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The nutritional constraint of sterols in the origin and evolution of bees

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Résumé

Contrairement aux guêpes apoïdes prédatrices dont elles sont issues, les abeilles se nourrissent exclusivement de ressources florales (nectar et pollen). Elles sont apparues il y a près de 130 millions d'années, et ont ensuite connu une radiation évolutive exceptionnelle associée à un élargissement des choix floraux de l'état spécialiste ancestral vers le généralisme alimentaire.

Parmi les contraintes évolutives imposées par ces transitions, la présence de stérols dans la diète (constituants essentiels des membranes cellulaires et précurseurs d'hormones de mue) semble être un élément important. Alors que la plupart des insectes, incapables de produire les stérols *de novo*, obtiennent du cholestérol par une alimentation carnivore, les abeilles n'ont souvent accès qu'à des phytostérols comportant des atomes de carbone supplémentaires. Si certaines espèces sont capables de synthétiser des hormones de mue alternatives à base de ces phytostérols, d'autres semblent contraintes à synthétiser des hormones classiques en utilisant des stérols à 27 carbones similaires au cholestérol.

Dans cette étude, nous avons collecté des provisions larvaires de 20 espèces d'abeilles provenant de 6 familles différentes, ainsi que des proies de 3 espèces de guêpes apoïdes, et avons analysé leur contenu en stérols. Nous avons étudié les relations entre stérols des diètes, phylogénie et choix floraux, afin de mieux comprendre les contraintes imposées par ces nutriments dans : **1)** la transition carnivore-pollinivore ; **2)** la transition spécialiste-généraliste.

Nos résultats ont montré de fortes similarités entre certaines proies de guêpes apoïdes et le pollen collecté par 3 espèces provenant de familles d'abeilles différentes. Ces similarités étaient dues au taux de cholestérol élevé dans le pollen d'Asteraceae récolté par ces espèces, et n'étaient pas spécifiques à la famille basale des Melittidae. Nous avons constaté des différences de tendances générales entre le pollen collecté par les espèces spécialistes et les espèces généralistes. Les premières étaient généralement associées à des stérols à 27 carbones, alors que les secondes présentaient des patterns plus variés, souvent associés à des phytostérols communs. Nos résultats supportent que les stérols pourraient avoir un rôle important dans les interactions plantes-pollinisateurs, et qu'ils représentent une contrainte à la transition évolutive vers le régime généraliste.

Summary

Unlike the predatory sphecoid wasps from which they are derived, bees feed exclusively on floral resources (nectar and pollen). They emerged almost 130 million years ago, and then underwent an exceptional evolutionary radiation, associated to a broadening of floral choices from the ancestral specialist state to a generalist diet.

Among the evolutionary constraints imposed by these transitions, the presence of sterols in the diet (essential constituents of cell membranes and precursors of moulting hormones) appears to be an important factor. While most insects, unable to produce sterols *de novo*, obtain cholesterol from a carnivorous diet, bees often only have access to phytosterols containing extra carbon atoms. While some species are able to synthetise alternative moulting hormones with these phytosterols, others appear to be forced to synthesise conventional hormones using 27-carbon sterols similar to cholesterol.

In this study, we collected larval provisions from 20 bee species from 6 different families, as well as prey from 3 sphecoid wasp species, and analysed their sterol content. We studied the relationships between sterols from the diet, phylogeny and floral choices, in order to gain a better understanding of the constraints imposed by these nutrients in: 1) the carnivorous-pollinivorous transition; 2) the specialist-generalist transition.

Our results showed strong similarities between sphecoid wasp prey and pollen collected by 3 species from different bee families. These similarities were due to high cholesterol levels in the Asteraceae pollen collected, and were not specific to the basal Melittidae family. We found differences in general trends between pollen collected by specialist and generalist species. The former were generally associated with 27-carbon sterols, whereas the latter showed more varied patterns, often associated with common phytosterols. Our results support the idea that sterols play an important role in plant-pollinator interactions, and that they represent a constraint on the evolutionary transition to generalist diet.

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Glossary and abbreviations

Glossary:

Clade:	Monophyletic taxon.
Diapause:	Phase genetically determined, of reduced metabolic activity in the development of an organism.
Monophyletic:	In the classification of living organisms, a taxon is monophyletic when it includes a common ancestor and all its descendants (nouns: monophyly).
Paraphyletic:	In the classification of living organisms, a taxon is paraphyletic when it includes a common ancestor and some of its descendants (nouns: paraphyly).
Polyphyletic:	In the classification of living organisms, a taxon is polyphyletic when it groups several taxa without including their common ancestor (nouns: polyphyly).

Abbreviations:

a.m.u.:	Atomic mass unit
SD:	Standard deviation
s. str.:	Sensus stricto
RT:	Retention Time
TMS:	Trimethylsilyl-

1. Introduction

1.1. What are Apoidea?

Within the Hymenoptera order, species with females possessing a stinger (modified ovipositor) capable of injecting venom form the Aculeata clade. The latter is divided in nine super-families: Apoidea (*e.g.*, bees), Chrysidoidea (*e.g.*, cuckoo wasps), Vespoidea (*e.g.*, common wasps and hornets), Pompiloidea (*e.g.,* spider wasps), Formicoidea (ants), Sierolomorphoidea, Thynnoidea, Tiphioidea and Scolioidea. These latter six taxa were previously included in the Vespoidea, until recent molecular data revealed the paraphyly of this group (Pilgrim *et al.*, 2008; Branstetter *et al*., 2017).

The Apoidea super-family is monophyletic and appeared 185 million years ago according to Sann *et al*. (2018). It includes Anthophila (bees *s. str.*) and the 'sphecoid wasps', which together represent over 30,000 described species (Pulawski, 2021; Ascher & Pickering, 2023).

1.1.1. The Sphecoid wasps

The 'sphecoid wasps', which refers to the taxa within Apoidea that do not belong to bees, form a paraphyletic group. The 10,100 species of sphecoid wasps described nowadays are traditionally spread in four families: Ampulicidae, Sphecidae *s. str.*, Crabronidae and Heterogynaidae (Melo, 1998; Brothers, 1999; Pulawski, 2021). Research by Sann *et al.* (2018; 2021) confirms monophyly of Ampulicidae and Sphecidae but supports polyphyly of Crabronidae (Fig. 1). The exact phylogenetic situation of Heterogynaidae has often been discussed and remains unclear (Ohl & Bleidorn, 2006; Lohrmann *et al.*, 2008; Sann *et al.*, 2018; 2021).

While adults feed mainly on flower nectar, most sphecoid wasps hunt arthropods to feed their larvae, and some are parasitic on other wasps. The only known exception is the genus *Krombeinictus* (Crabronidae, Crabroninae), in which larvae are fed with pollen and nectar (Krombein & Norden, 1997, cited in Michener, 2007). The type of prey targeted by sphecoid wasps varies greatly between species. Those of the genus *Aphilanthops* (Crabronidae, Philanthinae) are very specialised and only hunt ant queens from the genus *Formica* (Evans, 1962). Some others are generalist hunters like *Glenostictia scitula*

(Crabronidae, Bembicinae) which captures preys in at least 27 families of Hemiptera, Diptera and Hymenoptera (Evans, 1966, cited in O'Neill & Evans, 1982).

Sphecoid wasps are predominantly solitary, although different levels of sociality have been observed in some taxa (*e.g.*, Matthews, 1968; Eberhard, 1972). While most species build their nest in burrows that females excavate, some use pre-existing cavities (hollow stems, snail shells, ...) or build small external structures with mud. Each brood cell usually contains a single egg, which the female supplies with paralysed preys (O'neill, 2008).

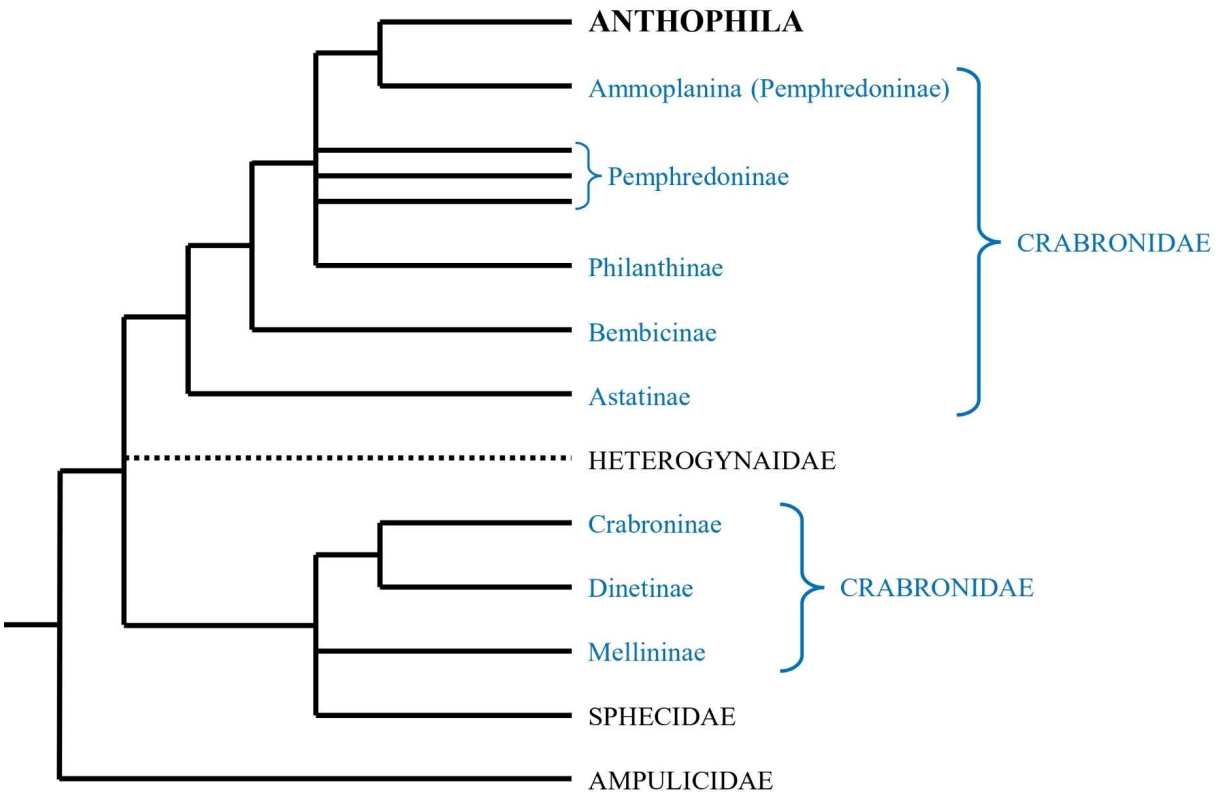

Figure 1: Apoidea phylogeny according to Sann *et al.* (2018; 2021). In blue, the different clades currently grouped in the family Crabronidae. Dotted lines indicate uncertain phylogenetic positions.

1.1.2. The Bees

Anthophila (= bees *s. str.*) is a clade strongly supported by ethological, morphological, and genetic evidence. More than 20,000 species are known and are classified into seven families (see section 1.2.4.). They emerged from within the sphecoid wasps (Fig. 1) about 128 million years ago (Sann *et al.*, 2018). One of the main characteristics of bees is their close mutualistic relationship with flowering plants. While the larvae of sphecoid wasps are

carnivorous, bees are exclusively phytophagous and feed their offspring with pollen and nectar (except for a few species of Meliponini, see section 1.2.3.) (Michener, 2007).

1.2. General information on bees

For most people, bees are honey-producer insects, living in large colonies with their queen. However, this is only true for very few species, including the well-known and domesticated western honeybee (*Apis mellifera*) (Michener, 2007). In fact, most of the other species have completely different lifestyles and remain barely studied.

1.2.1. Sociality

While a large majority of species is solitary or at most communal (*i.e.*, several females sharing a nest, but each are looking after their own offspring only), there is a marked tendency to sociality in bees (Michener, 2007). This behaviour emerged four times in independent clades (Santos *et al*., 2019). Species of the *Apis* genus and those of the Meliponini tribe (Apidae) display the highest level of sociality. They form colonies that can reach tens of thousands of individuals, with differentiated castes having very distinct morphologies and roles: the queen, which is exclusively responsible for egg-laying; the (sterile) workers, which are in charge of all other tasks (harvesting resources, feeding larvae, maintaining and protecting the nest); and the males, which are committed to reproduction only. Such an organisation, with sociality being essential at any time of the life cycle, is called highly eusocial (Michener, 2007). Social species in which part of the cycle is solitary are considered as primitively eusocial, like Bumblebees. In these species, the young queens establish their colonies and are in charge of all the tasks until the first workers emerge, and then devote themselves solely to laying eggs. Sociality is not always obligatory, some species may be solitary in some regions or conditions, and social in others (Michener, 2007).

1.2.2. Nesting behaviour and life cycle

The main structures of a bee nest are the brood cells, where the eggs are laid and develop. Except in species which feed their larvae progressively (*e.g*., *Apis, Bombus*), each brood cell will contain enough food provision for one larva to grow (Michener, 2007). Nesting patterns are very varied in bees (Fig. 2). Most species excavate burrows in the soil, some others dig in dead wood or in pithy stems, and many others settle in pre-existing cavities (*e.g.*, empty stems, snail shell, old nests of other species). Several taxa use foreign material to line or partition the brood cells (*e.g.*, leaf, petal, vegetal wool, resin, mud), and some build

simple external structures with mud. The most complex nest architectures are found in eusocial species of the subtribe Meliponini (Apidae) (Linsley, 1958; Michener, 2007). There are also several taxa of parasitic bees, which do not build nests but lay their eggs in those of other species. The larvae of the parasites usually kill those of the host and consume their provisions (Michener, 2007).

Like other holometabola, adult bees differ greatly in comparison to their juvenile stages. The egg hatches into a larva which grows by feeding on its brood cell provisions. At the end of the last larval stage, pupation begins and make the transition to the adult form (Michener, 2007). Most solitary bees emerge during spring or summer and are active for a short period of time. Firstly, during this activity period, males and females mate. Then, the females build their nests, lay eggs and provision the brood cells with pollen and nectar. The adults then die, and the offspring enter diapause until the following year. There are many variations from this archetypal life cycle: some species can have two generations per year, other can remain in diapause for several years in arid regions, waiting for favourable conditions (*e.g.*, Rozen, 1990; Houston, 1991). In many social species such as bumblebees, it is the fertilised females that enter diapause at the end of the season. Finally, some eusocial species such as the honeybee and other tropical bees simply do not enter diapause (Michener, 2007; Santos *et al*., 2019).

1.2.3. Relationship with flowering plants

Angiosperms (flowering plants) became the dominant plants in terrestrial ecosystems towards the end of the Cretaceous period and now reach 352,000 species described (The Plant List, 2013). Their evolutionary radiation was concomitant with that of bees, and these two clades seem to have benefited from each other's diversification (Grant, 1994; Cardinal & Danforth, 2013). Anthophila are the most specialised group of pollinators, with virtually all bees depending exclusively on floral resources and showing specific adaptations for their exploitation. This explains why bees constitute a major pollinator group, on which most wild and cultivated flowering plants depend (Ollerton, 2017; Hristov *et al*., 2020).

Flower nectar is rich in carbohydrates and is the main source of energy for bees. It is consumed by adults and mixed with pollen in the brood cell provisions. Bees have a proboscis (‘tongue’) which varies in size between taxa and is adapted to reach the nectaries of flowers (Michener, 2007). Certain orchid bees (Euglossini, Apidae) have a tongue twice as long as their body allowing them to visit flowers with very deep corollas, inaccessible to other bees

(Düster *et al*., 2018). As some flowers produce oils rather than nectar, some bee species show adaptations and use this energy source as a substitute or supplement to nectar in larval feeding (*e.g.*, Melittidae of the *Rediviva* genus, some of which have extremely elongated forelegs to collect oil from *Diascia* floral spurs) (Michener, 2007).

Pollen provides proteins, lipids, and macronutrient that are essential for the development of larvae. Specimens in the adult stage need little or no pollen, as they no longer grow (Day *et al*., 1990; Michener, 2007). Females have structures adapted to pollen transport called scopa (except in parasite taxa). These usually take the form of dense bristle brushes, located on the hind legs in most taxa and under the abdomen in the Megachilidae. Some Apinae (*e.g.*, honeybees, bumblebees) have instead a hairless, enlarged and concave surface at the end of the tibiae ('corbicula') which allows pollen to be transported in dense pellets. Several other secondary structures allow them to handle and transfer pollen from the body to the scopa (Thorp, 1979). However, a few species within the tribe Meliponini (Apidae) are exceptions and are known to be necrophagous (Camargo & Roubik, 1991). These species have a microbiota with a higher proportion of acidophilic bacteria, lowering the pH of their digestive tract and probably preventing infections (Figueroa *et al*., 2021).

Bees do not forage randomly, they can have well-defined floral choices. While some species are generalists and can use pollen from a wide variety of sources, others are restricted to a specific taxonomic group of flowers. This is true for many solitary species with a short life cycle, for which it is possible to forage on flowers that bloom during their active period only. Those species that collect pollen from only one plant family are called oligolectic. In contrast, those that forage on flowers from several different families are called polylectic (*e.g.*, Müller 1996). This is the case for some solitary species and almost all eusocial species. The latter are active for a longer period and must be able to exploit pollen from plants flowering at different times (Michener, 2007). Although the terms oligolectic and polylectic are widely used, they poorly reflect the continuum of floral preferences that exist between the most specialist and the most generalist species. Therefore, a detailed lexicon was proposed by Cane & Sipes (2006) to better described the different levels of floral preferences.

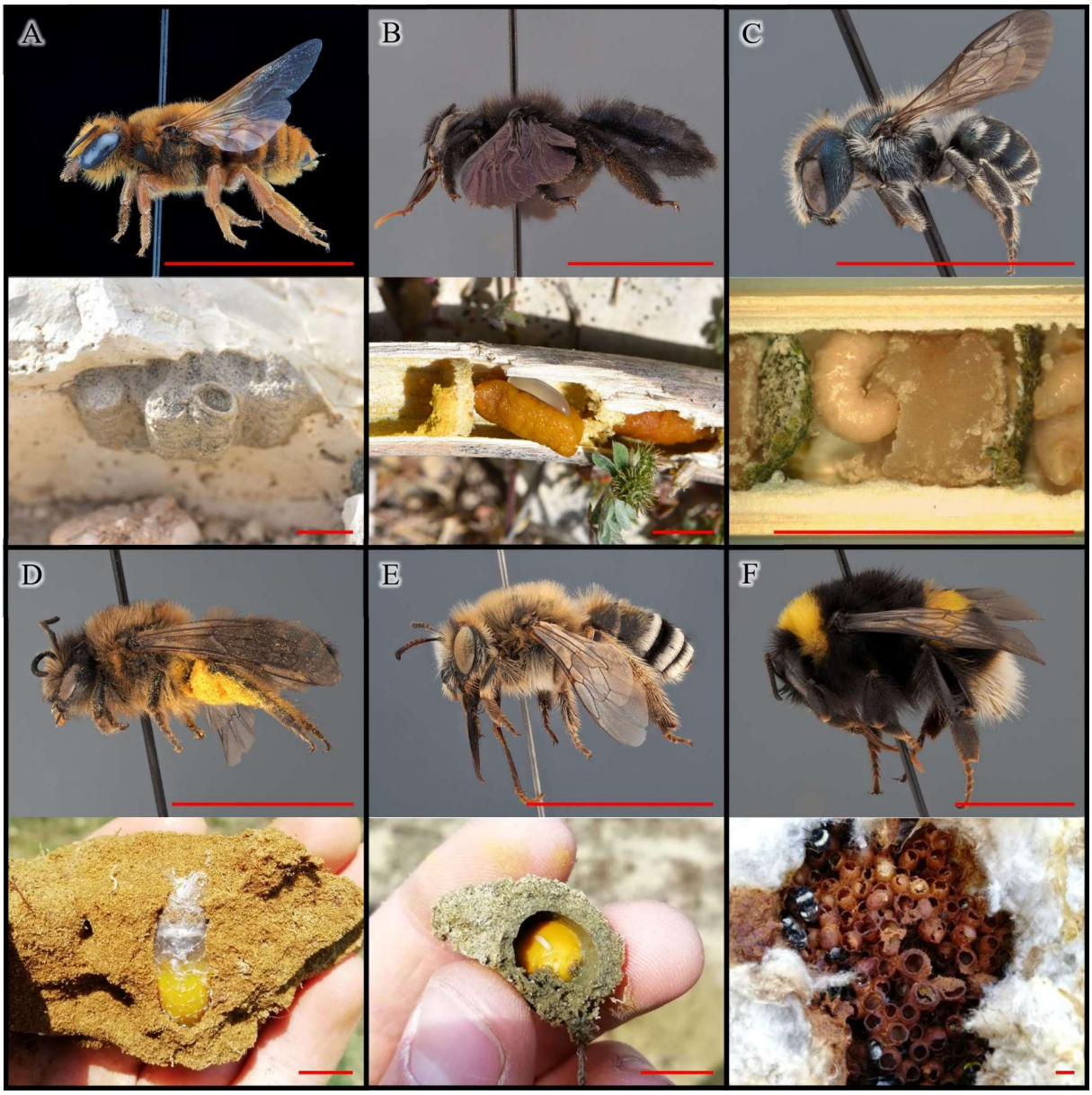

Figure 2: Diversity of nesting patterns in bees. In each box: a female specimen (top) and an example of nest from the species in question (bottom). Red lines = 1 cm. A: *Megachile cypricola* (Megachilidae), external mud nest consisting of brood cells built against each other; B: *Xylocopa iris* (Apidae), brood cells in an Apiaceae stem, partitioned with its pith; C: *Osmia caerulescens* (Megachilidae), brood cell in a hollow stem, partitioned with vegetal fibres; D: *Colletes cunicularius* (Colletidae), brood cell lined with polyester, from a ground nest; E: *Eucera dimidiata* (Apidae), brood cell from a ground nest; F: *Bombus terrestris* (Apidae), colony in late season, mainly composed of abandoned cocoons. Photo credit: Paolo Rosa (pined specimens), Jordan Benrezkallah (nests A and B), Rémi Santerre (nests C to F).

1.2.4. Systematics and diversity of contemporary bees

Over 20,000 bee species are described worldwide, with more than 2,000 of them occurring in Europe (Michener, 2007; Ascher & Pickering, 2023; Ghisbain *et al*., *in press*). They are divided into seven families, traditionally split into two major morphological guilds: the 'long-tongued bees' and the 'short-tongued bees' (Fig. 3). While the monophyletic origin of the first group is strongly supported by recent molecular studies, the short-tongued bees are paraphyletic due to the basal position of Melittidae (*e.g.*, Hedtke *et al.*, 2013; Branstetter *et al.*, 2017; He *et al.*, 2018; Husemann *et al.*, 2021). These studies also confirmed the monophyly of the seven families, including Melittidae which was suspected to be paraphyletic in the past (Danforth *et al*., 2006; Alexander, 1995, cited in Michez *et al*., 2009). Each of these families have particular features, which are briefly reviewed here below.

The **Melittidae** (Fig. 3F) form a small family of solitary ground-nesting bees that include many oligolectic species, distributed in Holarctic and in Africa (Michener, 2007; Michez *et al*., 2008; Ascher & Pickering, 2023). Three subfamilies exist, including two occurring in Europe (Dasypodainae and Melittinae) (Ghisbain *et al*., *in press*). Given their basal position in bee phylogeny, Melittidae are a subject of major interest for the understanding of bee evolution.

Of the remaining 'short-tongued bees', the **Andrenidae** (Fig. 3E) seem to have diverged first. These are also solitary ground-nesting bees, most of which are oligolectic. They are present on all continents excepted the Australasian ecozone. This clade is divided in three subfamilies, including two occurring in Europe (Andreninae and Panurginae). About half of the Andrenidae species belong to the widespread genus *Andrena* (Michener, 2007; Bossert *et al.*, 2022).

The **Colletidae** (Fig. 3D) have more diverse nesting behaviours (*e.g.*, underground burrows, pre-existing cavities, stems), and have the particularity of lining their brood cells with a polyester that they synthetise (Fig. 2) (Almeida, 2008). This globally distributed clade is divided into five subfamilies, represented in Europe only by the genera *Colletes* (Colletinae) and *Hylaeus* (Hylaeinae) (Almeida *et al*., 2012; Ghisbain *et al*., n.d.). The Colletidae and the Australian endemic **Stenotritidae** form together the sister clade of the following family.

The **Halictidae** (Fig. 3C) form the largest and most behaviourally diverse 'short-tongued' family. All levels of sociality exist in this group, it includes many parasites and

some nocturnal species (Michener, 1978; Wcislo *et al*., 2004; Schwarz *et al*., 2007; Gibbs *et al*., 2012). Most are ground-nesting, some prefer stems or pre-existing cavities, and many species are polylectic (Michener, 2007). The Halictidae have a global distribution and are divided in four subfamilies, three of which occur in Europe (Rophitinae, Nomioidinae and Halictinae) (Ghisbain *et al*., n.d.).

The **Megachilidae** (Fig. 3B) are solitary bees characterised by a scopa located under the abdomen (excepted in very basal taxa). They display highly diverse nesting behaviours: they exploit all types of cavities, some are ground-nesting and others build simple external mud nests. They also use a great variety of foreign materials to line and partition brood cells (Michener, 2007). The Megachilidae have a global distribution, and are divided in four subfamilies, two of which occur in Europe (Lithurginae and Megachilinae) (Litman *et al.*, 2011; Gonzalez *et al*., 2012; 2019; Ghisbain *et al*., n.d.).

The **Apidae** (Fig. 3A) form the largest Anthophila family and includes several familiar species like bumblebees (*Bombus* spp.) and the western honeybee (*Apis mellifera*). The greatest behavioural diversity is found in this group. It includes the species with the highest level of sociality, and nearly a third are parasites. They show various nesting patterns and have many polylectic species (Michener, 2007). The Apidae occur everywhere and can be divided in five monophyletic subfamilies, all present in Europe (Apinae, Anthophorinae, Eucerinae, Nomadinae and Xylocopinae) (Bossert *et al*., 2019; Ghisbain *et al*., n.d.).

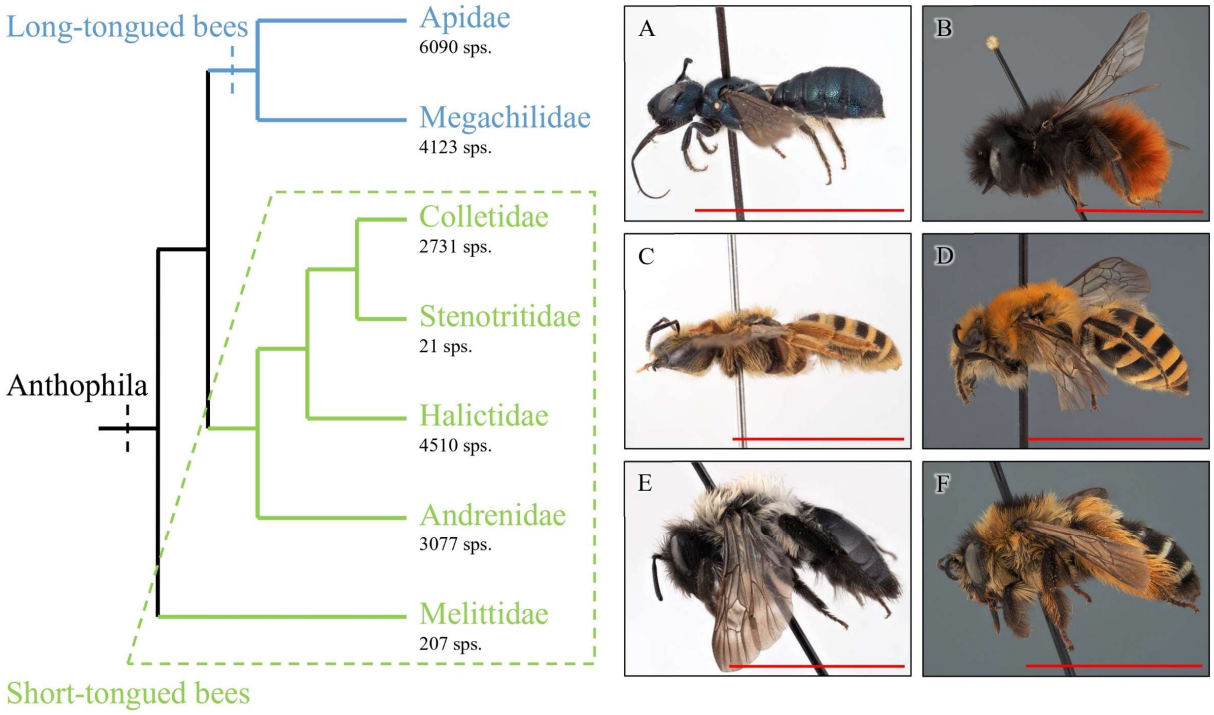

Figure 3: Consensus phylogeny of Anthophila families according to Danforth *et al*., 2006; Hedtke *et al.*, 2013; Branstetter *et al.*, 2017; He *et al.*, 2018; Sann *et al.*, 2018; Husemann *et al.*, 2021. The number of described species is reported under each family name (Ascher and Pickering, 2023). In blue, the monophyletic guild of long-tongued bees; in green, the paraphyletic guild of short-tongued bees. Red lines = 1 cm. A, *Ceratina chalybea* (Apidae); B, *Osmia cornuta* (Megachilidae); C, *Halictus scabiosae* (Halictidae); D, *Colletes hederae* (Colletidae); E, *Andrena vaga* (Andrenidae); F, *Dasypoda hirtipes* (Melittidae). Photo credit: Paolo Rosa.

1.3. Origin and evolution of bees

1.3.1. Evolutionary shift from carnivory to pollinivory

The emergence of bees in the early Cretaceous period is closely related to the diversification of Angiosperms, and more specifically to the diversification of Eudicotyledons (Cardinal & Danforth, 2013). A transition from carnivorous to phytophagous diet occurred in the ancestor of bees, which was probably facilitated by the increasing diversity of flowering plants. While the precise process of this transition is not yet known, Sann *et al.* (2018; 2021) have highlighted the subtribe Ammoplanina as the closest relative of bees. These small ground-nesting wasps (2 to 4 mm) are distributed across the Holarctic region and in Ethiopia

(Antropov, 2010; Bohart & Menke, 1976, cited in Sann *et al*., 2018). They hunt thrips which are known to aggregate on flowers and feed on pollen (Kirk, 1984; 1985).

These data allowed Sann *et al.* (2018) to reconstruct an evolutionary hypothesis. The bees would have diverged from a sphecoid ancestor which hunted thrips fed and covered with pollen. This behaviour would have facilitated the transition to a strict pollinivorous diet, both in terms of nutrition and ecology. Indeed, the recognition of visual and olfactory stimuli associated with flowers probably has a common origin between Ammoplanina and Anthophila. This scenario is consistent with the characteristics of the oldest known bee-related fossils, *Melittosphex burmensis* and *Discoscapa apicula*. These 100-million-year-old insects are each about 3 millimetres long, which has been associated by authors with the very small size of most flowers in the Cretaceous (Danforth & Poinar, 2011; Poinar, 2020).

1.3.2. From oligolectism to polylectism

Oligolectism is the ancestral state within Anthophila, and it remains widely represented in basal lineages from the different families (*e.g.*, Danforth *et al*., 2006; 2013; Sedivy *et al*., 2008). At the contrary, polylectism is a derived behaviour assumed to reduce the dependency on a particular floral group (Michener, 2007).

It seems that these floral choices have had a major influence on the evolution of bees, and especially on their diversification. Traditionally, the transition to pollinivory was assumed to be responsible for the exceptional radiation of bees. However, Murray *et al*. (2018) studied this phenomenon and found out that it was explained more by broadening of pollen diet than by pollinivory *per se*. These findings provide potential explanation of the low diversity in the basal and mostly host-specialised Melittidae family (Michez *et al.*, 2008).

Despite the advantages of a broad diet, the transition to polylecticism appears to present physiological and neurological barriers, related for example to specific nutrient requirements, digestive capacity or recognition ability (Sedivy *et al*., 2008; Praz et al., 2008a; 2008b).

1.4. The importance of sterols in bee nutrition

1.4.1. Generalities about sterols

Sterols are lipid molecules with an amphipathic character (Behmer & Nes, 2003). They are composed of three main domains: a tetracyclic structure composed of three 6-carbon

rings and one 5-carbon ring (Fig. 4, A), a C_3 hydroxyl group (Fig. 4, B), and a C_{17} side chain counting 8 to 10 carbon atoms (Fig. 4, C) (Behmer & Nes, 2003; Jing & Behmer, 2020).

Sterols are essential components of cell membranes and have an influence on their rigidity. Their hydrophobic part (side chain and tetracyclic nucleus) interacts with fatty acid tail of phospholipids inside the bilayer, while the C_3 hydroxyl group interact with their hydrophilic phosphate heads. They act as stabilisers by ordering carbon chains. Sterols are also the precursors of steroid hormones and, in the case of insects, of ecdysteroids. These moulting hormones are essential to control their development. Finally, sterols are involved in the Hedgehog signalling pathway, affecting growth and morphology (Behmer & Nes, 2003; Jing & Behmer, 2020).

A wide variety of sterols exist in the different kingdoms of life. In animals, cholesterol ($C_{27}H_{46}O$) is the main compound. Plants synthetise alternative sterols, with one or two additional carbons on C_{24} position. More than 200 different compounds have been identified in plants, varying in size of the C_{24} alkyl group, and in number and position of the double bonds. In fungi, ergosterol and related C_{28} sterols are the most abundant, although cholesterol and 'phytosterols' are also found in some groups (Behmer & Nes, 2003; Jing & Behmer 2020).

1.4.2. Sterol constraints of phytophagous diet in insects

As mentioned here above, cholesterol is the main sterol in animals and performs essential functions. Unlike vertebrates, insects (and other arthropods) are unable to synthetise sterols *de novo*. Therefore, they have to get these nutrients from their diet. For carnivorous insects, it does not appear to be a challenge as they can find cholesterol in their preys. In phytophagous insects however, phytosterols are often the only sterols available, as most plants contain no or very little cholesterol.

The metabolism of most insects allows them to convert the C_{28} and C_{29} phytosterols in C_{27} cholesterol by removing the C_{24}-methyl or ethyl group. This dealkylation ability is the ancestral way in insects and is, for example, maintained through the phytophagous order Lepidoptera. This allows them to synthetise C_{27} moulting hormones (ecdysone and 20-hydroxyecdysone) from cholesterol precursor, like carnivorous insects. At the contrary, this C_{24}-dealkylation pathway appears to be lost in several taxa, including derived lineages of Hemiptera, Diptera and Hymenoptera. A common adaptation in these groups is the use of alternative C_{28} or C_{29} moulting hormones (Makisterone A and C respectively) (Behmer &

Nes, 2003). In bees, which cannot dealkylate, the type of ecdysteroids used seem to depend on the taxa. In the Apidae species investigated so far, C_{27} or C_{28} sterols are used as precursors of 20-hydroxyecdysone and Makisterone A respectively. While *Apis mellifera* synthetise Makisterone A (Feldlaufer *et al*., 1985; 1986), *Diadasia rinconis* use mainly 20-hydroxyecdysone (Feldlaufer *et al*., 1993), and *Bombus terrestris* seems able modulate synthesis of C_{27} and C_{28} hormones depending on the sterol precursors available (Regali, 1996).

In general, sterol composition seems to have an influence on the pollen choices of bees. This may be particularly true for taxa that need rare C_{27} sterols, in contrast to those using common C_{28} like 24-methylenecholesterol (Vanderplanck *et al.*, 2017; 2020a; 2020b). However, floral choices are not the only factor that can modulate the sterol content of larval provisions. Paludo *et al.* (2018) demonstrated that *Scaptotrigona depilis* (Apidae) larvae need ergosterol produced by a symbiotic *Zygosaccharomyces* sp. (Saccharomycetaceae, Fungi) to synthetise their C_{28} Makisterone A. Although this does not necessarily imply such important nutritional alterations, this type of secondary consumption appears to be generalised in bees, whose actual trophic position corresponds to omnivory (Steffan *et al*., 2019). Furthermore, a behaviour of sterol addition to the pollen collected has been reported. This transfer from the endogenous sterols pool of adults would be related to glandular secretions (Svoboda *et al*., 1986; Vanderplanck *et al*., 2020b).

1.4.3. The sterols and the emergence of bees

In their review of the chemical ecology and evolution of bee-flower interactions, Dötterl & Vereecken (2010) suggested a relation between the cholesterol present in Asteraceae and the transition from carnivory to pollinivory. Indeed, it has been found that this plant family tend to contain cholesterol, sometimes in very high concentrations (*e.g.*, *Hypochaeris radicata*, *Picris hieracioides*) (Devys & Barbier, 1966; Vanderplanck *et al*., 2020b). In the meantime, oligolectism on Asteraceae is the putative ancestral state in the Dasypodainae (Melittidae), which has a very basal position in bee phylogeny (Michez *et al*., 2004; 2008; Dötterl & Vereecken, 2010). Moreover, *Dasypoda hirtipes* seems to add cholesterol from its endogenous pool to the pollen provided to the larvae (Vanderplanck *et al*., 2020b), and other bee species are still synthetising C_{27} ecdysteroids from cholesterol precursor (Feldlaufer *et al*., 1993; Regali, 1996).

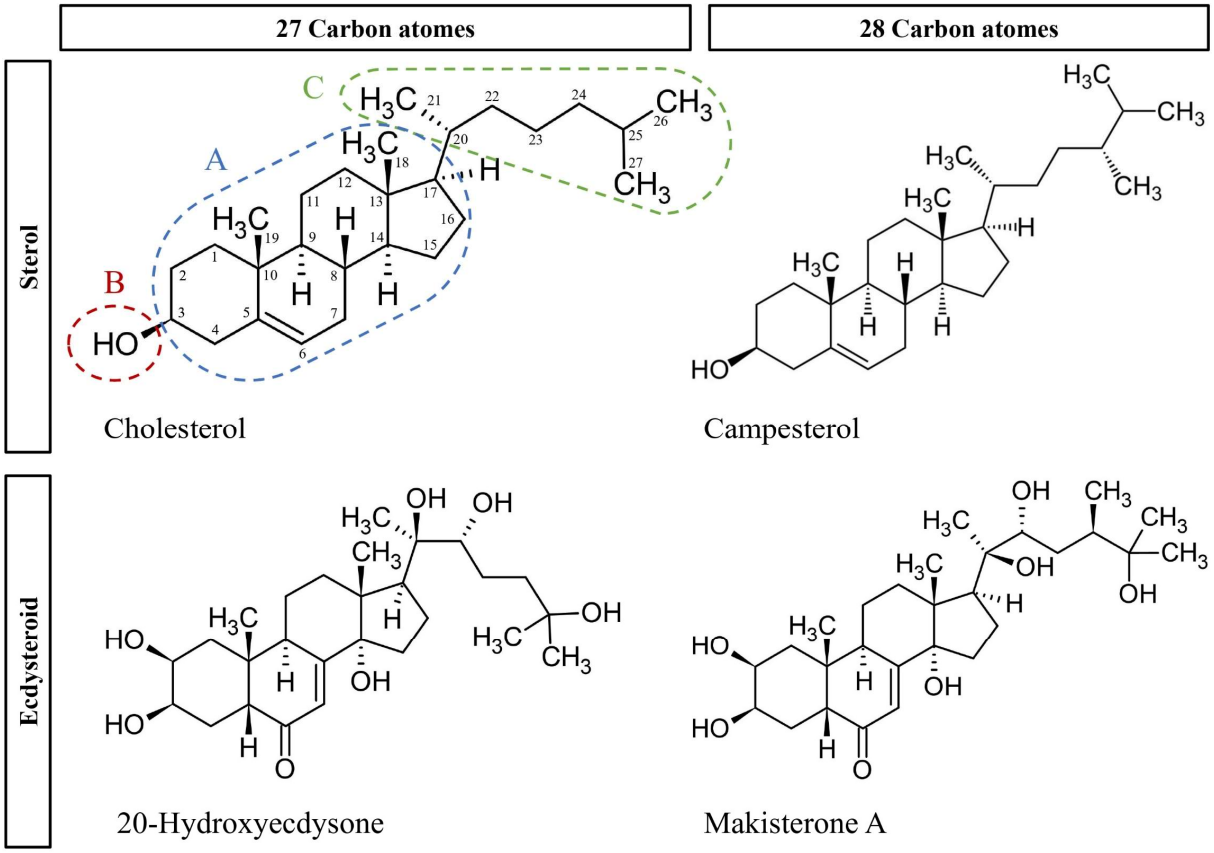

Figure 4: Chemical structure of common steroids. From top left to bottom right: cholesterol, the main animal sterol; campesterol, a common C_{28} phytosterol; 20-hydroxyecdysone, a common moulting hormone; makisterone A, a C_{28} moulting hormone synthetised in several phytophagous taxa. The different domains are highlighted on the cholesterol. A: tetracyclic nucleus; B: hydroxyl group; C: side chain.

2. Objectives

The main target of this work is to describe how sterol composition of bee-collected pollen varies with lineages and feeding specialisation, in order to highlight key elements in bee evolution. We aimed to gain a better understanding of the constraints imposed by these essential nutrients: **1)** in the evolutionary transition from carnivory to pollinivory which led to the emergence of bees; **2)** in the transition from oligolectism to polylectism which accelerated their diversification.

1) ***Are there similarities in sterol composition between the preys of sphecoid wasps and the pollen provisions of basal bee taxa?***

Our hypothesis is that the cholesterol contained in certain plants facilitated the shift from carnivory to pollinivory, and that the early bees used C_{27} sterol like their predator ancestors. The pollen collected in basal clades, especially in the Melittidae family, would therefore have a similar sterol composition than preys of sphecoid wasps.

2) ***Are there differences in sterol composition between pollen collected by specialist species and that collected by generalist species?***

Our hypothesis is that the physiological constraints related to the transition from oligolectism to polylectism include specific sterol requirements. The oligolectic species would depend on specific sterols, like rare C_{27} sterols, while polylectic species would be able to feed on pollen with common C_{28} phytosterols.

More generally, no studies on the sterol content of larval provisions have yet been carried out on a broad taxonomic scale. The collection and analysis of a large sample, including representatives of each bee family and presenting different pollen regimes, will provide data of major interest on the variability of sterol profiles within bees.

3. Material and Method

3.1. Sampling

3.1.1. Samples description

The sampling aimed to be as broad as possible in terms of taxonomic diversity, to cover the different lineages of Apoidea, and especially of Anthophila. The larval provisions of at least one representative of each of the bee families present in Europe were included in the analysis (Fig. 5). The samples studied in this work originate from Belgium, France, Cyprus, Spain, and Morocco (Tab. 1). They were collected by the author between spring 2021 and summer 2022, excepted *Dasypoda visnaga* (collected in the framework of the study by d'El Abdouni *et al*., 2021), *Halictus scabiosae* (collected by Tomas J. Wood in 2020) and *Osmia cornuta* (collected by Antoine Flandroit in 2021).

The brood cell provisions were targeted so that the samples would reflect the sterol composition as available to the larvae. Indeed, several factors can modify the sterol content during collection by the females and then in the nest (Svoboda *et al*., 1986; Eckhardt *et al*., 2014; Paludo *et al*., 2018; Vanderplanck *et al*., 2020b). As the two eusocial species represented in the sample (*Apis mellifera* and *Bombus terrestris*) feed their larvae progressively (Michener, 2007), the pollen was taken from the common stocks accumulated in the combs (*A. mellifera*) or former cocoons (*B. terrestris*) by the workers.

In sphecoid wasps, preys of *Isodontia mexicana* (Sphecidae) were collected directly from the nest, the same way as for bees. As no nest of other species was found (with enough provisions), free-living insects corresponding to the prey of taxa closely related to bees were collected and added to the study. Adult specimens of *Apis mellifera* have been considered as larval food of *Philanthus triangulum* (Crabronidae, Philanthinae), as this species hunts almost exclusively honeybees to provision its brood cells (*e.g.*, Beekhuis van Till, 1953; Simon Thomas & Veenendaal, 1978; Strohm, 1995). Aphids (Aphididae) are common prey in Pemphredoninae (Crabronidae) and are the exclusive larval provisions in virtually all the Pemphredonina subtribe (Bohart & Menke, 1976). Aphididae collected on *Cirsium arvense* have been considered as larval food of 'Pemphredonina indeterminate'.

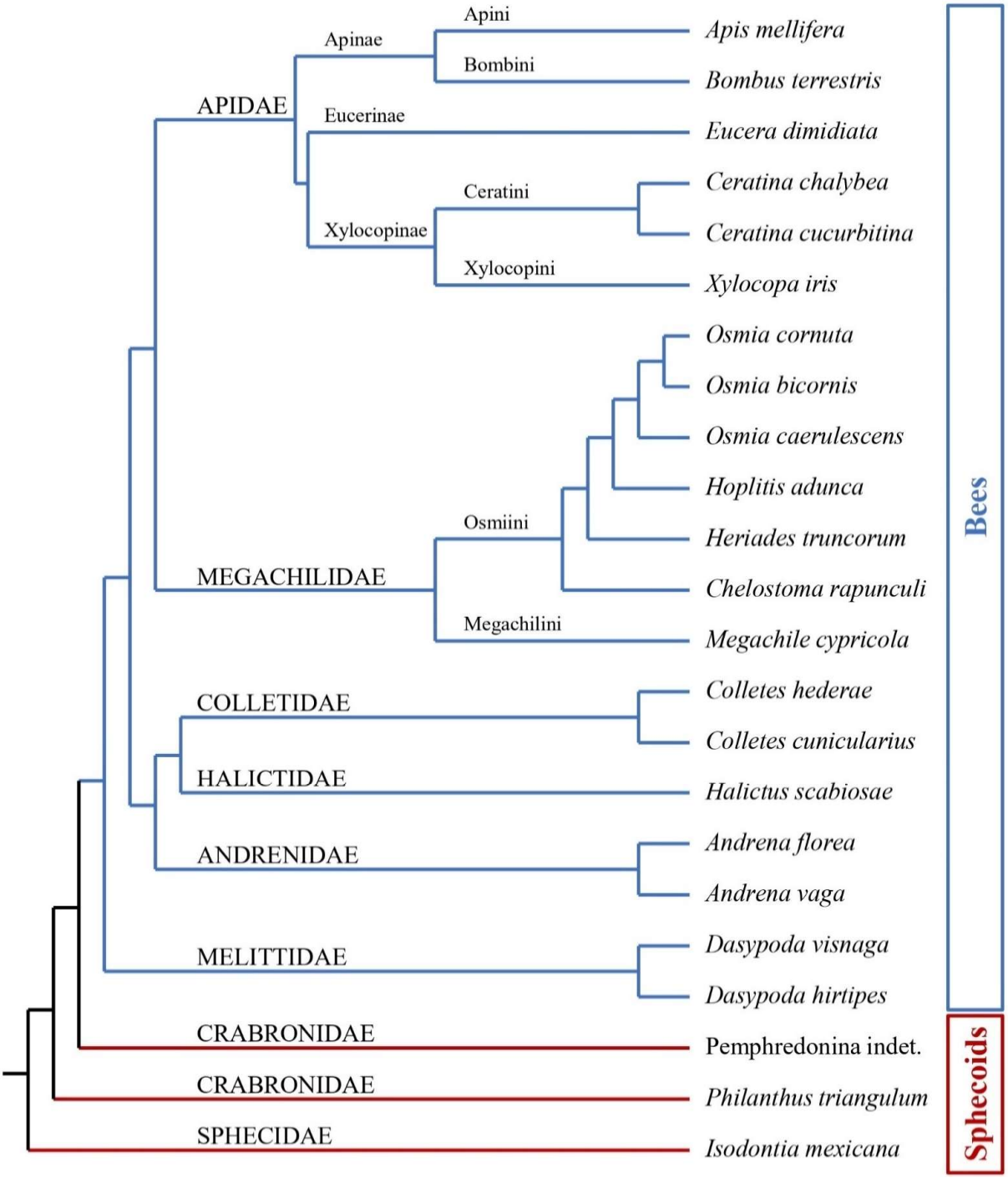

Figure 5: Cladogram of the Apoidea species included to the study, according to various sources (*e.g.*, Danforth *et al*., 2006; Praz *et al*., 2008c; Litman *et al*., 2011; Hedtke *et al*., 2013; Sann *et al.*, 2018; Bossert *et al*., 2019). In green, the branches belonging to the sphecoid wasps; in blue, the branches belonging to the bees (Anthophila). Families are indicated on the branches, as well as subfamilies and tribes when more than one is represented within the same family.

Table 1: Taxa included in the analysis, with related sampling information. BE: Belgium; FR: France; ES: Spain; CY: Cyprus. 1: Sample consist in free-living Aphididae collected on *Circium arvense*; 2: Sample consist in free-living *Apis mellifera*.

Taxon	Locality	Date	Collector
Sphecidae			
Isodontia mexicana	BE, Mons, *Campus Plaine de Nimy*	19 July 2022	R. Santerre
Crabronidae			
Pemphredonina indet.[1]	BE, Mons, *Gare de Mons*	29 September 2022	R. Santerre
Philanthus triangulum[2]	BE, Mons, *Campus Plaine de Nimy*	29 July 2022	R. Santerre
Melittidae			
Dasypoda (Dasypoda) hirtipes	BE, Beloeil, *Mer de Sable de Stambruges*	20 August 2021	R. Santerre
Dasypoda (Megadasypoda) visnaga	Morocco	2019-2020	El Abdouni *et al*., 2021
Andrenidae			
Andrena (Poliandrena) florea	FR, Mainsat, *Villebat*	7 July 2021	R. Santerre
Andrena (Melandrena) vaga	BE, Bernissart, *Grande Bruyère de Blaton*	28 April 2022	R. Santerre
Halictidae			
Halictus (Halictus) scabiosae	BE, Boussu	2020	T. J. Wood
Colletidae			
Colletes (gr. cunicularius) cunicularius	BE, Beloeil, *Mer de Sable de Stambruges*	27 April 2022	R. Santerre
Colletes (gr. succinctus) hederae	BE, Antoing, *Aérodrome de Maubray*	25 September 2021	R. Santerre
Megachilidae			
Chelostoma (Gyrodromella) rapunculi	BE, Mons, *Campus Plaine de Nimy*	23 July 2021	R. Santerre
Heriades (Heriades) truncorum	BE, Mons, *Campus Plaine de Nimy*	23 July 2021	R. Santerre
Hoplitis (Hoplitis) adunca	BE, Mons, *Campus Plaine de Nimy*	23 July 2021	R. Santerre

Megachile (Chalicodoma) cypricola	CY, Moni, *SE of Water Treatment Facility*	5 April 2022	R. Santerre
Osmia (Helicosmia) caerulescens	ES, Murcia, *Campus Espinardo*	19 April 2021	R. Santerre
Osmia (Osmia) cornuta	BE, Quévy, *Blaregnies*	20 May 2021	A. Flandroit
Osmia (Osmia) bicornis	FR, Halluin, *La Motte*	21 June 2021	R. Santerre
Apidae			
Apis (Apis) mellifera	BE, Mons, *Campus Plaine de Nimy*	19 July 2022	R. Santerre
Bombus (Bombus) terrestris	BE, Arlon, *Sterpenich*	30 May 2022	R. Santerre
Ceratina (Ceratina) cucurbitina	FR, Mainsat, *Villebat*	7 July 2021	R. Santerre
Ceratina (Euceratina) chalybea	FR, Mainsat, *Villebat*	7 July 2021	R. Santerre
Eucera (Eucera) dimidiata	CY, Pissouri, *Dog's Beach*	6 April 2022	R. Santerre
Xylocopa (Copoxyla) iris	CY, Koúklia, *1.5km E of Aphrodite's Beach*	6 April 2022	R. Santerre

3.1.2. Sampling method

Part of the samples were collected from sites known for their natural nest aggregations (*Andrena vaga*, *Dasypoda hirtipes*, the two species of *Colletes*), others from artificial nests (*Isodontia mexicana*, all species of Osmiini) including domestical colonies (*Apis mellifera*, *Bombus terrestris*). All other samples are from opportunistic field searches of wild nests.

The sampling procedure depended on the type of nest (Fig. 6). For those in tubes or stems, the whole structure was collected and opened in the lab. It was split lengthwise with a scalpel or a knife, taking care not to damage the larvae inside. The external mud nest of *Megachile cypricola* was opened directly on the field (Fig. 6, C). For eusocial species, additional protective equipment such as beekeeper's clothing was worn, and pollen was taken from the common storages. For ground-nesting bees, digging was required. The excavation was started with a shovel about 50 centimetres from the targeted burrow. The vertical side of the hole was cut cleanly to a depth of at least 50 cm where the soil allowed. The cutting face was then progressively advanced towards the nest, by removing thin slices of soil. When detected, brood cells were extracted carefully, and were coarsely cleaned on the field with a thin tweezers if necessary. Brood cells of most species can be collected with this method, as they are located on average between 17 and 35 cm deep according to Cane & Neff (2011).

For each species, pollen provisions from at least three distinct brood cells were collected[1] and placed in individual labelled Eppendorf tubes, to perform the three biological replicates. These samples were always collected with a control adult for identification purposes, except when the identity of the species was completely certain (*e.g.*, honeybee hive). The pollen samples were carefully inspected under the stereomicroscope and cleaned with fine tweezers when necessary. A small amount of each sample was set aside for palynological analyses (see section 3.2.). The tubes were conserved at -80°C.

[1] Excepted for *Megachile cypricola*, a Cyprus endemic species listed as critically endangered (Nieto *et al*., 2014). A single brood cell was opened, and its content divided into three for analysis. Sterol variability between pollen provisions is expected to be low for this species, as it is highly oligolectic and forage almost exclusively on *Onobrychis venosa* (Fabaceae) (R. Santerre and J. Benrezkallah, *unpublished data*, based on palynological analysis of scopal pollen of 35 females from 4 distinct locations).

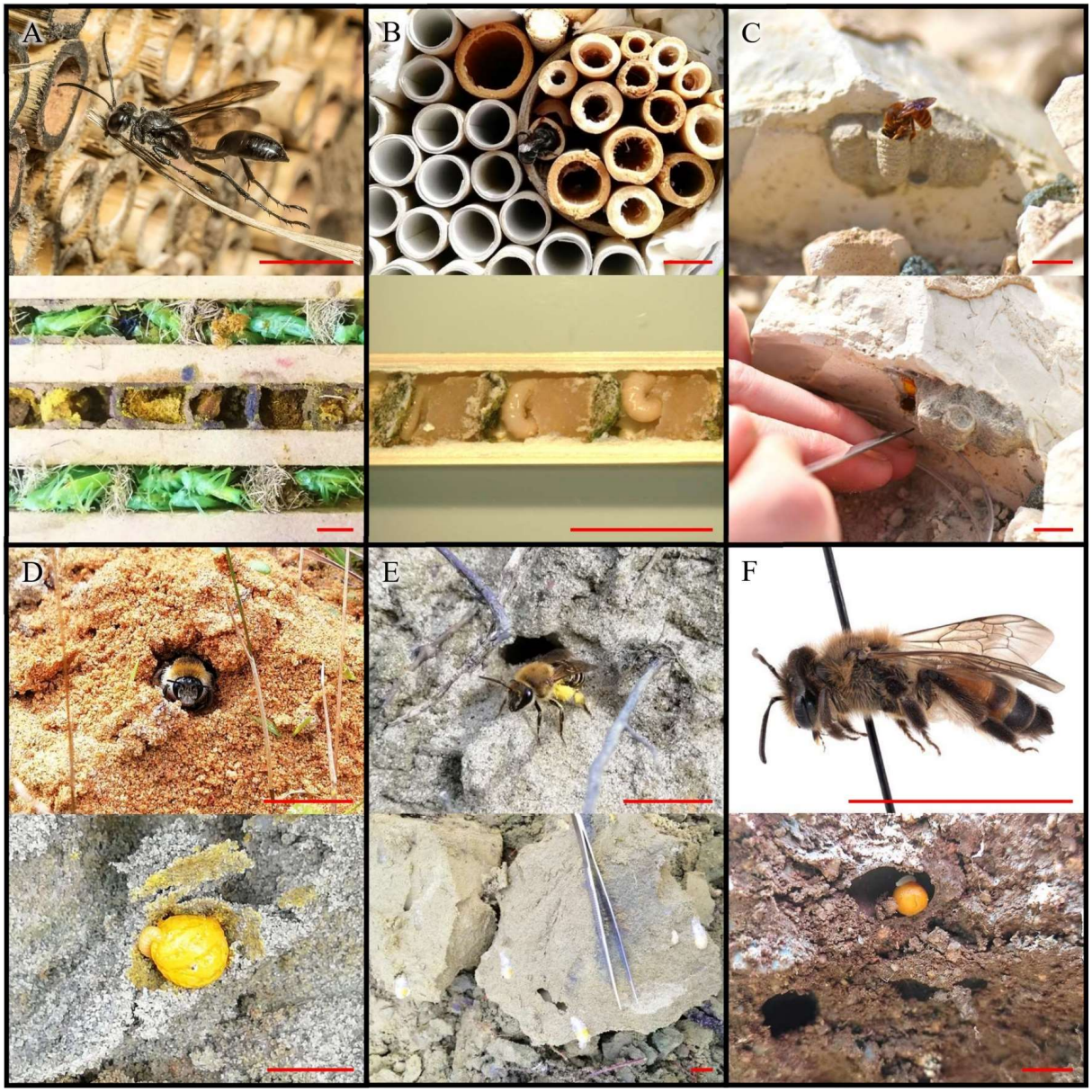

Figure 6: In each box: a female specimen (top) and a nest of the species in question during the sampling process (bottom). Red lines = 1 cm. A: *Isodontia mexicana* (Sphecidae), artificial nest provisioned with Acrididae; B: *Osmia caerulescens* (Megachilidae), nest in a hollow stem from a trap nest built for this study; C: *Megachile cypricola* (Megachilidae), external mud nest dissected on the field; D, E, F respectively: *Dasypoda hirtipes* (Melittidae), *Colletes hederae* (Colletidae) and *Anderna florea* (Andrenidae), ground nests collected by excavation. Photo credit: Jordan Benrezkallah (*Megachile cypricola* and its nest), Paolo Rosa (pined *Andrena florea*), Pjt56[1] (*Isodontia mexicana*, CC BY-SA 4.0[2]), Rémi Santerre (all other photos).

[1]Creator's page: https://commons.wikimedia.org/wiki/User:Pjt56/gallery

[2]Link to the CC BY-SA 4.0 license: https://creativecommons.org/licenses/by-sa/4.0/deed.en

3.2. Palynological analyses

The pollen was prepared and analysed following Wood *et al.* (2016). A small amount of pollen from each sample (around 1 to 2 mg) was transferred to a drop of water on a microscope slide. The pollen was homogenised, left for a few minutes for any dehydrated grains to rehydrate, and the slide was gently heated to allow the remaining water to evaporate. Molten fuchsin-stained glycerine jelly (Brunel Microscopes Ltd, UK) was added, and the slide was sealed with a coverslip. Pollen grains were identified to the family level under a light microscope Brunel SP100 (Brunel Microscopes Ltd, UK) at magnifications ×400. The relative area of the slide occupied by each plant family was estimated along several randomly selected transects. This method better reflects the total volume of the different pollen types collected by bees than counting an absolute number of grains. This correction is important in mixed samples where grain size can vary considerably (Cane & Sipes, 2006). Given the small number of replicates, the palynological data obtained is not expected to be representative of the general diet of the species examined. These analyses were carried out to ensure a better understanding of the material studied.

In order to classify the species according to their level of pollen specialisation, the literature was reviewed. We used the three categories: *Polylectic* for species collecting pollen from several plant families, '*Polylectic with strong preference*' for species collecting pollen from several plant families but showing a strong specialisation in one of them (*i.e.*, 70-95% of the pollen collected) (Müller & Kuhlmann, 2008), and *Oligolectic* for those collecting pollen within a single plant family. The more precise sub-categories of oligolectism and polylectism proposed by Cane & Sipes (2006) were not considered due to the lack of literature data for certain species.

3.3. Sterol analyses

The analytical method presented below is largely based on that described by Vanderplanck *et al*. (2011). Prior the extraction, the samples were lyophilised for 24 hours and homogenised. Their fresh and dry weights were both measured with an analytical balance (KERN & SOHN GmbH, DE).

3.3.1. Sterol extraction

For each replicate (three per species), 20.0 to 30.0 mg of dry sample were precisely weighted with an analytical balance (KERN & SOHN GmbH, DE). A saponification was

performed for one hour in a water bath at 80°C, with methanolic KOH (2 M). For sphecoid wasp prey, body parts too rich in chitin (*i.e.*, antennae, legs, wings) were removed with tweezers before weighing, and the remaining bodies were crushed with a glass rod in the methanolic KOH prior the saponification.

After cooling, 1 ml of an ethanolic solution of betulin (0.05 mg/ml) was added to each sample as an internal standard. The addition of this standard at the beginning of the process allows the subsequent quantification of the sterols, because even in case of losses during the process, the proportions between the sterols to be analysed and the standard will be preserved.

The sterols, which are unsaponifiable, were separated from the saponified fraction in separatory funnels, by three consecutive extractions with 5 ml diethyl ether. The ether fraction containing the sterols was rinsed three times with 5 ml milli-Q water to remove all of the saponified residues. It was then filtered through anhydrous sodium sulfate to remove residual water. The diethyl ether was evaporated under reduced pressure and replaced with 250µl of chloroform.

The samples were then deposited on silica plates for fractionation by thin layer chromatography. Three drops of a standard cholesterol/betulin solution were also deposited in line with the samples on each side of the plate. This serves to delimit the area of interest containing sterols and betulin after elution (while observing under UV light, see below). The mobile phase was composed of chloroform, diethyl ether and ammonia water 28% (90:10:0.5). After elution, dried plates were sprayed an ethanolic solution of 2',7'-dichlorofluorescein (0.2%), to make sterols observable under UV light (254 nm). The area containing the sterols (delimited by the cholesterol and betulin standards) was scraped and suspended in chloroform. The silica was finally removed from the solvent by filtering with filter paper.

3.3.2. Identification and quantification by gas chromatography (GC-FID)

The chloroform was evaporated under a nitrogen stream, and the sterols were derivatised by adding 50µl of anhydrous pyridine as solvent and 50µl of silylation reagent (SILYL-991) composed of BSTFA and trimethylchlorosilane (99:1). To accelerate the reaction, it was performed at 90°C for 30 minutes. The reagents were then evaporated under a nitrogen stream and the samples were re-suspended in 0.2 ml of n-hexane. This silylation reaction, which replaces the active oxygens of the hydroxide groups with trimethylsilyl groups, improves the resolution of the peaks in the GC-FID analysis (Goad & Akihisa, 2012).

The samples in n-hexane solution were analysed with a GC-FID GC-2010 Shimadzu (Kyoto, Japon) under analytic conditions described in Appendix 1Appendix 1: GC-FID analytic conditions. This analytical method separates compounds using gas chromatography, and measures their ionic response per unit time using a flame ionisation detector. Chromatograms were visualized and processed with the software LabSolutions version 5.99, and the peak integration was corrected manually when necessary (*e.g.*, peaks not detected by the software, erroneous integration of peaks outside the range corresponding to sterols, pooling of 24-methylenecholesterol and campesterol [see below]). Peaks were identified by comparing relative retention times with sterols identified and corroborated by GC/MS by Vanderplanck *et al.* (2011). The concentration of the different sterols was determined using the ratio of the area under their peaks to that of the internal betulin standard of known concentration. Under analytic conditions applied, 24-methylenecholesterol and campesterol are nearly impossible to distinguish, their results are therefore pooled.

3.4. Data processing and statistical analyses

Data extracted from LabSolutions was compiled in Excel version 2304 (Microsoft Corporation, 2018). All analyses were then performed with R version 4.2.3 (R Core Team, 2023) in RStudio (Posit team, 2023).

A summary table showing the relative abundance of each sterol (%) and the total sterol content (mg/g of dry sample) for each species was generated, and interspecific differences in total sterol content were assessed using ANOVA followed by a Tukey HSD pairwise comparison test.

To highlight potential similarities in sterol profiles between the prey of sphecoid wasps and the pollen collected by bees, an agglomerative hierarchical clustering (AHC) and a principal component analysis (PCA) were performed on the data transformed in percentage of total sterols. The AHC was performed with the complete-linkage method, using a Euclidean distance matrix. The dataset used for these analyses contained relative values (expressed as percentage of total sterols), in order to preserve the proportions between the different sterols while eliminating the influence of interspecific differences in total sterol concentration.

To explore the relation between bee floral specialisation and pollen sterol composition, several PCA were performed on data standardised to the z-score (mean = 0, SD = 1) after the sphecoid wasps had been removed. The aim of this standardisation was to give

the same relative importance to all compounds, and therefore capture variation in secondary sterols. Indeed, some rare sterols can be of major importance in the diet of certain bee species (Vanderplanck *et al*., 2020a). The three data sets that were standardised and used for PCA were as follows:

1) The original data;
2) A data set in which the sterols were pooled by number of carbon atoms (C_{27}, C_{28}, C_{29} sterols);
3) A data set reduced to the C_{27} and C_{28} sterols only (C_{29} sterols were dropped as they are not used as precursor of moulting hormones in bees, according to current knowledge).

4. Results

4.1. Pollen specialisation

After reviewing the literature, we report that eight of the 20 species studied were oligolectic, ten polylectic and two 'polylectic with strong preference' (Tab. 2).

All samples from the specialised species (oligolectic and 'polylectic with strong preference') were indeed pure pollen of their main host plant family, which was Asteraceae for four of them. All samples from strict polylectic species were composed of mixed pollen, except those from *Osmia caerulescens* which were pure Lamiaceae pollen (Tab. 2).

Table 2: Results of the palynological analysis and pollen specialisation according to literature. The values are as percentage of the area occupied by pollen from each plant family. Ara: Araliaceae; Ast: Asteraceae; Bor: Boraginaceae; Bra: Brassicaceae; Car: Caryophyllaceae; Cis: Cistaceae; Cuc: Cucurbitaceae; Ger: Geraniaceae; Fab: Fabaceae; Fag: Fagaceae; Lam: Lamiaceae; Mal: Malvaceae; Oxa: Oxalidaceae; Ran: Ranunculaceae; Ros: Rosaceae; Sal: Salicaceae; Oth: Other pollen types. 1: Based on literature data. 2: The precise palynological data was not recorded for these species due to the excessive number of different pollen types.

Taxa (n = 3)	Microscopic analysis results (%)	Host plant specialisation [1]	References
Melittidae			
Dasypoda hirtipes	Ast 100	Oligolectic	Michez *et al*., 2004; 2008
Dasypoda visnaga	Ast 100	Oligolectic	Michez *et al*., 2008; El Abdouni *et al*., 2021
Andrenidae			
Andrena florea	Cuc 100	Oligolectic	Westrich, 1996; Wood *et al.*, 2016
Andrena vaga	Sal 100	Oligolectic	Vanderplanck *et al*., 2009
Halictidae			
Halictus scabiosae	Ast 100	Polylectic with strong preference	Fortunato & Zandigiacomo, 2012; Westrich, 2019
Colletidae			
Colletes cunicularius	Sal 75, Ros 25	Polylectic	Müller & Kuhlmann, 2008; Vanderplanck *et al*., 2009
Colletes hederae	Ara 100	Polylectic with	Müller & Kuhlmann, 2008;

		strong preference	Hennessy *et al*., 2021
Megachilidae			
Chelostoma rapunculi	Cam 100	Oligolectic	Müller, 2015
Heriades truncorum	Ast 100	Oligolectic	Käpylä, 1978; de Almeida, 1980; Müller, 2022
Hoplitis adunca	Bor 100	Oligolectic	Sedivy *et al*., 2013; Müller, 2022
Megachile cypricola	Fab 100	Oligolectic	Mavromoustakis, 1938; Santerre & Benrazkallah, *unpubl. data*
Osmia bicornis	Fag 58, Ran 38, Oth 4	Polylectic	Haider *et al*., 2014; Müller, 2022
Osmia caerulescens	Lam 100	Polylectic	Tasei, 1976; Raw, 1974; Müller, 2022
Osmia cornuta	Ros 62, Sal 35, Oth 3	Polylectic	Haider *et al*., 2014; Müller, 2022
Apidae			
Apis mellifera	Various types[2]	Polylectic	Linsley, 1958; Thorp, 1979
Bombus terrestris	Various types[2]	Polylectic	Rasmont *et al*. 2008
Ceratina chalybea	Fab 58, Ast 16, Car 13, Ger 4, Lam 4, Oth 5	Polylectic	Terzo *et al*., 2007
Ceratina cucurbitina	Fab 70, Lam 15, Ast 5, Mal 5, Oth 5	Polylectic	Terzo *et al*., 2007
Eucera dimidiata	Bra 80, Oxa 20	Polylectic	Nachtigall, 1994
Xylocopa iris	Bra 53, Ast 21, Cis 9, Fab 7, Lam 5, Oth 5	Polylectic	Terzo *et al*., 2007

4.2. Sterol composition of larval provisions

4.2.1. Total sterols concentration

Average total sterols ranged from 0.16 ± 0.02 mg/g in prey of Pemphredonina indet. (aphids) to 25.21 ± 1.46 mg/g in pollen from *Halictus scabiosae* (pure Asteraceae). In bees, *Hoplitis adunca* was the species with the lowest sterol content. With a mean of 0.72 ± 0.10 mg/g, its provisions were nearly 5 times more concentrated than those of Pemphredonina indet. Provisions of *Halictus scabiosae* were twice as concentrated than those of *Eucera dimidiata* (12.34 ± 1.92 mg/g), the species with the second highest content. Apart from these two exceptions, the total sterol formed a continuous gradient between the species (Fig. 7).

Significative differences in total sterol concentration were detected (ANOVA $F_{(22,47)} = 65.969$, $p = < 2\times10^{-16}$), and occurred in several species pairs according to the multiple

comparisons. However, no clear structuration related to feeding behaviour or phylogeny could be observed. While the pollen from the two Melittidae species (*Dasypoda hirtipes* and *D. visnaga*) had similar sterol concentrations, it differed significantly from the preys of each sphecoid wasp species (Appendix 2).

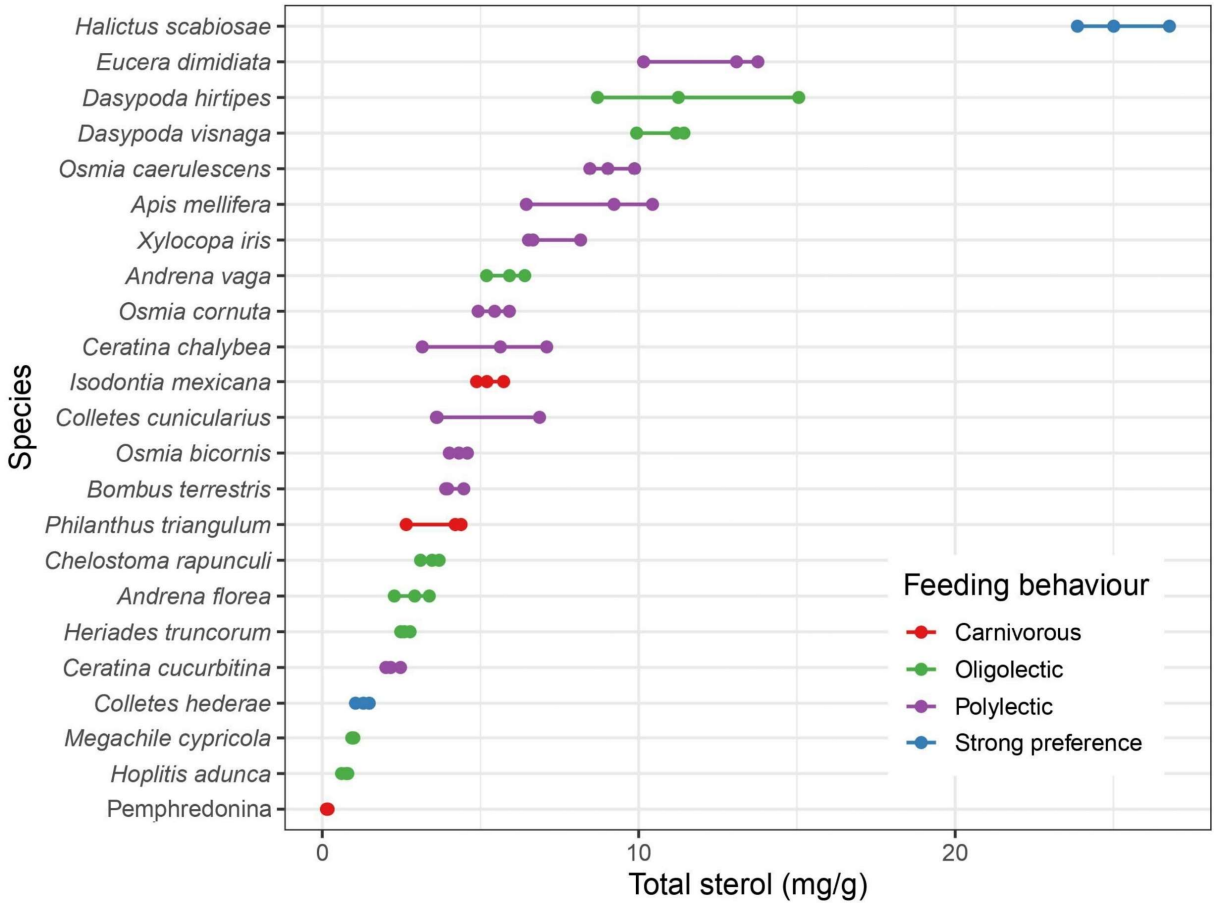

Figure 7: Total sterol content of larval provisions from the different species studied. Replicas are represented by the points.

4.2.2. Sterol profiles

Among the larval provisions of the 23 studied species, twelve main sterolic compounds were detected in GC-FID analyses (*e.g.*, Fig 8). Nine of them were identified: cholesterol, desmosterol, and cholestenone (C_{27} sterols); 24-methylenecholesterol & campesterol pooled together (C_{28} sterols); stigmasterol, β-sistosterol, Δ5-avenasterol, Δ7-stigmasterol, and Δ7-avenasterol (C_{29} sterols).

Two unknown sterols were also detected. The first, referred to here as 'Unknown 1', was also detected by Vanderplanck *et al.*, (2011) who measured a mass of 484 a.m.u. for this

compound. This corresponds to the mass of a C_{29} sterol (TMS derivative) (*e.g.*, PubChem, s. d.). The mass and number of carbon atoms of the second compound, referred to here as 'Unknown 2', are not known. Under the analytical conditions applied, it had a retention time of approximately 26.3 min, its peak being located between Δ5-avenasterol (RT: 25.9 min) and cholestenone (RT: 26.6 min). Its concentration was very low (or zero) in all samples, except those from *Chelostoma rapunculi* (pure Campanulaceae pollen), where it represented on average 38.83 ± 6.00% of total sterols (Tab. 3).

Finally, a compound that appeared to be brassicasterol (C_{28}) was detected. It was abundant only in samples from *Eucera dimidiata* and *Xylocopa iris*, which were the only samples with a large proportion of Brassicaceae pollen (Tab. 2). Brassicasterol is known to be of the main sterols in many common plants from this family (*e.g.*, Ingram *et al*., 1968; Appelqvist *et al.*, 1981; Shukla *et al*., 2002; Antova *et al.*, 2017). The relative retention time of brassicasterol, which is shorter than that of campesterol, seems to correspond to what we observed (Kalo & Kuuranne, 2001, Shukla *et al*., 2002; Yücel *et al.*, 2017). However, GC-MS analyses would be required to confirm the identity of this compound. The sum of unidentified minor compounds was assigned to a variable called 'Not Identified' (N.I.). This did not include 'Unknown 1' and 'Unknown 2', which were each assigned to their own variable as they were of major importance in at least one species. The average sterol content of larval provisions is detailed for each species in table 3.

In sphecoid wasps, the orthoptera collected by *Isodontia mexicana* contained almost exclusively cholesterol (90.79 ± 1.54%), while the *Apis mellifera* typically hunted by *Philanthus triangulum* contained mainly phytosterols, with 24-methylenecholesterol + campesterol reaching 46.18 ± 3.50%. Aphids analysed as larval food for Pemphredonina indet. contained 31.59 ± 4.72% cholesterol, but also a high relative content of unidentified minor compounds (26.64 ± 5.49%) due to the low content of total sterols (0.16 ± 0.02 mg/g).

In bees, sterol profiles were highly variable. Cholesterol was detected in all species, generally at low concentration except in *Halictus scabiosae* (75.13 ± 0.64%), *Dasypoda hirtipes* (56.41 ± 4.49%) and *Heriades truncorum* (52.06 ± 8.82%) where it was largely dominant. Its overall inter-species average was 11.51 ± 21.66%. Other C_{27} sterols were generally rarer, although desmosterol reached over 40% in *Bombus terrestris* and *Ceratina chalybea* (Apidae) and cholestenone reached over 10% in *Megachile cypricola*. The pooled 24-methylenecholesterol and campesterol were abundant in most of the samples, especially in Apidae and Megachilidae families, and were totally absent only from *Colletes hederae*. The

inter-species average of these two pooled compounds was 28.94 ± 26.53%. As mentioned above, the sterol suspected to be brassicasterol was very rare except in *Eucera dimidiata* (8.11 ± 1.00%) and *Xylocopa iris* (7.83 ± 0.9%). Among the C_{29} sterols, β-sistosterol and Δ5-avenasterol were the most abundant and were detected in all species. Their inter-species averages were 16.64 ± 14.87% and 16.39 ± 9.22% respectively. The other compounds were present in varying amounts but were more rarely dominant. However, the rare 'unknown 1' was the main sterol in *Andrena florea* pollen (34.31 ± 1.00%), which also had a high level of N.I. (16.6 ± 3.16%). Finally, as mentioned above, the rare 'unknown 2' was specific to *Chelostoma rapunculi* in our sample.

Regarding to the carbon backbones, C_{29} sterols were the most abundant in pollen provisions of bees overall, with an across-species average of 45.81 ± 25.57% of the total sterols. They were the dominant sterols for nine of the 20 bee species studied. The C_{28} sterols came after, with an average of 29.95 ± 27.04% overall. They were the dominant sterols in six bee species, all from the Apidae and Megachilidae families. Finally, the C_{27} sterols had an average of 18.39 ± 23.89%, and were dominant in five species from 4 different families (Fig. 9; Appendix 3).

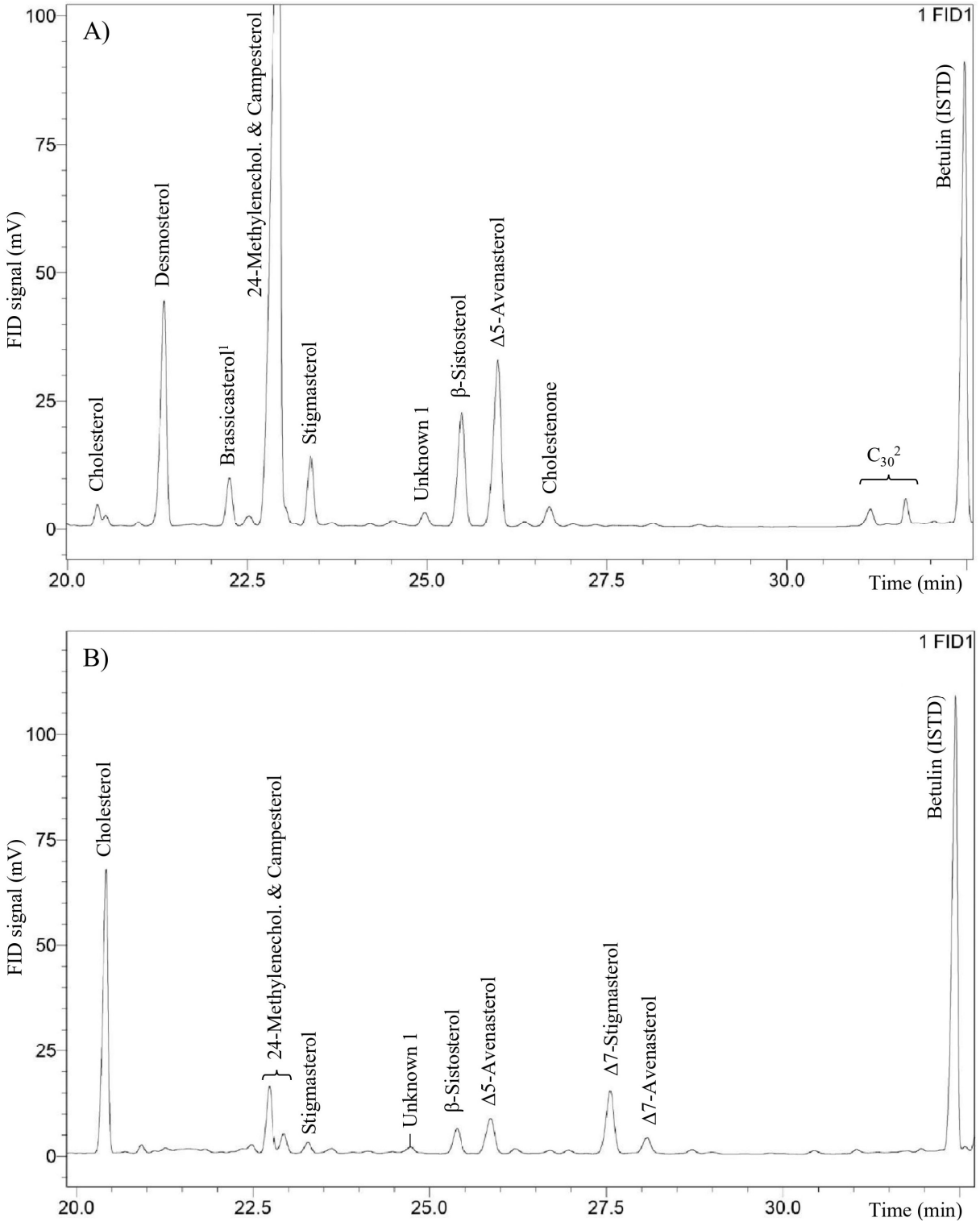

Figure 8: Examples of GC-FID chromatograms. Sterol profiles of pollen provisions from: A) *Apis mellifera*, B) *Heriades truncorum*. 1: Compound identity not yet corroborated with GC-MS. 2: C_{30} compounds.

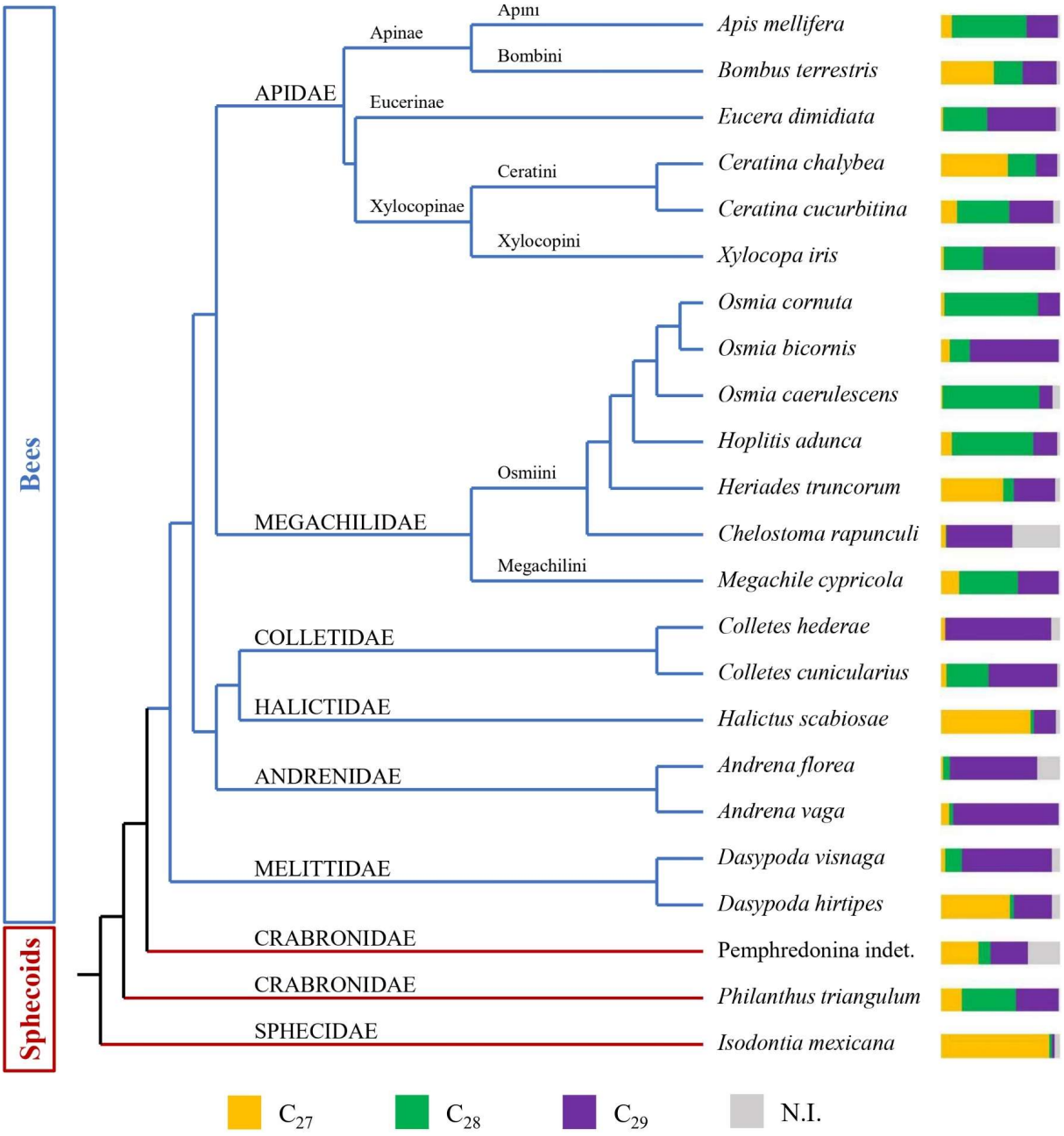

Figure 9: Variations in C_{27}, C_{28} and C_{29} sterol proportions across the Apoidea. N.I.: Sum of the minor unidentified compounds + 'Unknown 2'. The suspected brassicasterol and 'Unknown 1' are respectively included in C_{28} and C_{29}.

Table 3: Sterolic composition of brood cell provisions from 20 bee species and 3 sphecoid wasp species. The concentrations of individual sterols are expressed as percentage of total sterolic content (mean ± SD), which is expressed in mg/g of dry sample. Major compounds (>5%) are indicated in bold, with the most abundant of each species underlined. Desmo = desmosterol; Cholesten = cholestenone; Brassica = brassicasterol; 24-Meth & Camp = 24-methylenecholesterol and campesterol pooled together; Stigma = Stigmasterol; β-Sisto = β-Sistosterol; Δ5-Avena = Δ5-Avenasterol; Δ7-Stigma = Δ7-Stigmasterol; Δ7-Avena = Δ7-Avenasterol; N.I. = Not Identified: sum of the minor unidentified compounds. 1: Four replicas were analysed for Pemphredonina indet. (n = 4). 2: Compound identity not yet corroborated with GC-MS.

Taxa (n = 3)[1]	C_{27} sterols			C_{28} sterols		C_{29} sterols								Total sterol (mg/g)
	Cholesterol	Desmo.	Cholesten.	Brassica.[2]	24-Meth. & Camp.	Stigma.	β-Sisto.	Δ5-Avena.	Δ7-Stigma.	Δ7-Avena.	Unknown 1	Unknown 2	N.I.	
Melittidae														
Dasypoda hirtipes	**56.41 ± 4.49**	0 ± 0	1.56 ± 0.53	0 ± 0	3.37 ± 0.54	0.8 ± 0.26	**5.53 ± 0.85**	**13 ± 0.91**	**9.01 ± 2.77**	3.17 ± 0.06	0.39 ± 0.35	0.53 ± 0.15	**6.24 ± 1.01**	11.67 ± 3.2
Dasypoda visnaga	0.62 ± 0.39	0 ± 0	2.48 ± 0.15	0 ± 0	**14.09 ± 1.4**	**22.69 ± 1.18**	**7.35 ± 0.12**	**20.69 ± 0.96**	**15.33 ± 0.6**	**9.1 ± 0.91**	0.74 ± 0.05	0.75 ± 0.04	**6.15 ± 0.14**	10.85 ± 0.8
Andrenidae														
Andrena florea	1.27 ± 0.41	0 ± 0	0 ± 0	0.98 ± 0.2	4.86 ± 1.07	1.12 ± 0.48	**20.47 ± 1.42**	**7.27 ± 1.16**	**8.54 ± 2.16**	2.74 ± 0.35	**34.31 ± 1.4**	1.85 ± 0.15	**16.6 ± 3.16**	2.86 ± 0.55
Andrena vaga	4.58 ± 0.46	0 ± 0	1.91 ± 0.27	0 ± 0	3.43 ± 0.42	0 ± 0	**49.96 ± 0.59**	**35.25 ± 1.17**	0 ± 0	1.49 ± 0.2	2.51 ± 0.47	0 ± 0	0.85 ± 0.76	5.84 ± 0.61
Halictidae														
Halictus scabiosae	**75.13 ± 0.64**	0.6 ± 0.04	0 ± 0	0 ± 0	2.7 ± 0.15	0.28 ± 0.02	3.1 ± 0.04	**8.97 ± 0.28**	**5.38 ± 0.5**	0.57 ± 0.01	0.32 ± 0.03	0.34 ± 0.05	2.61 ± 0.27	25.21 ± 1.46
Colletidae														
Colletes cunicularius	3 ± 1.87	0 ± 0	1.16 ± 0.38	0 ± 0	**35.96 ± 7.51**	0 ± 0	**30.17 ± 4.72**	**25.07 ± 3.2**	0.13 ± 0.23	0.6 ± 0.01	2.12 ± 0.48	0.66 ± 0.1	1.14 ± 1.28	4.7 ± 1.87
Colletes hederae	3.31 ± 0.41	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	**31.62 ± 1.71**	**12.41 ± 1.06**	**30.79 ± 0.29**	**12.71 ± 0.85**	2.18 ± 0.24	0 ± 0	**6.99 ± 0.57**	1.28 ± 0.22
Megachilidae														
Chelostoma rapunculi	1.76 ± 1.93	1.76 ± 0.32	0 ± 0	0 ± 0	1.02 ± 1.25	0 ± 0	**25.1 ± 5.54**	**11.34 ± 4.06**	0 ± 0	**12 ± 6.88**	**7.34 ± 2.06**	**38.83 ± 6**	0.85 ± 0.97	3.42 ± 0.3

Heriades truncorum	**52.06 ± 8.82**	0 ± 0	0 ± 0	0 ± 0	**9.13 ± 5.94**	2.06 ± 1.34	4.67 ± 1.04	**10.11 ± 0.79**	**13.15 ± 7.09**	4.13 ± 1.15	0.74 ± 0.8	0 ± 0	3.95 ± 3.84	2.62 ± 0.15
Hoplitis adunca	**9.07 ± 4.62**	0 ± 0	0 ± 0	0 ± 0	**69.06 ± 4.31**	1.62 ± 2.81	4.36 ± 0.95	**13.76 ± 6.21**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2.13 ± 2.46	0.72 ± 0.1
Megachile cypricola	2.85 ± 0.79	0.82 ± 1.42	**10.95 ± 1.05**	0 ± 0	**50.15 ± 1.12**	0 ± 0	**14.11 ± 0.32**	**20.05 ± 0.4**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.08 ± 1.87	0.96 ± 0.03
Osmia bicornis	4.08 ± 1.86	1.11 ± 0.28	2.07 ± 1.23	0 ± 0	**16.67 ± 1.03**	0 ± 0	**53.09 ± 5.23**	**19.85 ± 0.75**	0 ± 0	0.98 ± 0.28	1.33 ± 0.3	0 ± 0	0.83 ± 0.83	4.31 ± 0.29
Osmia caerulescens	0.49 ± 0.17	0 ± 0	0.19 ± 0.33	0 ± 0	**82.36 ± 2.79**	0.9 ± 0.26	4.3 ± 1.82	**5.52 ± 0.34**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	**6.24 ± 0.86**	9.12 ± 0.71
Osmia cornuta	1.77 ± 0.74	0 ± 0	0.87 ± 0.19	0.88 ± 1.53	**78.35 ± 5.17**	0 ± 0	**10.54 ± 3.5**	**7.17 ± 1.46**	0 ± 0	0.42 ± 0.08	0 ± 0	0 ± 0	0 ± 0	5.43 ± 0.49
Apidae														
Apis mellifera	1.06 ± 0.59	**6.32 ± 5.66**	1.62 ± 0.54	2.07 ± 1.87	**60.85 ± 5.57**	3.21 ± 2.97	**9.9 ± 2.73**	**12.76 ± 1.65**	0 ± 0	0 ± 0	0.68 ± 0.62	0 ± 0	1.55 ± 1.61	8.7 ± 2.04
Bombus terrestris	1.3 ± 0.43	**43.29 ± 14.97**	0 ± 0	0 ± 0	**24.21 ± 2.37**	0.53 ± 0.51	**14 ± 3.96**	**14.31 ± 6.07**	0 ± 0	0 ± 0	0 ± 0	0.59 ± 1.03	1.76 ± 2.71	4.11 ± 0.31
Ceratina chalybea	**8.03 ± 5.41**	**46.47 ± 2.1**	1.32 ± 0.2	0.37 ± 0.65	**24.06 ± 3.63**	0.32 ± 0.55	**7.27 ± 0.7**	4.79 ± 1.17	4.93 ± 4.3	0.53 ± 0.92	0 ± 0	0.88 ± 0.33	1.04 ± 1.07	5.3 ± 1.99
Ceratina cucurbitina	1.01 ± 0.34	**11.02 ± 4.76**	0.9 ± 0.17	0 ± 0	**44.37 ± 6.75**	0 ± 0	**7.02 ± 0.47**	**26.1 ± 5.57**	0 ± 0	3.14 ± 3.38	0.85 ± 0.37	1.17 ± 0.96	4.43 ± 2.22	2.22 ± 0.24
Eucera dimidiata	1.29 ± 0.53	0 ± 0	0.49 ± 0.07	**8.11 ± 1**	**28.77 ± 3.63**	**12.05 ± 2.34**	**10.21 ± 15.06**	**33.1 ± 6.82**	0.55 ± 0.64	0 ± 0	2.52 ± 0.39	0 ± 0	2.91 ± 0.81	12.34 ± 1.92
Xylocopa iris	1.12 ± 0.03	0 ± 0	0.8 ± 0.07	**7.83 ± 0.9**	**25.44 ± 1.11**	**10.18 ± 0.89**	**20.11 ± 4.42**	**26.29 ± 1.91**	1.67 ± 1.29	0.48 ± 0.17	2.2 ± 0.37	0 ± 0	3.89 ± 0.38	7.11 ± 0.91
Sphecidae														
Isodontia mexicana	**90.79 ± 1.54**	0.38 ± 0.05	0 ± 0	1.14 ± 0.09	1 ± 0.22	0 ± 0	1.62 ± 0.31	0.84 ± 0.13	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4.23 ± 1.09	5.27 ± 0.43
Crabronidae														
Pemphredonina indet.	**31.59 ± 4.72**	0 ± 0	0 ± 0	**8.4 ± 2.48**	1.64 ± 3.29	0 ± 0	**8.92 ± 4.57**	**22.81 ± 2.47**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	**26.64 ± 5.49**	0.16 ± 0.02
Philanthus triangulum	**5.55 ± 4.08**	**9.3 ± 4.5**	1.87 ± 0.62	0 ± 0	**46.18 ± 3.5**	0 ± 0	**14.72 ± 0.22**	**19.6 ± 1.25**	1.82 ± 1.81	0 ± 0	0 ± 0	0 ± 0	0.94 ± 1.33	3.75 ± 0.95

4.3. Sterol profiles across Apoidea and prey-pollen similarities

The data set used in this section contained relative values (expressed as percentage of total sterols). The proportions between compounds in each sample were thus preserved, but the influence of interspecific differences in total sterol concentration were eliminated. The sterol 'profiles' could therefore be compared.

Three main groups were highlighted by the AHC analysis. The first one (Fig. 10, A) brought together two species of sphecoid wasp and three bee species from different families. Larvae of these three species, *Halictus scabiosae*, *Dasypoda hirtipes* and *Heriades truncorum*, all have a diet based on Asteraceae pollen (Tab. 2). The second group (Fig. 10, B) brought together long-tongued bee species from the Apidae and Megachilidae families with *Philanthus triangulum*, whose larval diet consists of *Apis mellifera* (Apidae). The third group (Fig. 10, C) included all the remaining species, and was therefore composed of the Colletidae and Andrenidae, as well as the Apidae, Megachilidae and Melittidae that were not included in the first two groups. The two *Dasypoda* species were therefore split in distant branches. Overall, most of the individuals were correctly grouped according to their species, but the taxonomic families were not reconstituted.

The same groups could be observed with the PCA performed on the same data set (Fig. 11). Three main variables seemed to explain the formation of these groups: cholesterol, 24-methylenecholesterol + campesterol, and β-sistosterol. These compounds were respectively the most abundant C_{27}, C_{28} and C_{29} sterols in the overall sample (Tab. 3). The distribution of the group A) on the figure 11 (upper left-hand section) indicates that the prey - pollen similarities highlighted by the AHC were indeed linked to the cholesterol content.

4.4. Relation between floral specialisation and pollen composition

While no clear phylogenetic signal was observed, interesting grouping patterns related to dietary specialisation were observed. All the polylectic species were distributed on the right-hand side of the PCA graph, while more specialised species (oligolectic and 'polylectic with strong preference') were mainly distributed to the left-hand side. *Megachile cypricola* and *Hoplitis adunca* were the exception and were mixed in with the polylectic species. This separation occurred along the principal component 1, which is correlated with cholesterol and 24-methylenecholesterol + campesterol, but not with β-sistosterol.

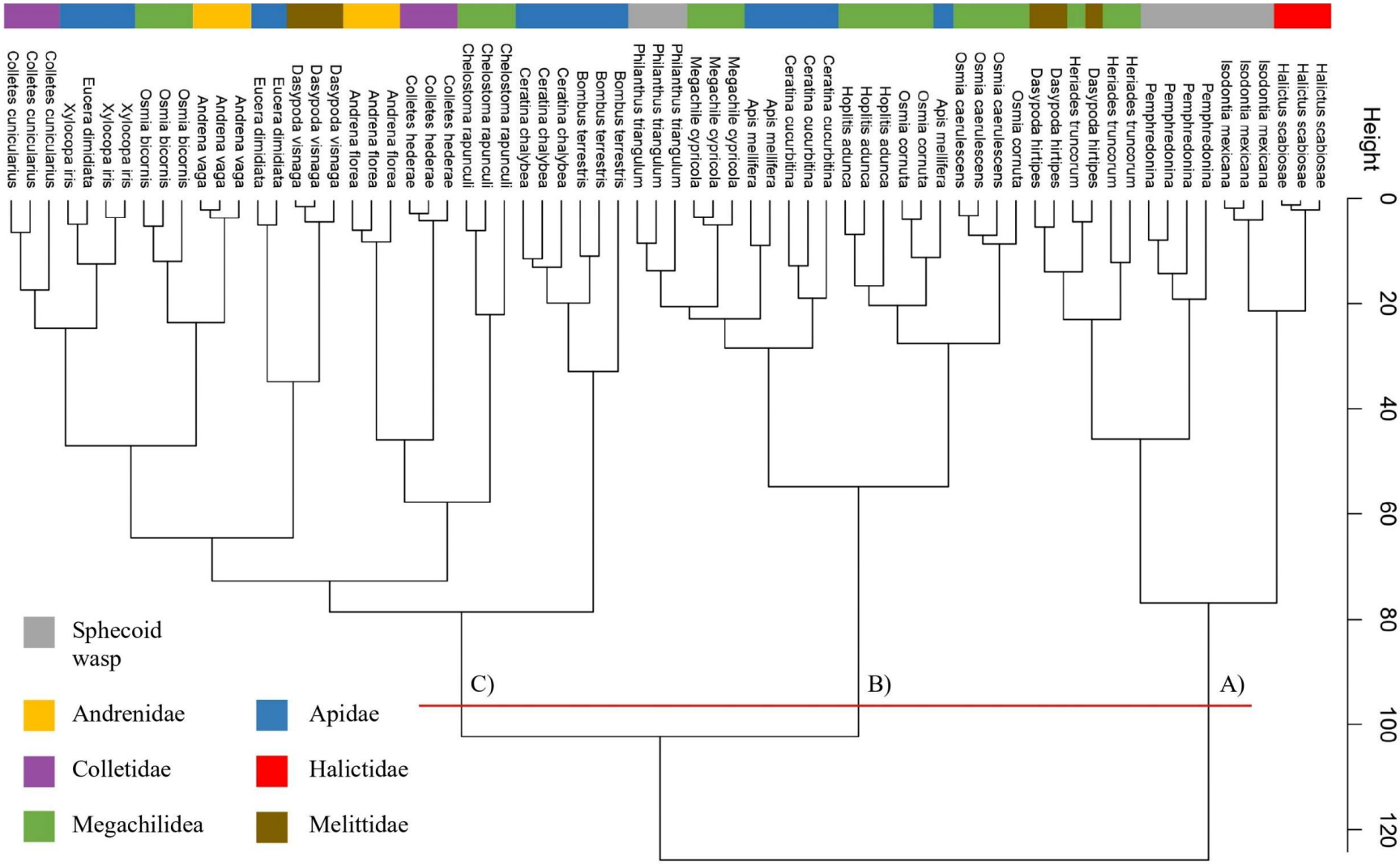

Figure 10: Agglomerative hierarchical clustering using the complete-linkage method, based on a Euclidean distance matrix calculated with the data transformed into percentage of total sterols. The taxon to which each species belongs is colour coded. A, B & C: the 3 main groups formed.

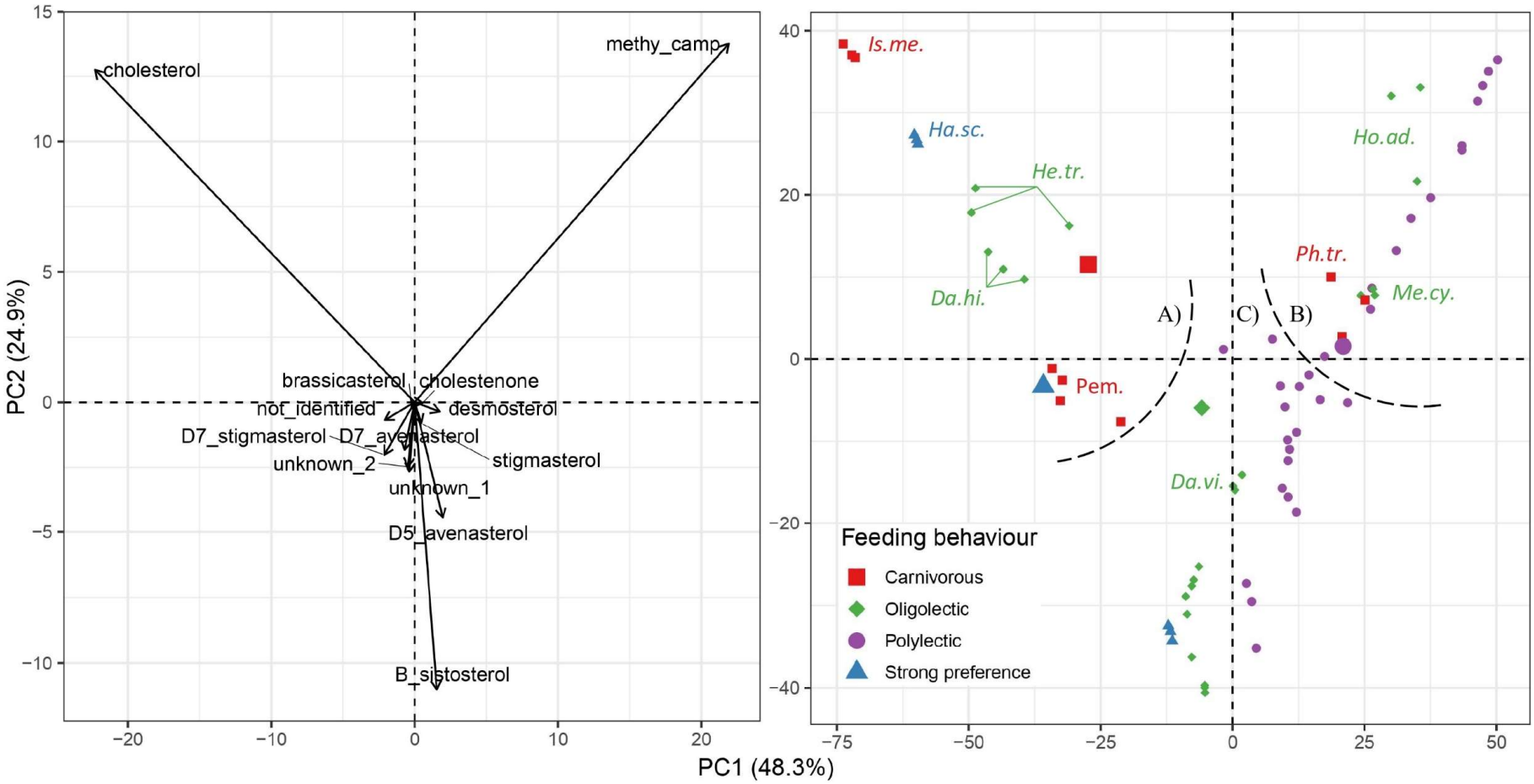

Figure 11: Principal component analysis based on data transformed into percentage of total sterols. The two principal axes explain together 73,2% of the total variance. Left: variables; right: individuals, represented by the small-sized points and coloured according to their feeding behaviour. The four larger shapes represent the centroids of each category. A, B, C: the 3 main groups formed by the AHC analysis, separated by dotted lines. *Da.hi.*: *Dasypoda hirtipes*; *Da.vi.*: *Dasypoda visnaga*; *Ha.sc.*: *Halictus scabiosae*; *He.tr.*: *Heriades truncorum*; *Ho.ad.*: *Hoplitis adunca*; *Is.me.*: *Isodontia mexicana*; *Me.cy.*: *Megachile cypricola*; Pem.: Pemphredonina indet.; *Ph.tr.*: *Philanthus triangulum*

The PCA performed with standardised data provided a better representation of the variables masked by the dominant sterols in the previous PCA (Fig. 12). Despite a low proportion of variance explained, a similar pattern tending to separate species with a wide dietary range from more the more specialised ones was observed. However, none of the two principal components clearly made the separation. The polylectic species were mainly distributed to the left and upwards, and the more specialised species were mainly distributed to the right and downwards. Cholesterol, Δ7-Stigmasterol, Δ7-Avenasterol and 'Unknown 2' appeared mainly associated to specialised species, while 24-methylenecholesterol + campesterol, brassicasterol and β-sistosterol appeared mainly associated with polylectic species. Nevertheless, the high number of variables made interpretation complex.

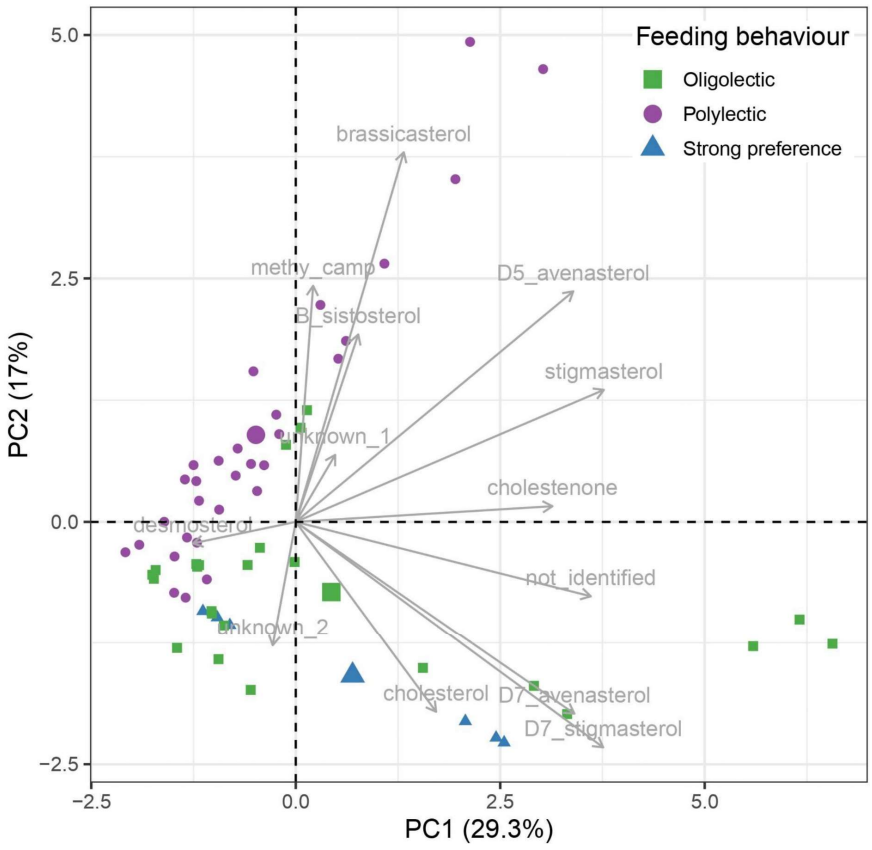

Figure 12: Principal component analysis based on data standardised to the z-score (mean = 0, SD = 1). The two principal axes explain together 46.3% of the total variance. Variables are represented in grey. The individuals are represented by the small-sized points and are coloured according to their feeding behaviour. The four larger shapes represent the centroids of each category.

The PCA performed with the second standardised data set (in which the sterols were pooled by number of carbon atoms, and 'Unknown 2' was included to 'Not Identified'), showed an even more clear diet-related pattern (Fig. 13). As in the previous analysis, none of the two principal components clearly made the separation. The polylectic species were mainly distributed to the left and upwards, while the more specialised species were mainly distributed to the right and downwards. The sterol differences between pollen provisions of polylectic and more specialised species appeared to be driven mainly by the content of C_{27} and C_{28} sterols. The sum of the C_{29} sterol contents, while explaining a large part of the variability, was not particularly associated to one group or another. However, it was strongly associated with *Dasypoda visnaga*.

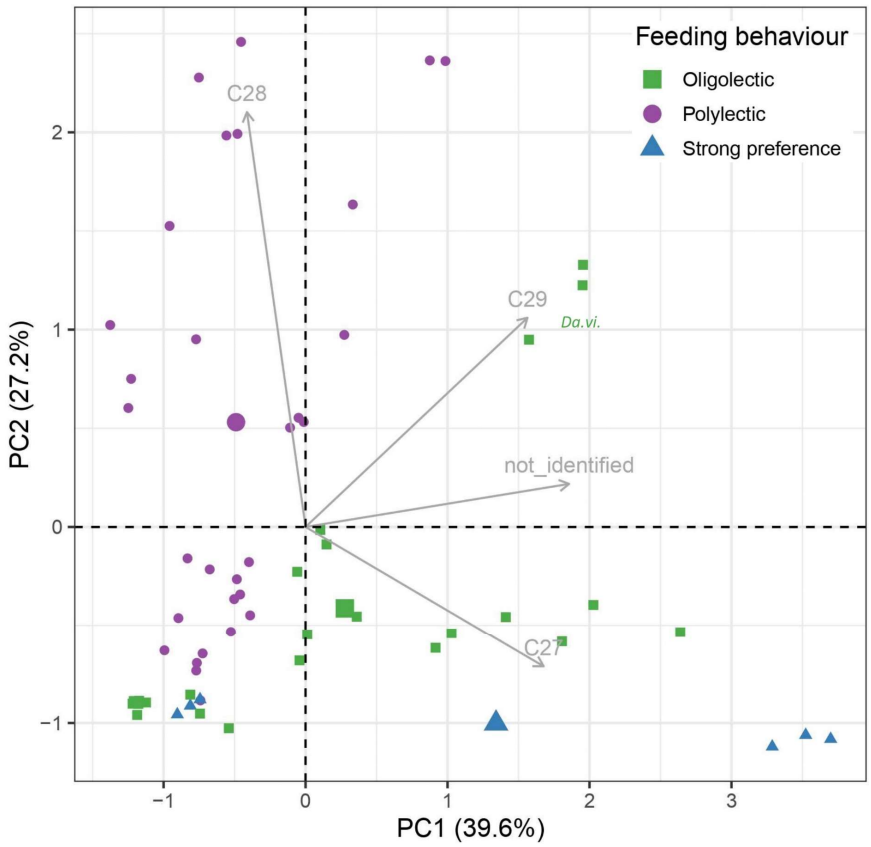

Figure 13: Principal component analysis based on a dataset with sterols pooled by number of carbon atoms (C_{27}, C_{28} and C_{29} sterols), and with 'Unknown 2' added to the 'Not Identified' pool. Data standardised to the z-score (mean = 0, SD = 1). The two principal axes explain together 66.8% of the total variance. Variables are represented in grey. The individuals are represented by the small-sized points and are coloured according to their feeding behaviour. The four larger shapes represent the centroids of each category. *Da.vi.*: *Dasypoda visnaga*.

These C_{29} sterols, which did not appear to be clearly linked to differences between polyleges and specialists, were not included in the last tested dataset. The same pattern as in previous analyses could be observed with this last PCA: a clear trend towards the separation of polylectic and specialist groups (Fig. 14). The specialised species were grouped at the bottom left while the polylectic species were way more spread. While *Dasypoda visnaga* was associated to cholestenone (C_{27}), the remaining specialised species were mainly associated with cholesterol. In polylectic species, the trends were diverse: most were associated with the 24-methylenecholesterol + campesterol and brassicasterol (C_{28}), while *Bombus terrestris* and *Ceratina chalybea* were associated with desmosterol (C_{27}). *Osmia bicornis*, *Ceratina cucurbitina* and *Colletes cunicularius* were not clearly associated to a variable.

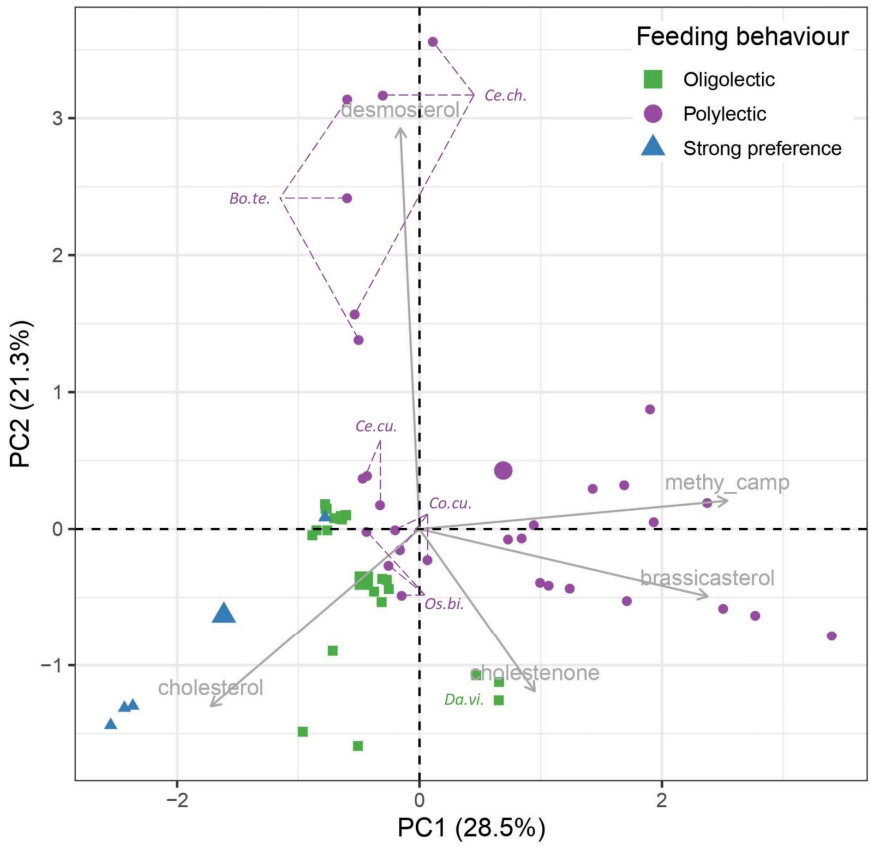

Figure 14: Principal component analysis based on a dataset including only C_{27} and C_{28} sterols. Data standardised to the z-score (mean = 0, SD = 1). The two principal axes explain together 49.8% of the total variance. Variables are represented in grey. The individuals are represented by the small-sized points and are coloured according to their feeding behaviour. The four larger shapes represent the centroids of each category. *Bo.te.*: *Bombus terrestris*; *Ce.ch.*: *Ceratina chalybea*; *Ce.cu.*: *Ceratina cucurbitina*; *Co.cu.*: *Colletes cunicularius*; *Da.vi.*: *Dasypoda visnaga*; *Os.bi.*: *Osmia bicornis*.

5. Discussion

In this work, sterol composition of larval provisions from 20 species of bees and three species of sphecoid wasps were analysed. The target was to describe how these essential compounds varied with lineages and feeding specialisation, in order to better understand the emergence and evolution of bees.

5.1. Sterol composition across bee diet

In a recent survey, Zu *et al*. (2021) examined the sterol profiles of pollen from 122 plant species belonging to 51 families. They found out that 24-methylenecholesterol, Δ5-avenasterol (= isofucosterol) and β-sitosterol were the most abundant phytosterols across species, with averages of 23%, 21.5% and 20.7% of the total sterols respectively. Our results for bee pollen provisions were consistent and showed the same three dominant phytosterol: in average, the pooled 24-methylenecholesterol and campesterol represented 28.94%, the Δ5-avenasterol 16.39%, and the β-sitosterol 16.64% of the total sterols across species. Although the orders of magnitude were similar, we could observe that the main C_{29} sterols presented a lower relative content in bee-collected pollen overall.

However, our results diverged more sharply from those of Zu *et al*. (2021) with regard to cholesterol content: while it represented less than 1% of the average plant sterols, it was the fourth most abundant sterol in bee-collected pollen, with a mean of 11.51%. This is mainly due to the high frequency of Asteraceae specialists, which are well represented in the sample. Indeed, several Asteraceae have a cholesterol-rich pollen (Devys & Barbier, 1966; Vanderplanck *et al*., 2020b). This could be exacerbated by the phenomenon of addition of endogenic C_{27} sterols highlighted by Vanderplanck *et al.* (2020b).

Pollen provisions of *Andrena vaga* and *Colletes cunicularius* displayed similar profiles than those described Vanderplanck *et al.*, (2020a), while strong differences in proportions between the different sterols were observed in *Osmia cornuta*: 24-methylenecholesterol + campesterol were nearly twice more concentrated in our samples (78.35% against 40.46%), and other sterols (cholesterol, β-sitosterol and Δ5-avenasterol) were therefore substantially lower. In *Apis mellifera* as well, 24-methylenecholesterol + campesterol was more concentrated in pollen provisions analysed in this study than in pollen loads from Vanderplanck *et al.* (2011) (60.85% against 2.5%). This probably reflects the

extent of intraspecific variability in polylectic species. In contrast, the sterol content of *Apis mellifera* adults analysed as larval food for *Philanthus triangulum* was consistent with Vandeplanck *et al.* (2011). Pollen provisions of *Colletes hederae* and *Hoplitis adunca* showed no major differences with pollen from their host plants (*Hedera helix* and *Echium vulgare* respectively) (Vanderplanck *et al.*, 2020b). Finally, the very low sterol levels measured in aphids are consistent with the general trend in this group (Jing & Behmer, 2020).

5.2. Similarities between prey and pollen from basal bee clades

While we found strong similarities between preys of sphecoid wasps and pollen collected by certain bee taxa, these similarities were not restricted to the basal Melittidae family. The pollen provisions from *Heriades truncorum* (Megachilidae) and *Halictus scabiosae* (Halictidae), which are not particularly 'basal' taxa (*e.g.*, Danforth *et al.*, 2008; Praz *et al.*, 2008c; Hedtke *et al.*, 2013), also displayed high cholesterol content and therefore clustered with prey. Moreover, these similarities did not seem to be maintained within the Melittidae family either: *Dasypoda visnaga*, despite being oligolectic on the Asteraceae just like *Dasypoda hirtipes*, showed no particularly high cholesterol content. These findings are consistent with the relatively low cholesterol content of pollen from host plants of the *Melitta leporina* group (Melittidae) (Vanderplanck *et al.*, 2017).

However, pollen from *Dasypoda visnaga* contained cholestenone in similar proportions to those found for *Andrena vaga* and *Colletes cunicularius* by Vanderplanck *et al.* (2020a): 2.40% and 2.36% respectively, whereas we obtained 2.48% for *D. visnaga*. In this study, the authors brought evidence that the two species in question use this rare C_{27} sterol for moulting hormone synthesis. We do not rule out the possibility of a similar metabolism in *D. visnaga*.

The similarities in phytosterol composition between the typical prey of *Philanthus triangulum* (*Apis mellifera* specimens) and the pollen of several species of long-tongued bees were naturally expected. However, it is interesting to consider that several other taxa of sphecoid wasps probably also feed on phytosterol-rich prey: the Astatinae mainly hunt phytophagous Heteropterans (Parker, 1962; 1969; 1972) and all species from the Philanthini tribe hunt bees (Bohart & Grissell, 1975). As these preys lack the C_{24}-dealkylation pathway (Nes & Behmer 2003), sphecoid wasps from these groups could therefore be subject to the same phytosterol constraints as bees, despite being carnivorous.

5.3. Sterols and lectism

Groupements peut-être associés au signal phylogénétique des plantes

Our analyses revealed a clear pattern tending to separate polylectic species from the more specialised ones, mainly on the basis of the C_{27} and C_{28} sterols in their pollen provisions.

The specialised species were mainly associated with cholesterol (C_{27}), except *Dasypoda visnaga* which was associated with cholestenone (C_{27}). This corroborates at larger scale what was previously observed in *Dasypoda hirtipes* by Vanderplanck *et al*. (2020b). While species with the highest cholesterol rates were Asteraceae foragers, the trend towards isolation from polylectic species also affected the other specialised taxa to some extent.

Previous studies have shown that even with low levels of C_{27} sterols, some species appear to be constrained to produce C_{27} moulting hormones instead of metabolising abundant phytosterols (Feldlaufer *et al*., 1993; Vanderplanck *et al.*, 2020a). Our results suggest that these constraints could be mainly associated with specialised species. At present, evidence of the use of Makisterone A (alternative C_{28} moulting hormone) has only been provided for common and highly polylectic species (Feldlaufer *et al*., 1985; Regali, 1996; Vanderplanck *et al.*, 2020a), while the two only oligolectic species investigated, *Andrena vaga* (Andrenidae) and *Diadasia rinconis* (Apidae), appear to rely on C_{27} moulting hormones (Feldlaufer *et al*., 1993; Vanderplanck *et al.*, 2020a).

In polylectic species, we observed a clear association with the C_{28} sterols, and especially with 24-methylenecholesterol + campesterol which are among the most common sterols in plants (Zu *et al*., 2021). This is consistent with Vanderplanck *et al*. (2020a), who suggested that the ability of polylectic species to metabolise such sterols could facilitate pollen harvesting from a wide range of host plants. However, *Ceratina chalybea* and *Bombus terrestris* were mainly associated with desmosterol (C_{27}). The latter species is in fact capable of using both C_{27} and C_{28} sterols as precursors of ecdysteroids (Regali, 1996). The use of C_{27} sterols has also been suggested in *Colletes cunicularius*, which does not appear to be able to use 24-methylenecholesterol, unlike *Bombus terrestris* (Vanderplanck *et al*., 2020a).

While C_{29} sterols pooled together were strongly associated with *Dasypoda visnaga* and explained an important part of the sterolic variability overall, they appeared not to be associated to a particular category of flower specialisation. Therefore, we provided no evidence for a specific selection of sterol with C_{29} backbone. This is consistent with the

current knowledges, as only C_{27} and C_{28} are known to be used for moulting hormone synthesis in bees (Feldlaufer *et al.*, 1985; 1986; 1993; Regali, 1996; Vanderplanck *et al.*, 2020a).

Unexpectedly, Δ7-avenasterol and Δ7-stigmasterol (C_{29}) appeared strongly associated with the specialised species. An accumulation of evidence suggests that Δ7-sterols are not adapted to the growth of many non-specialist insects, and could even be toxic and act as protection against phytophagy (*e.g.*, Ritter & Nes, 1981; Behmer & Elias 1999a; 1999b; Vanderplanck *et al.*, 2014; 2018; 2020c).

The presence of these rare Δ7-sterols in Asteraceae pollen has recently been suggested as a factor potentially responsible for the 'Asteraceae paradox' (Vanderplanck *et al.*, 2020b; 2020c; Zu *et al.*, 2021). This phenomenon highlighted by Müller & Kuhlmann (2008) refers to the fact that polylectic bees seem to avoid Asteraceae despite their ubiquitous distribution. The pollen from this family has shown to have negative impact on growth of several bee species (Praz *et al.*, 2008b; Vanderplanck *et al.*, 2018), and Vanderplanck *et al.* (2020c) provided evidence for a chemical factor related to phytosterols.

In our sample, pollen from specialised species contained high levels of Δ7-sterols (>5%), except in the three species specialised on Fabaceae, Salicaceae and Boraginaceae, which are common families in diet of polylectic species (*e.g.*, Müller 1996; Müller & Kuhlmann, 2008; Sedivy *et al.*, 2013; Haider *et al.*, 2014; Wood *et al.*, 2016). Our results therefore support the idea that Δ7-sterol could be the chemical factor avoided by polylectic species.

Overall, the results strongly support previous findings suggesting that the sterols shape bee-plant interactions and host specialisation (Vanderplanck *et al.*, 2017; 2018; 2020a; 2020b). The evidence provided supports that the sterol content of pollen could be a limiting factor in the transition from oligolectism to polylectism. Although the hypothesis of similarities between the prey and pollen collected by basal Melittidae has not been validated, oligolectism, which is the ancestral state in bees, carries an intrinsic phylogenetic signal. The pattern of specialised species being associated with cholesterol therefore seems to support that C_{27} sterols produced by certain early flowers facilitated the emergence of bees from sphecoid wasps.

5.4. Perspectives

Despite the accumulation evidence that sterols are important to understand the evolution of bee-plant interactions, these molecules and their metabolisation remain under-studied in bees. Further research focussing on how bees are using sterols will provide a better understanding of the constraints that guide their floral choices.

Although the analysis of ecdysteroids would provide the most informative data, the concentration of these molecules fluctuates greatly during insect development (*e.g.*, Sehnal *et al.*, 1981; Rachinsky *et al.*, 1990), which makes it very difficult to study them in wild species whose cycle is not controlled. The preliminary GC-MS/MS analysis carried out in the framework of this study on different larval stages of reared bumblebees failed to detect a signal corresponding to ecdysteroids.

Therefore, the approach of Vanderplanck *et al.* (2020a), who compared sterol levels between larvae at the last stage of development and non-emerged adults, would be a more realistic method for assessing the probable sterols used as moulting hormone precursors in wild species. Conducting these analyses on certain taxa highlighted by this study (*e.g.*, *Dasypoda hirtipes*, *Heriades truncorum*, *Chelostoma rapunculi*, *Hoplitis adunca*, *Colletes hederae*) would allow to test the hypothesis of widespread use of C_{27} sterols in specialised species.

Similar research should be carried out on sphecoid wasps hunting herbivorous insects that are unable to dealkylate phytosterols (*e.g.*, Astatinae, Philanthinae). Such predators could be subject to the same constraints as bees and have a capacity to metabolise phytosterols similar to some of them. Studying whether this ability to use phytosterols as precursors of moulting hormones may have appeared several times in Apoidea, or whether it could be shared between bees and closely related wasps, is of major interest for understanding the evolution of this clade.

Finally, the question of the potential Δ7-sterols avoidance by polylectic species should be deeply examined through laboratory experiments. Bioassays evaluating the effects of plants with high levels of these sterols on the growth of polylectic species (*e.g.*, reared *Apis mellifera*, *Bombus terrestris* and *Osmia* spp.) could verify this hypothesis.

6. Conclusion

By characterising the sterol composition of pollen provisions from twenty phylogenetically diverse bee species, this study provides the most comprehensive overview of sterol profiles in bee-collected pollen to date. The overall proportions of the different sterols collected by bees were similar to those observed in plants, with the exception of cholesterol which was more the than times higher in average in bee pollen provisions.

Although we detected strong similarities between the pollen collected by certain bees and the prey of sphecoid wasps, these similarities were neither specific to the Melittidae nor maintained within this basal family, contrary to our hypothesis. These similarities were associated to the cholesterol-rich pollen collected by certain Asteraceae specialist species, belonging to different families.

We observed clear differences in general trends between polylectic species and more specialised species. These trends corroborated our hypothesis: pollen collected by specialised species tended to be associated with C_{27} sterols (mainly cholesterol, more rarely cholestenone), while polylectic species displayed more variable patterns. Most exploited pollen associated with common C_{28} phytosterols and some were associated cholestenone (C_{27}). Our results therefore support the existence of constraints related to sterols, which shape plant-pollinator interactions and could limit the transition from oligolectism to polylectism.

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8. Appendices

8.1. Appendix 1: GC-FID analytic conditions

Column	SLB-5MS (length: 30 m; inner diameter: 0.25 mm; film thickness: 0.25 µm)
Injection volume	1 µl
Injection mode	Splitless at 280°C
Temperature	60°C for 1 min, increasing to 290°C (30°C/min), isotherm at 290°C for 22 min, thermal peak at 320°C for 5 min
FID Temperature	350°C
Carrier gas	Helium
GC-FID device	GC-2010 (Shimadzu)

8.2. Appendix 2: ANOVA results

R code and result:

```r
#ANOVA: total_sterol ~ species

anova_res <- aov(total_sterol ~ species,
  data = data_total)

summary(anova_res)
```

```
            Df Sum Sq Mean Sq F value Pr(>F)
species     22 1982.7   90.12   65.97 <2e-16 ***
Residuals   47   64.2    1.37
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Post hoc results for *Dasypoda* spp. and Preys:

Species	diff	lwr	upr	p adj
Dasypoda visnaga - Dasypoda hirtipes	-0.81978098	-4.47888955	2.83932758	0.9999994
Isodontia mexicana - Dasypoda hirtipes	-6.39838965	-10.05749821	-2.73928109	0.0000052
Pemphredonina - Dasypoda hirtipes	-11.51054421	-14.93332686	-8.08776157	0.0000000
Philanthus triangulum - Dasypoda hirtipes	-7.92243381	-11.58154237	-4.26332525	0.0000000
Isodontia mexicana - Dasypoda visnaga	-5.57860867	-9.23771723	-1.91950011	0.0000988
Pemphredonina - Dasypoda visnaga	-10.69076323	-14.11354587	-7.26798058	0.0000000
Philanthus triangulum - Dasypoda visnaga	-7.10265283	-10.76176139	-3.44354427	0.0000004

8.3. Appendix 3: C_{27}, C_{28} and C_{29} sterols content

Table of the average content of C_{27}, C_{28} and C_{29} sterols per species. Values are expressed as percentage (%) of total sterols. N.I.: Sum of the minor unidentified compounds + 'Unknown 2'. Dominant compounds are underlined.

Species	**C_{27}**	**C_{28}**	**C_{29}**	**N.I.**
Andrena florea	1,27	5,84	<u>74,45</u>	18,45
Andrena vaga	6,50	3,43	<u>89,22</u>	0,85
Apis mellifera	8,99	<u>62,92</u>	26,54	1,55
Bombus terrestris	<u>44,59</u>	24,21	28,84	2,36
Ceratina chalybea	<u>55,81</u>	24,44	17,83	1,92
Ceratina cucurbitina	12,93	<u>44,37</u>	37,10	5,60
Chelostoma rapunculi	3,52	1,02	<u>55,79</u>	39,67
Colletes cunicularius	4,15	35,96	<u>58,08</u>	1,81
Colletes hederae	3,31	0,00	<u>89,70</u>	6,99
Dasypoda hirtipes	<u>57,97</u>	3,37	31,89	6,77
Dasypoda visnaga	3,11	14,09	<u>75,90</u>	6,91
Eucera dimidiata	1,78	36,88	<u>58,43</u>	2,91
Halictus scabiosae	<u>75,73</u>	2,70	18,61	2,95
Heriades truncorum	<u>52,06</u>	9,13	34,86	3,95
Hoplitis adunca	9,07	<u>69,06</u>	19,75	2,13
Isodontia mexicana	<u>91,17</u>	2,14	2,46	4,23
Megachile cypricola	14,62	<u>50,15</u>	34,16	1,08
Osmia bicornis	7,26	16,67	<u>75,25</u>	0,83
Osmia caerulescens	0,68	<u>82,36</u>	10,72	6,24
Osmia cornuta	2,64	<u>79,23</u>	18,13	0,00
Pemphredonina indet.	31,59	10,05	<u>31,73</u>	26,64
Philanthus triangulum	16,73	<u>46,18</u>	36,15	0,94
Xylocopa iris	1,92	33,27	<u>60,93</u>	3,89