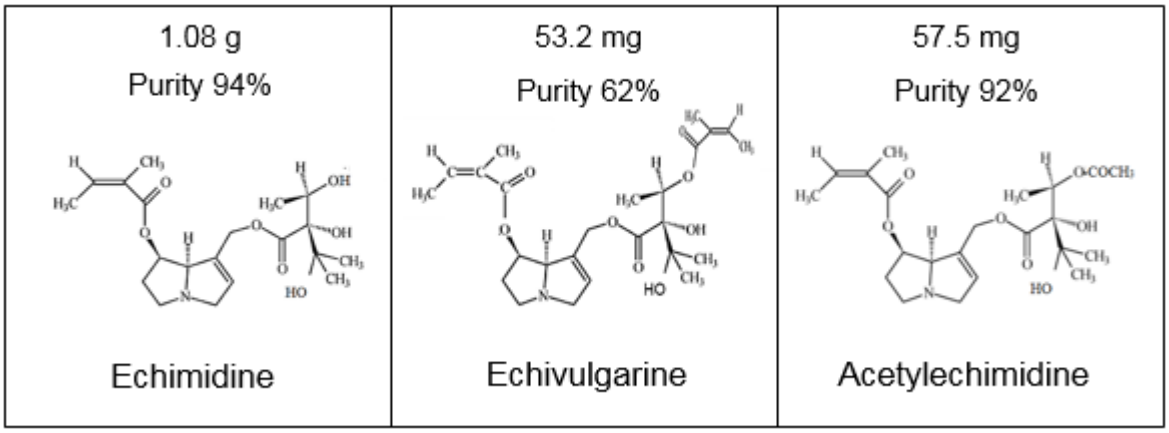


**Figure 1** Amounts and estimated purity of the echimidine, acetylechimidine and echivulgarine extracted.

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## Publications

Lucchetti MA, Kast C, Glauser G. 2017 *Pyrrolizidine alkaloid profiling in plant pollen by UHPLC-HRMS*. In: The Coloss Beebook, Vol. 3, International Bee Research Association, Cardiff, UK. (In press)

Lucchetti MA, Glauser G, Kilchenmann V, Dübecke A, Beckh G, Praz C, Kast C. 2016 Pyrrolizidine alkaloids from *Echium vulgare* in honey originate primarily from floral nectar. *J. Agric. Food Chem.* 64, 5267–5273. (doi:10.1021/acs.jafc.6b02320)

Lucchetti MA, Kilchenmann V, Glauser G, Praz C, Kast C. 2017 Larval nursing protects honeybee larvae from pollen secondary metabolites. *Proc. R. Soc. B* (submitted)

## Presentations & Posters

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| May 2014 | Annual Ph.D. students meeting 2014 (poster) |
| September 2014 | International symposium on bee products (IHC), 3rd edition, Opatija, Croatia (poster and oral presentation) |
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# Curriculum Vitae

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