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Specialisation and adaptation in pollen sterol use by wild bees

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SUMMARY

Sterols are stabilising components of membranes and hormone precursors in eukaryotes. Honeybees incorporate a subset of pollen-derived sterols into their tissues, rather than converting phytosterols to cholesterol as occurs in most insect herbivores. To establish whether this approach to sterol acquisition was typical of all bees, we measured the sterolome of 56 bee species representing all major bee families to identify and quantify which pollen sterols they used. The Δ5 sterols, 24-methylenecholesterol, isofucosterol and β-sitosterol, which are common components of pollen sterolomes were also the main sterols in most bee species, but this was not so in a minority of species suggesting they had different sterol requirements. Generalists contained more 24-methlyenecholesterol than their specialist congeners, suggesting an adaptation to use pollen sterols that occur widely in plants whereas Asteraceae specialists use pollen sterols that are not used by generalists, which may explain the Asteraceae paradox. Overall, our data suggests an ecological rather than phylogenetic driver of bee sterol composition.

Keywords Bee sterolome, wild bee nutrition, pollen sterols, 24-methylenecholesterol, isofucosterol, campesterol.

INTRODUCTION

The co-evolution of bees and flowers has led to an inter-dependency whereby many flowers are reliant on animal pollination and the flower visiting bees depend on floral nectar for carbohydrate and pollen for their protein and lipid requirements[1]. However, the chemical composition of pollen with respect to nutrient ratios, amino acid profiles, defence compounds and lipids including sterols is highly variable, potentially influencing the suitability of the pollen for palynivores[2, 3, 4, 5, 6, 7, 8].

Sterols are essential micronutrients serving key functions as membrane components and steroid hormone precursors in animals and plants. Sterols vary significantly in pollen with most variation explained by the position of a double bond in the steroid B ring and substitution at C-24 [8]. For example, six pollen sterols are required to optimise brood production in honeybees and these are characterised by a double bond at C-5 (Δ5 sterols)[9]. Since pollen sterols vary widely across taxa and most pollen contains only a few major sterols some pollen may not be a suitable food source for all bees [8]. Many of these pollen sterols such as 24-methylenecholesterol, isofucosterol, and desmosterol are not found in animal tissues and are scarce in terrestrial plant vegetative tissues [5,8,10]. Honeybees have lost the capacity to synthesise cholesterol, and unlike other insects cannot dealkylate phytosterols at C-24 to produce cholesterol[11]. Instead, honeybees rely on phytosterols derived from pollen for tissue sterol composition, in place of cholesterol[9,12]. Moore et al., [9] showed that 6 pollen sterols including 24-methylenecholesterol, isofucosterol and desmosterol supported substantial increases in brood production while Bogaert et al.[13] also reported that 24-methylenecholesterol and isofucosterol were required by honeybees to produce offspring. This contrasts with many insect herbivores that can dealkylate dietary phytosterols to cholesterol, although sterol metabolic capacity varies substantially across taxa and some herbivores retain appreciable phytosterols in their tissues[14].

Whether or not all bee species share the sterol metabolism of honeybees is unknown, though the few studies that have reported sterols from the tissues of other bees indicate that they have several of these sterols in common (e.g., 24-methylenecholesterol)[14,15,16]. Given the diversity of sterols in pollen[8], it is possible that other bee species show similarities between the sterolome of their food and the sterols they use in their tissues. The tissues of solitary bees contain phytosterols in common with honeybees but in different proportions. For example, *Diadasia rinconis* is dominated by 24-methylenecholesterol, while isofucosterol is three times higher in *Megachile rotundata* than honeybees, and β-sitosterol accounted for over 50% of total sterol content in *Colletes cunicularius* [15,16,17]. This suggests that they either ingest a different sterol content to honeybees and/or that these bee species have specific sterol transporters in the gut that favour particular sterols in food[18].

Wild bees provide an important pollination service for many wildflower species as well as contributing to pollination of many crops[19]. Some wild species also exhibit dietary specialisation in pollen foraging. Monolectic bee species for example forage from a single plant species for pollen, although this degree of specialisation is not common[20,21] while oligolectic species collect pollen from species belonging to a single genus or family[22]. Specialisation may limit distribution and phenology of wild bees. For instance, *Melitta dimidiata* (sainfoin bee) is confined to Salisbury plain in the south of England due to its reliance on *Onobrychis viciifolia*[21]. Asteraceae pollen is associated with oligolectic bee species and is rarely gathered by polylectic species such as honeybees, a phenomenon referred to as the 'Asteraceae paradox'[22,23,24]. Polylectic bee species collect pollen from multiple families and are often referred to as generalist foragers. In the UK where the present study was undertaken all bumblebees and some solitary bee species are polylectic[21]. The UK also hosts approximately 70 kleptoparasitic bee species, including six cuckoo bumblebee species which do not have any morphological adaptations for collecting pollen on their bodies and rely on host bee species to provision their young[21] and so present an interesting target for nutritional studies.

There are over 270 bee species native to the UK, which range in their distribution owing to the availability of different flowering plants, nesting requirements and climate[21]. Florally homogenous landscapes may be nutritionally incapable of supporting a diverse bee community with a wide range of nutritional requirements, notably specialist species. In nutritionally homogenous arable landscapes, seed mixes designed to support bee populations are often targeted at common, generalist taxa such as bumblebees, however the nutritional value of these flowers for other bee species is currently unknown. Bumblebees are often target taxa of seed mixes designed to increase floral resources in amenity and agricultural settings[25]. However, plant selection for these mixes is not based on an understanding of the nutritional value of specific flowers to a range of different bee species[26]. Since sterols vary across plant taxa[8] specific sterol compounds may not be available in pollen from plants in all habitats. Therefore, we sought to determine how sterols varied in wild bees and how this related to pollen sterols.

Specifically the study addressed the following questions: 1) Which sterols are found in wild bee species and do they differ across taxa and in different floral landscapes?; 2) How variable is the sterolome of related generalist and specialist solitary bees and do kleptoparasites have similar sterolomes to their hosts?; 3) How variable are the sterolomes of bumble bees?; 4) Do bees regulate or bias their sterol incorporation?; 5) Can bees adapt to the pollen sterols of different plants? In addressing these questions, we present sterol data from taxonomically diverse solitary bee species and bumblebees representing all bee families worldwide except for the Stenotritidae which is restricted to Australia. We examined the data for differences based on lecty and on phylogenetic relatedness and present a comprehensive overview of the patterns in sterol use across these species. In doing so we also reveal how the sterolome in bees is related to their pollen food (e.g., in monolectic species and Asteraceae specialists); and how the sterols vary between bee tagmata and between sexes of the same bee species.

RESULTS

The sterol profile of bees is largely defined by the three pollen sterols

Total sterol content and sterol profile (sterolome) varied both within and between bee species (Figures 1 and 2). Nineteen sterols were detected across 56 wild bee species of which 13 sterols were assigned using standards. The dataset included sterol analysis for seven eusocial *Bombus* species and 49 solitary bee species and included 21 genera representing all bee families except for Strenotritidae which are restricted to Australia.

The sterolomes of bees analysed here contained isofucosterol, β-sitosterol and 24-methylenecholesterol at a median percentage of >10%. The relative abundance of these three sterols varied as a function of bee species (Figure 1, Table S1). For example, the relative quantities of 24-methylenecholesterol within the genus *Lasioglossum* ranged from 3% in the shaggy furrow bee (*L. villosulum*, species mean) to 72% in Smeathman's furrow bee *L. smeathmanellum*.

The relative amount of β-sitosterol was never lower than 2% and it was recorded < 10% for only six species. In addition, 24-methylenecholesterol and β-sitosterol were the only sterols that were recorded at >70% of the sterolome of any given species (e.g., β-sitosterol: *Hylaeus signatus*, 24-methylenecholesterol: *Halictus rubicundus*, *Lasioglossum smeathmanellum*). Fourteen of the nineteen sterols recorded accounted for less than 2% of the total sterols on average (Table S1). In Asteraceae specialists, cycloartanol was an important sterol (>15%: *Andrena denticulata*, *A. humilis*, *A. fulvago*, *Colletes daviesanus*, *Heriades truncorum*, *Osmia leaiana*, *Dasypoda hirtipes*) (Figure 1). The fungal sterol, ergosterol was recorded in 39 bee species and was likely recorded as a result of fungal contamination of pollen as it is recorded widely on plant pollen[8] (Table S1).

Most bee sterolomes contained at least 17 out of the 19 sterols we recorded (Table S3). Only three species contained fewer than 10 sterols: *Halictus rubicundus* (9), *Ceratina cyanea* (8), *Nomada flavopicta* (8). Five species contained no measurable cholesterol: *Andrena wilkella*, *Nomada flavopicta*, *Halictus rubicundus*, *Lasioglossum smeathmanellum* and *Coelioxys inermis*. Furthermore, campesterol was not detected in either *Halictus rubicundus* nor *Ceratina cyanea*. The occurrence of campesterol and cholesterol in relatively small quantities almost universally in bees was expected since they are precursors to key hormones, ecdysone and makisterone A [11] thus their absence from two species was surprising.

Only the proportions of four out of nineteen sterols in the bee sterolome showed significant phylogenetic signal (Figure 1): desmosterol (K=0.325, p<0.050, λ=0.781, p<0.005), campesterol (K=0.523, p=0.001, λ=1.012, p=0.001), salisterol (K=0.946, p<0.050, λ=1.005, p=0.001) and ST(28:1)B (K=0.479, p<0.050, λ=0.758, p=0.001) (Figure S1). Two additional sterols showed significant phylogenetic signal (i.e., $p < 0.050$) for one metric (Blomberg's K or Pagel's λ):

ST(28:1)A and 24-methylenecholesterol. We also grouped the sterols by carbon chain and B-ring saturation and found that the 28 carbon chain sterols group also showed significant phylogenetic signals for a single metric (Table S4).

We analysed separately the relationship between sterolome and phylogeny in the genus *Bombus* (Figure 2). None of the sterols, carbon number/B-ring substitution or total sterol (µg/g) were associated with a significant phylogenetic signal in this genus. Across the bumblebee dataset (18 species), whole-body tissues were dominated by 24-methylenecholesterol, isofucosterol and β-sitosterol (Supplementary Table 2), reflecting a sterolome like that of honeybees[9]. Cholesterol was present in very low proportions across all species (< 0.5% median) compared to campesterol (median 6.6%). Ergosterol was detected in all species at a median proportion of 2.1%.

Total sterol quantities in the bees varied more than the sterol profile. For example, the shaggy furrow bee, *Lasioglossum villosulum,* showed the widest range with a maximum of 1143 µg/g and a minimum of 87 µg/g. The species with the highest mean values (*Nomada rufipes* = 551 µg/g and *Lasioglossum villosulum* = 489 µg/g) were both driven by outliers. For *Lasioglossum villosulum,* the two highest total sterol samples also had elevated quantities of the unassigned sterol, ST(28:1)B; these samples were collected from a different site than the other four samples. The red-thighed epeolus, *Epeolus cruciger,* had a less variable but higher total sterol content (293 µg/g) and was also better sampled.

The sterolomes of solitary bees and bumblebees differ.

In the nine most sampled bee species in our dataset, the sterolome differed significantly among species except for one of the kleptoparasites and its host (Figure 3A, PERMANOVA, $F_{8,118}$=25.306, *p*=0.001, NMDS stress value = 0.151, pairwise comparisons, $p < 0.05$). In this case, the ashy mining bee, *Andrena cineraria,* and its kleptoparasite, *Nomada fucata*, had a distinct sterolome from other bee species but the two species were not significantly different from each other ($p>0.100$). It is notable that the sterolome of the heather colletes, *Colletes succinctus,* and its kleptoparasite, *Epeolus cruciger,* shared similar sterolomes, presumably due to their parasite-host relationship but nevertheless still differed significantly.

Species identity explained over 50% of the variation in the data (R^2=0.632). However, species groups also displayed significantly different variances (Fig 3, $F_{8,118}$=6.825, $p<0.001$). All species were associated with a difference in at least one sterol except for *Epeolus cruciger* (Figure 3B), a parasite of *Colletes* and which had a sterolome that was similar to *Colletes hederae* (Figure 3B). The pantaloon bee, *Dasypoda hirtipes,* an Asteraceae specialist, showed the most distinct sterolome which included strong associations with cycloartanol and cholesterol (Figure 3B).

In comparison to the larger dataset, the sterolomes of bumblebees exhibited only small differences in the proportions of sterols (Figure S2, PERMANOVA: $F_{5,145}=2.938$, $p=0.001$, dispersion: $F_{5,145}=2.095$, $p>0.050$, NMDS stress value = 0.128). Species identity explained a low proportion of the variation in the data ($R^2=0.092$). Pairwise comparisons showed the garden/ruderal bumblebee, *Bombus hortorum/ruderatus* and the brown-banded carder bee, *B. humilis*, which are species with characteristically long tongues and thus typically distinctive floral preferences, had the most distinct sterolomes. Both differed significantly from *B. pascuorum* ($p<0.050$) and *B. terrestris/lucorum* ($p<0.050$) as well as each other ($p<0.050$). In addition, *B. humilis* differed significantly from the red-tailed bumblebee, *B. lapidarius* ($p<0.050$).

Comparing all bumblebee species, the red-shanked carder bee, *B. ruderarius,* was more associated with campesterol (IV=0.317, $p<0.050$). It is worth noting the indicator value for this association is very low (<0.4) suggesting there is little difference between species that can be attributed to individual sterols. Furthermore, *B. ruderarius* was represented only by a single sample.

The sterolomes of generalist and specialist bees

While all bumblebees (*Bombus*) are generalist foragers, other genera display a wider variety of foraging types. For instance, the genus *Andrena* includes polylectic, oligolectic and monolectic species. Generalist (polylectic) *Andrena* species showed a significantly different sterol profile from specialist *Andrena* (oligo/monolectic) species (Figure 4, NMDS: stress = 0.077, PERMANOVA: $F_{1,11}=3.417$, $p<0.050$, dispersion: $F_{1,11}=1.105$, $p>0.100$). Generalist bees in the genus *Andrena* were strongly and significantly associated with 24-methylenecholesterol (IV= 0.852, $p=0.010$) and the unassigned sterol ST(28:1)B (IV=0.858, $p<0.050$). In contrast, specialist species were associated with cholesterol (IV=0.924, $p<0.050$) and the unidentified sterol ST(30:1)A (IV=0.764, $p<0.050$). However, foraging strategy only explained a small proportion of the variation in the data ($R^2=0.237$).

In addition to differences between generalist and specialist bees, it should also be expected that specialist bees displayed distinct sterol profiles as a result of their host plant pollen. Two specialist bees in the genus *Colletes* illustrate this relationship well; *C. succinctus* which targets heather (especially *Calluna vulgaris*) pollen, and *C. hederae*, the ivy bee, which is a specialist of ivy pollen (*Hedera helix*) (Figure 5, stress= 0.105). These bees display significantly different sterolomes despite being closely related (Figure 5A, PERMANOVA, $F_{1,31}=37.418$, $p=0.001$, dispersion: $F_{1,31}=27.761$, $p<0.001$), with species identity accounting for over 50% of the variation in the data ($R^2=0.547$).

Colletes hederae, an ivy flower specialist, was most strongly associated with cycloartenol (IV=0.946, $p=0.005$) and avenasterol (IV= 0.856, $p=0.005$) which are both prominent sterols in ivy pollen (Baker et al., 2025). In contrast, the profile of the heather specialist, *Colletes succinctus,* was strongly associated with β-sitosterol (IV= 0.778, p=0.005) and isofucosterol (IV= 0.757, p=0.005) (full list of associations not shown). These associations reflect sterols which are at higher proportions in their respective pollens, indicating that diet strongly influences bee sterolome (Figure 5C).

Bumblebees maintain a consistent sterol profile

Bumblebees are generalist foragers and have been reported collecting pollen from a wide range of plants[21]. We compared the sterolome of bumblebee corbicular pollen to the mean sterolome of eighteen bumblebee species and to the sterolome of 295 different UK pollens[8]. The sterol profile of corbicular pollen was more similar to bumblebees than the hand collected pollens suggesting bumblebees target pollen specifically. Corbicular pollen and bumblebees showed higher 24-methylenecholesterol than hand collected pollen which had higher cycloartenol and which is either absent or a minor sterol in two bumblebees (Figure S3A, NMDS stress value= 0.179, PERMANOVA: $F_{2,350}$=13.030, p=0.001). There was also a significant difference in the variance of the groups ($F_{2,350}$=28.015, p <0.001; Note: as the pollen sample types covered a much wider taxonomic breadth than the bumblebees, this difference in variation was expected). Hand collected pollen was strongly associated with sterols containing a cyclopropane ring, cyclolaudenol and cycloartenol, whereas corbicular pollen was strongly associated with the Δ5 sterols, desmosterol and cholesterol (Figure S3 B).

To understand whether bumblebees maintained the same sterol profile in their tissues under different floral landscapes, a set of widely distributed and common *Bombus* species was sampled from at least three floristically distinct sites, each in a different UK county. Intraspecies variation in sterolomes between sites could indicate that bees are flexible in the sterols they can use. Only the common carder bee, *Bombus pascuorum*, exhibited a significantly different sterol profile at different collection sites (NMDS stress = 0.177, PERMANOVA: $F_{3, 22}$=1.818, p<0.050, dispersion: $F_{3,22}$=0.504, p>0.500). However, this was not highly significant (p=0.046), and the variance explained by collection site was low (R^2=0.199). Both the red-tailed bumblebee, *B. lapidarius,* and the buff-tailed bumblebee, *B. terrestris/lucorum,* showed no significant difference in sterolome between sites (*B. lapidarius*: NMDS stress = 0.123, PERMANOVA: $F_{2,13}$=1.540, p>0.100, dispersion: $F_{2,13}$=1.103, p>0.100. *B. terrestris/lucorum*: NMDS stress = 0.172, PERMANOVA: $F_{3, 35}$=1.162, p>0.100, dispersion: $F_{3,35}$=0.609, p>0.500). All NMDS biplots are shown in Figure S4. This suggests that bumblebee foraging can fulfil sterol needs in landscapes where floral resources differ.

The sterol use by bumblebees was consistent among species and across different habitats. In addition, the sterol requirements of bumblebees appeared also consistent between sexes and body tagmata. Analysis of individual head, thorax and abdomens of bumblebees showed that the sterol profile of the head differed significantly from the abdomen (p<0.050) and thorax (p<0.010). However, the variance explained by both was very low and differences varied by species (Figure S5, NMDS: stress=0.150, PERMANOVA: Tagmata: $F_{2,181}$=4.581, *p*=0.001, Species: $F_{5,181}$=2.382, *p*=0.001, Variance explained: Tagmata: R^2=0.045, Species: R^2=0.059). *Bombus vestalis* had the highest comparable number of male/female specimens and there was no difference in sterolome between these groups (NMDS stress= 0.121, PERMANOVA: $F_{1,17}$= 2.064, *p*=0.100, dispersion: $F_{1,17}$=0.339, *p*>0.500). When all eight species with male and female specimens were compared there was also no difference between sexes. NMDS biplots are shown in Figure S6.

Bee sterolomes diverge from pollen sterol profiles

The sterolomes of specialist bees analysed in this dataset do not directly resemble those in their pollens indicating, as in honeybees[9] (9 Moore et al., 2025), that bee sterolomes do not directly mirror pollen sterol composition.

For example, the sterolomes of the monolectic bees *Melitta dimidiata*, *Macropis europaea*, *Melitta tricincta*, *Andrena florea* and two other species heavily dependent on a single pollen source (*Colletes hederae*, *C. halophilus*) differed significantly from their pollen ($F_{1,56}$=53.733, R^2=0.231, *p*=0.001). There were also significant differences between bee-pollen paired groups ($F_{5,56}$=18.635, R^2=0.401, *p*=0.001). The interaction between these effects was also significant ($F_{5,56}$=5.854, R^2=0.126, *p*=0.001), indicating that the relative difference between bee and its host pollen depended on the pairing of specialist and host (Figure S7 NMDS: stress = 0.162).

Furthermore, some of the specialist bee species contained sterols which were not detected in their pollen. For example, the bryony mining bee, *Andrena florea,* and the sainfoin bee, *Melitta dimidiata,* both contained ergosterol (0.706% and 0.012% of total sterols respectively) while their pollen did not. The yellow-loosestrife bee, *Macropis europaea,* and the ivy bee, *Colletes hederae,* both contained cholesterol, which did not occur in their pollen (0.265% and 0.926% respectively) while desmosterol was absent from ivy (*Hedera helix)* but present in the ivy bee, *Colletes hederae* (1.299%). One interpretation of this is that these bees convert pollen sterols to cholesterol. However, the relative amounts were very low and bees likely obtain these minor sterols elsewhere. *M. europea*, for example, is an oil collecting species so may obtain cholesterol from floral oils or from microbial activity in the larval provisions. Only *Melitta tricincta,* and *Colletes halophilus* contained sterols that were totally represented in their host species' pollen, *Odontites vernus* and *Tripolium pannonicum* respectively.

Bees can adapt to the pollen sterols of different plants

The adaptation to using phytosterols in pollen may have benefits to bees from reducing the metabolic cost of sterol production. However, since honeybees are limited in the phytosterols they can use and restricted to acquiring these from pollen [9,13], this may also be the case for other species of bee. Plants could produce pollens that are 'undesirable' so to deter palynivory by producing higher quantities of unusable sterols. It would, therefore, be beneficial to bees if they could tolerate some of these 'undesirable' phytosterols in their tissues. Asteraceae was highlighted in Baker et al. [8] as producing lower proportions of Δ5 sterols and higher proportions of sterols with a B-ring cyclopropane substitution but still has specialist bee species associated with its pollen. To determine whether these bees have adapted to these sterols, we compared the sterolomes of generalist bee species, Asteraceae specialist bee species, and non-Asteraceae specialists to the pollen sterols of all three Asteraceae tribes which differ greatly in their pollen sterolomes (Cichorioideae, Asteroideae, and Carduoideae) (Figure 6A). While bee and pollen sterolomes were distinct, those of the Asteraceae specialist bees were most similar to Asteraceae pollen, specifically Cichorioideae (PERMANOVA, stress=0.151; $F_{5,96}=16.041$, $p=0.001$). Generalist bee species were significantly associated with 24-methylenecholesterol (ISA, Figure 6C). Non-Asteraceae specialist bees were significantly associated with β-sitosterol and Asteraceae specialists with cholesterol. Different Asteraceae tribe pollens were associated with at least one sterol with a B-ring cyclopropyl substitution (CPR) each: Asteroideae with cycloartenol (IV=0.564, $p=0.005$), Carduoideae with cyclolaudenol (IV=0.568, $p=0.005$) and Cichorioideae with cycloartanol (IV=0.576, $p=0.010$). Carduoideae was associated with the most individual sterols (Figure 6C).

When comparing all 56 bee species, Asteraceae specialists showed significantly lower proportions of Δ5 sterols and higher proportions of sterols with a B-ring cyclopropane substitution (CPR) than non-Asteraceae specialist, generalist and parasitic bee species (CPR: $H(3)=19.077$, $p<0.005$, Δ5: $H(3)=12.743$, $p<0.050$, *post-hoc* Dunn tests: CPR ($p<0.005$), Δ5 ($p<0.050$)) (Figure 6B).

DISCUSSION

This study represents the largest survey of wild bee sterolomes to date. The whole-body tissues of all 56 bee species analysed were dominated by phytosterols rather than cholesterol, indicating that in general, bees do not convert dietary sterols into cholesterol. Instead, they use a range of phytosterols that they acquire from pollen. Overall, bee sterolomes were characterized by the presence of 24-methylenecholesterol, β-sitosterol and isofucosterol, which is consistent with previous work [16,17]. These three sterols were the major sterolome components in *Bombus* spp. which was similar to that in honeybees [9,27]. These sterols occur in high proportions across a wide taxonomic range of floral pollen [5,8] indicating that many flowering species may provide suitable sterols for many bees. Our data implies that these three sterols are therefore best suited to bee physiological requirements, perhaps in part due to their Δ5 B-ring substitution. However, some sterols including cycloeucalenol and obtusifoliol that occur in the pollen of plants commonly visited by bees were not recorded as being used by bees, suggesting that sterol incorporation is regulated rather than passive. The lack of phylogenetic signal in most sterols across the dataset indicates the bee sterolome is driven by other forces such as foraging choice and life history, creating significant differences between even closely related bees.

Sterols are a stabilising compound in cell membranes which impact membrane permeability and fluidity (Reviewed in Dufourc, 2008 [28]). The phytosterols stigmasterol and β-sitosterol can form rafts and maintain a stable membrane fluidity across a wider temperature range than cholesterol [29]. Abundance of these specific sterols in bees could therefore be an adaptation to colder temperature conditions, as observed in *Drosophila*. Flies are flexible in their uptake of specific sterols; when they are reared in cold conditions (7.1°C), they took up proportionally more β-sitosterol and stigmasterol than cholesterol from food than flies reared at 28.6°C [30]. Although not as widely abundant as more common sterols, campesterol, cycloartanol and avenasterol were present in proportions (>30%) in some bee species suggesting their incorporation into membranes. Cycloartenol for instance has been shown to substitute for cholesterol when maintaining membrane dynamics in model tissues[28].

Insects also require sterols for the synthesis of steroid hormones: in most species, cholesterol is converted into moulting hormones[14]. Honeybees need cholesterol and campesterol, respectively, to synthesize the hormones 20-hydroxyecdysone and makisterone A[15]. Other dietary sterols can also be essential to successful development in bees, as seen with the reliance of a stingless bee on ergosterol to pupate[31]. The few bee species that have been studied for their steroid hormone precursors show that bees may share the trait of using campesterol as the basis for production of the alternative moulting hormone, makisterone A [15,31]. In our data, the phylogenetic trend seen in campesterol, where closely related species showed similar proportions, may therefore be due to its importance in hormone production. Further, the absence of cholesterol or campesterol from some species (*Andrena wilkella*, *Nomada flavopicta*, *Halictus rubicundus*, *Lasioglossum smeathmanellum*, *Coelioxys inermis* and *Ceratina cyanea*) could indicate that these species use other moulting hormone synthesis pathways [11,15]. It is however important to note that these species were all represented by a single sample. Therefore, it is possible cholesterol and campesterol were present in concentrations too low to be detectable in these samples.

It was shown from the analysis of bees at different collection sites that locations with different plant communities did not affect sterolomes in two of three bumblebee species analysed. This suggests that bumblebees maintain a relatively consistent sterolome even when foraging on different pollen sources, implying regulated use of dietary sterols even in generalist species. The seemingly stable ratio between sites may therefore represent a compromise between readily available dietary sterols and a physiological optimum [30]. This strategy may contribute to the ecological success of bumblebees, allowing them to forage in an extremely wide range of habitats, using pollen from many different plant species. Within the bumblebee dataset, the interspecies differences and species-sterol associations were the result of small variations in proportions of different sterols, likely a result of their similar foraging and life history strategies. As such, the ecological relevance of these differences may be limited.

Corbicular pollen collected from bumblebees showed a profile distinct from hand-collected pollens, correlating with higher 24-methylenecholesterol. It is therefore possible that bees are targeting certain sterols in pollen, though there is currently no evidence to suggest they can detect sterols directly. There are also other potential causes of variation between hand and bee collected pollens; bumblebees can change their pollen foraging target during a single trip [32] and they add saliva to collected pollen to aid compaction. Further, the bees analysed in this study will likely have collected different pollen to those they were raised on.

Despite some similarities, the sterolomes of congeneric generalist and specialist bees were distinct. For instance, polylectic *Andrena* species had higher proportions of 24-methylenecholesterol than oligo/monolectic species in this genus. The sterolome of honeybees is comprised of the sterols they require including 24-methylenecholesterol along with isofucosterol and β-sitosterol [9,13] and therefore the sterols we find in other bee species may also be the sterols they need. Thus, where generalists forage across a range of flowers to ensure access to desirable sterols such as 24-methylenecholesterol and isofucosterol, they may do so at a cost of competition for resources. Specialist foraging strategies may be driven by several factors and specialist bees may benefit from adapting their sterolome to make best use of their pollen host, as seen in *Colletes.* That said, there is no evidence that specialists experience less competition than generalists[33] although specialisation could have benefits that outweigh its cost (e.g., greater foraging efficiency). Our data, however, do suggest there is flexibility in sterol use in some specialist bees that have developed specific sterol needs and concurs with the concept of sterol nutritional niches [34]. This is illustrated in highly specialised bees, which exhibit sterolomes that are distinct from those of their host pollen, consistent with regulated incorporation of dietary sterols, as recently shown for honeybees [9]. Some bee species contained sterols that were absent from their pollen, indicating that the specialist plant-bee relationship is flexible and bees are using pollen from additional plant species or other sources such as plant oils and fungal contaminants. It could alternatively be explained by the fact that these sterols were not detected in our samples. In addition, the sterol requirements of specialist bees could be fulfilled by plants other than their preferred species; for this reason, we expect that the sterols are not what are driving specialization in host-pollinator relationships. Given that relying on a single pollen host may mean a bee is more vulnerable to changes in flowering time and distribution, other nutritional or ecological factors must be driving this relationship.

We were able to identify potential specialist sterol adaptation most distinctly in Asteraceae specialist bees due to their frequency in the dataset. The pollen sterolomes of Asteraceae flowers display lower levels of Δ5 sterols such as 24-methylenecholesterol, β-sitosterol and isofucosterol which are found commonly in generalist bees and higher levels of sterols with Δ8 B-ring bonds and cyclopropane rings[8]. This may be a potential reason for the infrequent use of Asteraceae pollen by generalist bee species of sterols [22,24] as these sterols do not appear suitable for the majority of bee species and may prevent successful growth and development [35,36]. Our results showed that this trend was reflected in the sterolomes of Asteraceae specialist bees which often had higher proportions of cycloartanol and cholesterol and lower proportions of Δ5 sterols. The ability to incorporate much higher levels of cycloartanol in their tissues than most other bee species suggests that these species have adapted to Asteraceae pollen. However, these bees still maintained a dominance of Δ5 sterol, indicating there are limits to sterolome adaptation in bees. The ability to overcome the nutritional deficiencies of Asteraceae pollen may have allowed these bees to benefit from the widespread and abundant availability of these flowers.

Cichorioideae flowers produced some of the highest cholesterol proportions reported in Baker et al.[8] (*Helminthotheca echioides*: 60.05%, *Crepis capillaris*: 44.21%, *Leontodon saxatilis*: 31.33%, *Crepis vesicaria*: 27.77%). The use of cholesterol as a dietary sterol has been proposed as facilitating the transition from carnivory to palynivory in Apoidea evolution [37]. As bees evolved from carnivorous ancestors to pollinivorous diets, cholesterol rich pollens may have provided a suitable source of useable sterols before bees adapted to use a wider range of phytosterols, as suggested by Dötterl and Vereecken (2010)[38]. *Dasypoda* species, which occupy a basal position in bee evolution, are also ancestrally oligolectic on Asteraceae flowers [39] and their sterolome had strong associations with cholesterol.

Our data does not reveal a singular clear trend in sterol profiles of wild bees. There is no evidence for a strong phylogenetic signal in most sterols, therefore, the drivers of sterol profile in bees are likely to be ecologically, rather than phylogenetically, constrained but with some plasticity. However, there are multiple instances of sterol profiles differing from those expected by ecological drivers, for instance where monolectic bee species sterol profiles do not match the sterol proportions of their pollen host. Proportional differences between pollen and bee sterol profiles could arise from several non-mutually exclusive processes, including regulated incorporation or retention of sterols, as well as other nutritional or ecological factors that influence pollen foraging choices. Future work can build on these data by experimentally manipulating the diets of bees to determine if they are fixed to a profile of specific sterols or if they demonstrate greater flexibility. The ecological and economic importance of wild bees makes a comprehensive knowledge of their nutritional requirements an important step in understanding the impact of floral community change on bee populations.

LIMITATIONS OF THE STUDY

We present chemical analysis of bees to describe their sterol profile. As it stands the data while extremely valuable in their own right—are not sufficient to draw conclusions about how bees forage to optimize their sterol profiles. Bees may have specific host-plant preferences that lead to specific sterol profiles, but whether or not the sterols are the reasons underlying their host-plant preferences is still unclear. Furthermore, there is no direct evidence that the observed sterol profiles are optimal, and further experimental work is needed for this.

RESOURCE AVAILABILITY

Lead contact.

- Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Philip C Stevenson p.stevenson@kew.org)

Materials availability

- This study did not generate new unique reagents.

Data and code availability

- All data generated or analysed during this study are included in this published article and supplemental information
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.
- This paper does not report original code.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTION

ECB, GAW, PCS and WEK designed the study, performed the analyses, and drafted the first version of the manuscript. ECB, EL and KB undertook sampling and prepared samples for analysis. All authors contributed to analyses, interpretation of the results, and the final form of the manuscript.

FIFURE LEGENDS

Figure 1. Phylogeny of 56 bee species subset from the supermatrix phylogeny after Henríquez-Piskulich et al. [40] showing family clades and foraging status. Species mean sterol percentages for seven key sterols are plotted along with total sterols (µg/g) for all samples (species means are shown by grey bars, error bars show ± standard error). 24-methylenecholesterol, isofucosterol and β-sitosterol account for the majority of sterols in most bee species. However, their proportions are highly variable between species. Bee families do not appear to show conserved sterolomes and only 3/19 sterols tested showed significant phylogenetic signal across the tree. Higher levels of cycloartanol appear across the tree and are often associated with species which are specialist (oligolectic) on Asteraceae flowers.

Figure 2. Bombus species phylogeny subset (from Hines[41]) with subgenera highlighted. Heatmap shows mean species proportion for the most commonly dominant five sterols plus cholesterol. Bar graph shows mean species total sterol production ±SEM. Points show individual samples of each species with different colours for each county the species was sampled from. The kleptoparasitic *Bombus* species do not show a sterolome distinct from other *Bombus* species. All bumblebee species analysed were dominated by 24-methylenecholesterol, isofucosterol and β-sitosterol, with no phylogenetic signal in any of the nine sterols analysed. *Bombus sylvestris* was omitted from this tree and so not included in phylogenetic analyses. Ruder = *ruderalis* & Hort = *hortorum*.

Figure 3. Interspecies comparison of sterols among bee species

A) Interspecies comparison of sterol profile among species with ≥ 10 samples demonstrating significant interspecies differences in sterolome (PERMANOVA, $F_{8,118}=25.306$, $p=0.001$). Grey lines indicate significant ($p<0.010$) and strong (absolute value >0.7) correlations. Outlined points represent means for each group and ellipses show 95% confidence interval. There are clear similarities in the sterol profile of ecologically linked species such as *Epeolus cruciger* with *Colletes succinctus* and *Nomada fucata* with *Andrena cineraria*, both of which are kleptoparasite-host pairs. All pairwise comparisons were significantly different ($p < 0.05$) except *Nomada fucata* and *Andrena cineraria*. There were significant differences in variance between species with *Colletes succinctus* displaying the most conserved sterolome and *Nomada fucata* the most variable. NMDS stress value = 0.151.

B) Indicator Species Analysis of species with ≥ 10 samples as shown in Figure 3A. Of the 19 sterols tested, 15 were indicators of individual species groups. Indicator values (IV) and p-values are shown for significant sterols. *Dasypoda hirtipes* was associated with the most sterols and shows a distinct grouping in Figure 3A. *Epeolus cruciger* was not associated with any individual sterols, potentially as a result of it's high similarity in sterolome with *Colletes succinctus* as well as other bee species.

Figure 4. Distribution of phytosterols among *Andrena* species.

A) NMDS of generalist (polylectic) and specialist (oligolectic/monolectic) *Andrena* species (stress value =0.077) which showed a significant difference between groups (PERMANOVA: $F_{1,11}=3.417$, $p<0.050$). Grey lines indicate significant ($p<0.010$) and strong correlations (absolute value >0.7). Outlined points represent means for each group and ellipses show 95% confidence interval. Generalist species were correlated with higher 24-methylenecholesterol and specialist species are associated with higher cholesterol.

B) Proportions of dominant sterols across all *Andrena* species included in Figure 4A. All remaining sterols are collapsed into a single group. Generalist species show higher proportions of 24-methylenecholesterol and specialist species that target Asteraceae species (*A. denticulata*, *A. fulvago* and *A. humilis*) show higher proportions of cycloartenol. *Andrena florea*, which specialises on *Bryonia cretica*, contains very high proportions of un-named sterols suggesting this bee’s sterolome requires further characterisation. *Andrena fuscipes* and *A. hattorfiana* specialise on Ericaceae and scabious flowers (*Knautia* and *Scabiosa* spp.) respectively.

Figure 5. Sterol composition of *Colletes* species and their main pollen food plants.

A) Sterol composition of all replicates of *Colletes hederae* and *C. succinctus* (*Hedera helix* and heather species respectively). Only major sterols are named with the remainder collapsed into single group. *Colletes succinctus* demonstrated much higher proportions of β-sitosterol whereas *C. hederae* contains higher proportions of avenasterol. Both species show a sterolome that is largely consistent across samples.

B) NMDS of *Colletes hederae* and *C. succinctus* (stress= 0.105). Species show distinct sterolomes (PERMANOVA, $F_{1,31}$=37.418, p=0.001) despite their relatedness. *C. hederae* is associated with higher avenasterol and cycloartenol whereas *C. succinctus* is associated with higher β-sitosterol. *Colletes hederae* shows a larger variation in sterolome, potentially due to its broader foraging habit. Grey lines indicate significant (p<0.010) and strong correlations (absolute value >0.7). Outlined points represent means for each group and ellipses show 95% confidence interval.

C) Sterol composition of the main pollen hosts of *Colletes hederae* and *C. succinctus* (*Hedera helix* and heather species). Only major sterols are named with the remainder collapsed into single group. The three Ericaceous heather species show a similar sterol profiles, dominated by isofucosterol and β-sitosterol. *Hedera helix* sterol profile differs more from that of *C. hederae* than pollen heather sterols do from C. *succinctus.*

Figure 6. Comparison of sterols among Asteraceae specialists and congeneric generalist bee species.

A) NMDS comparing the sterol profile of bees which specialise on Asteraceae pollen to those which do not (stress = 0.151). Bees and pollen species appear distinct in NMDS space. Most Asteraceae specialists target Cichorioideae and can be seen to cluster closer to this pollen than other Asteraceae. Groups were significantly different (PERMANOVA, stress=0.151; $F_{5,96}$=16.041, p=0.001) and group identity explained nearly 50% of the variation in the dataset (R^2=0.455). All pairwise comparisons between groups were significant (p<0.050) except Asteroideae versus Carduoideae pollen (p>0.100). Grey lines indicate significant (p<0.010) and strong correlations (absolute value >0.7). Outlined points represent means for each group and ellipses show 95% confidence interval.

B) Proportion of sterols belonging to different double bond position groups across solitary bee species. Asteraceae specialists show a lower proportion of Δ5 sterols and higher proportion of sterols with a B-ring cyclopropane substitution (CPR) than the rest of the dataset. (CPR: H(3)=19.077, p<0.005, Δ5: H(3)=12.743, p<0.050, post-hoc Dunn tests: CPR (p<0.005), Δ5 (p<0.050)). No other comparisons were significantly different.

C) Summary of Indicator Species Analysis results showing associations between groups in Figure 6A and individual sterols. All bee groups were associated with Δ5 sterol whereas each pollen group was associated with a sterol containing a cyclopropane ring.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

KEY RESOURCES TABLE

EXPERIMENTAL MODEL AND STUDY PARTICIPANT

Two datasets were collected and analysed for this paper. The first was a set of bumblebee specimens, covering 18 species, comprising whole bodies, tagmata and corbicular pollen loads. The second was a set of 56 wild bee species, comprising only whole-body samples. Processing, sample analysis and data analysis for both sets of samples is detailed below.

Sample collection for bumblebees

Female workers of all UK bumblebee species were targeted for collection. A minimum collection target of 5 individuals per species was set and specimens collected with landowner permission. Rare species (*Bombus distinguendus* and *B. sylvarum*) were collected only with prior arrangement with the Bumblebee Conservation Trust. Queens from abundant and common species (*Bombus hortorum*, *B. hypnorum*, *B. lapidarius*, *B. lucorum*, *B. pascuorum*, *B. pratorum* and *B. terrestris*) were collected to assess tagma composition. Female worker *Bombus lapidarius*, *B. terrestris/lucorum* and *B. pascuorum* were collected from at least three different sites to facilitate intra-species analysis. For kleptoparasitic bumblebee species, which do not have workers, queens were collected, with males collected when this was not possible. For *Bombus ruderarius* and *B. campestris*, no female workers could be collected and so males were collected instead. To ensure there was not a difference between the sterols of male and females of the same species, males were opportunistically collected from well sampled common species (*Bombus hortorum*, *B. lapidarius*, *B. lucorum*, *B. ruderatus*, *B. rupestris*, *B. vestalis*) to enable statistical comparison.

Bees were captured using a net and euthanized in a -20°C freezer. They were then stored at -20°C and moved to -80°C storage in batches. After sample collection, bees were defrosted for dissection and the full gut, crop, sting and venom sacs removed from the abdominal cavity. This was to prevent misinterpretation of sterols detected from gut contents with those incorporated into body tissues. Any visible parasites were also removed. Ovaries, where present, were retained. A hind leg was also removed from all samples for future barcoding of specimens with indeterminate characters. For any male specimens, genitalia were retained, and guts removed. A set of queen bumblebees were also separated into body tagmata (head, thorax, abdomen). Any loose pollen in corbiculae or on the body was removed by brushing and full corbicular pollen loads collected into 1.5ml glass vials. Dissected specimens were then refrozen at -80ºC.

Species with indeterminate characters were barcoded to confirm identification as follows. Hind legs were stored at -80°C in a 1.5ml Eppendorf. DNA was extracted from legs using a Qiagen DNeasy Blood & Tissue Kit following the supplementary protocol 'Purification of total DNA from insects using the DNeasy® Blood & Tissue Kit'. Legs were homogenised using four 2.3mm ceramic beads in an MP FastPrep-24™ 5G bead beating grinder and lysis system in five cycles of 35s at 6 m/second followed by 180s rest. The genomic DNA was then sent to SureScreen Scientifics for PCR, barcoding and species identification.

Sample collection for wild solitary bees

Species were chosen to cover a wide phylogenetic range and included all foraging strategies. These included monolecty a foraging strategy whereby bees feed from a single species [20], oligolecty, polylecty and kleptoparasitism. Close relatives that had different foraging strategies were prioritised to understand the effects of phylogeny and ecology. A sample size of three individuals for each species was targeted initially but this was not possible for some species. Very common bee species were collected at least three times, and from different locations to best capture intra-species variation. In total, 56 bee species were collected, with ten of these represented by a single sample. All samples were female except for *Melecta albifrons* which was represented by two male specimens. Collection was primarily focused in the South of England, from Royal Botanic Gardens (RBG), Kew and Wakehurst. Other species procured from specific sites were done so with the permission of landowners. Bees were collected with a net then euthanised and stored at -20°C. Pollen was brushed from the outside of the body and removed from any pollen-collecting structures such as corbiculae or scopae. Guts were retained within specimens as removal would have likely damaged other internal tissues for the smallest specimens and so processing was kept consistent.

METHOD DETAILS

Sample preparation for bumblebees and wild solitary bees

All whole-body and tagma samples were covered in 5ml or 3ml of deionised water respectively and re-frozen at -80°C before being freeze dried. Samples were then transferred to plastic bead-beating vials containing four 2.3mm steel beads and homogenised at 30rpm in a Qiagen tissue ruptor for 3 minutes in two 1.5 minute intervals. Homogenised tissue was then transferred back to their glass vial in a 1:50 (mg:µl) dilution of GCTU (Guanidine (6 M guanidinium chloride) and thiourea (1·5 M) dissolved in deionised H_2O together and stored at room temperature out of direct sunlight) and re-frozen at -80°C. For each sample, 60µl of homogenate was transferred to a 96-well plate for sterol extraction. GCTU was used as a buffer as previously reported [42] because it suppresses lipase activity, is antimicrobial, aids in the break-up of cellular structure without damaging target lipid metabolites and provides a pipettable solution.

Paired corbicular pollen loads weighing >20mg were separated and those weighing 10-20mg were combined for sterol analysis. As previously described[7], pollens were suspended in 200µl KOH and vortexed to disperse. They were then boiled at 75°C for two hours. Twelve which had dried out due to methanol evaporation during that time were re-suspended in 150 µl methanol. All saponified material was then transferred to a 96-well plate for sterol extraction.

For wild solitary bees specimens were covered in deionised water, frozen at -80°C and freeze dried until all water had been removed. Samples were then stored at -80°C before being homogenised. Specimens were homogenised in GCTU using a BioSpec Tissue Tearor with a 14mm head attachment.

Sample data collection

All plates included controls to monitor instrument functioning: Blanks (60µl GCTU) were used to detect and remove background noise and at the start of each plate. A stock solution of reference materials verified by multi-nuclear NMR (QA) was used to calculate coefficient of variance. Reference materials were available for: 24-methylenecholesterol, 24-methylenecycloartenol, anthelsterol (a sterol found in *Baccharoides anthelmintica*, ST(28:3), that is active under 330 nm UV but whose structure has not been determined formally using NMR)[7], avenasterol, β-sitosterol, brassicasterol, campesterol, cholesterol, cycloartenol, cycloeucalenol, cyclolaudenol, desmosterol, episterol, ergosterol, isofucosterol, schottenol, sitostanol, spinasterol and stigmasterol. The *m/z* and retention time for each sterol is shown in Table S5. Aliquots of 40 µl were used in the run. A QC solution of homogenised commercially available honeybee collected pollens (*Helianthus annus*, *Nymphaea* sp., *Fagopyrum esculentum*) and bumblebee adults, larvae and pupae in GCTU (6M guanidine + 1.5M thiourea to make 1 L dissolved in deionised water) was run at a series of concentrations to test the range of linearity of the instrument. Three concentrations of QC were used: 100%, 50%, 25%, corresponding to 40 µl, 20 µl and 10 µl aliquots of the QC stock solution. These were analysed in the same way as the samples to calculate the correlation between analyte concentration and signal size. Variables whose ratio was <0.75 were discarded.

All samples were then extracted as 96-well plates by SF (96-well plate, Esslab Plate+™, 2.4 ml/well, glass-coated) using a 96-channel pipette (liquid-handling robot, Integra Viaflow 96/384 channel pipette) as follows. First 150µl of internal standard (d_7-cholesterol) was added to all samples. Concentration differed between plates as detailed below.

Then 500µl DMT (250µl ×2) was added to all samples. DMT comprised dichloromethane (DCM) (3 parts), methanol (1 part) and triethylammonium chloride (0.0005 parts, i.e. 500 mg/l), mixed and stored at room temperature out of direct sunlight. Then 500 µl of deionised water was added before agitating using the multi-channel pipette. Layers were separated by spinning for 2 min (methanol + water, Solid, DMT + sterol) and 50 µl of extract (DMT & sterol) transferred to a 384-well plate and left for 30 min for the DMT to evaporate. Once the 384-well plate was full, 150 µl of LCMS quality methanol was added and the plate sealed with foil.

For bumble bees, samples were run across two 384-well plates comprising eight individual 96-well plates. Sample order was randomised within each 96-well plate. For plate A, whole-body bumblebee samples, the concentration of internal standard (d_7-cholesterol) was 5 mg/l (1.99098e^{-9} Moles/sample). For plate C, body tagmata and pollen samples, it was 1 mg/l (3.98196e^{-10} Moles/sample).

For wild solitary bees each sample, 60µl of homogenate was transferred to a 96-well plate for sterol extraction. Samples were run across two 384-well plates comprising eight individual 96-well plates. Sample order was randomised within each 96-well plate. The concentration of d_7-cholesterol for all plates in this run was 5 mg/l (1.99098e^{-9} Moles/sample).

QUANTIFICATION AND STATISTICAL ANALYSIS

Sample analysis and data processing

The liquid chromatography- mass spectrometry analysis of samples was completed following Furse et al.[7] and Baker et al.[8]. Instrument output was also processed as established in Furse et al. [7]. Relative abundance was calculated by dividing the signal area for each metabolite by the signal of internal standard (d_7-cholesterol).

These values were used for the majority of data analysis. For total sterol analysis, instrument output (*m/z*) was converted to µg/g (dry weight of the sample) first by dividing the signal area for each metabolite by the signal of internal standard (d_7-cholesterol) then using the following formula:

$$\frac{Sterol\ (\mu g)}{Tissue\ (g)} = \frac{sterol\ molecular\ mass\ \left(\frac{g}{mol}\right) \times\ (signal\ \times\ mol\ of\ d7chol.)\ \times\ 1{,}000{,}000}{Starting\ weight\ (mg)/1{,}000}$$

The amount of mol of d_7-cholesterol was:

- Bumblebee plate (whole bodies): 1.99098e^{-9} Moles/sample

- Bumblebee plate (tagmata and pollen): 3.98196e^{-10} Moles/sample
- Wild bee plates (whole bodies): 1.99098e^{-9} Moles/sample

Due to the impact of the COVID-19 pandemic a second analysis run was needed to analyse samples of *Bombus distinguendus* and *B. jonellus* which were collected a year later than intended. Ten samples which had also shown much higher proportions of un-named sterols than the rest of the samples in the first run were also re-analysed as part of this run. The second analysis detected a different selection of low-abundance sterols compared to the previous run. As the primary aim of this work was to enable comparison between species and sites, any sterols which were not detected in both runs were therefore removed from data analysis to enable the most accurate comparisons. This led to the removal of two sterols from the second run (stigmasterol, ST(28:2)C, ST(31:2)A, ST(30:3)) and ten from the first (24-MCA, sitostanol, ST(27:2)C, ST(28:2)C, ST(29:0)A, ST(29:0)B, ST(29:2)A, ST(29:2)B, ST(29:2)C, ST(30:2)A, ST(30:2)C and ST(31:2)A). Proportions were re-calculated using only sterols detected in both runs (24-methylenecholesterol, β-sitosterol, campesterol, cholesterol, cycloartenol, cyclolaudenol, desmosterol, ergosterol and isofucosterol). Total sterol values for this dataset are therefore lower than if all detected sterols were included. Inclusion of this second data set did not alter the significance of any findings regarding phylogenetic and interspecies analysis.

Phylogenetic analysis of bumblebees and wild solitary bees

Phylogenetic signal was calculated separately for each dataset following Zu et al.[5]. Phylo4d objects were created using phylo4d() from PHYLOBASE [43]. Phylosignal(reps=999) from PHYLOSIGNAL[44] was used to calculate Pagel's λ and Blomberg's K. These measures of phylogenetic signal were calculated for the individual proportions of all 19 sterols, total sterol (μg/g) and proportion of sterols belonging to different carbon chain lengths (27, 28, 29, 30, 31) and double bond saturation categories (cyclopropane ring(CPR), 0, 5, 7, NA). Ergosterol was classed as NA for double bond position as it has double bonds at both 5 and 7. All unidentified sterols were also classed as NA.

The phylogenetic trees were plotted by creating a phy-data object using treedata() from GEIGER v.2.0.11. [45]. Family and sub-genus clades were identified using getMRCA() from APE v.5.8. [46]. Phylogeny was plotted with ggtree() from GGTREE v.3.12.0. [47,48,49]. Heatmap and bar graph associated with phylogeny were plotted using ggplot() from TIDYVERSE v.2.0.0. [50]. The composite graph was then plotted using APLOT v.0.2.2. [51].

The most comprehensive phylogeny of the *Bombus* genus to date was used with permission from Hines (2008) [41]. Phylogeny was trimmed using keep.tip() from APE v.5.8.[46] to only include sampled species. *Bombus sylvestris* was not available in the phylogeny and so not included in these analyses. Subgenera were defined following Williams et al. (2008) [52]. Individuals of *Bombus terrestris* and the

B. lucorum complex were combined into *Bombus terrestris*/*lucorum* for analysis as many samples had indeterminate characters. Similarly, *Bombus hortorum* and *B. ruderatus* were combined into *B. hortorum*/*ruderatus*. To plot these species groups on the phylogeny branch, tips for *Bombus terrestris* and *B. hortorum* were used.

The supermatrix phylogeny produced by Henríquez-Piskulich, Hugall and Stuart-Fox (2024)[40] was used to assess phylogenetic signal in the sterol dataset for the wild bee dataset. The 4,586 bee species dated chronogram with outgroups removed was selected for analysis. The tree was downloaded from Dryad, imported into R and pruned to 56 species using APE v.5.8. [46]. Our dataset contained six out of the seven currently recognised families within the clade of Anthophila (bees): Andrenidae, Apidae, Colletidae, Halictidae, Megachilidae and Melittidae. Supplementary taxonomic data from Henríquez-Piskulich, Hugall and Stuart-Fox (2024) [40] for all 56 species is available in the supplementary resources (Table S6).

All data analysis was done in R version 4.4.0 (2024-04-24 ucrt) [53] and R studio (2024.9.1.394) [54]. For each data type (whole-body, individual tagma, corbicular pollen), a Bray-Curtis dissimilarity matrix was used to identify outliers using disana() from the LABDSV package v.2.1-0 [55]. Any data point with a minimum dissimilarity over 0.5 compared to all other points for that group was identified as an outlier, as per author guidance, and so was removed. No samples exceeded the outlier threshold in the bumblebee data, so all data was included in the analysis. For the wild bee dataset, each species with at least three replicates was tested for outliers. This led to the removal of five samples in total: One *Andrena flavipes*, two *Andrena florea*, one *Anthophora plumipes* and one *Colletes hederae*. Six specimens were removed from total sterol analysis as their post-freeze drying weight suggested an error, meaning their signals data could not be converted into μg/g units. This was likely due to incorrectly calibrated weighing equipment.

Summary statistics were calculated from species means using the STATS package from R [53]. To carry out statistical comparisons of sterol profile between groups, proportion data (0-1) was used, where zero denotes a sterol is absent from a sample, and one denotes it is completely dominant. Groupings used in individual analyses are specified below. Data were arcsine square root transformed and used to calculate a Bray-Curtis dissimilarity matrix using vegdist(method=”bray”). The matrix was used to plot an NMDS using metaMDS(autotransform=FALSE). The lowest number of axes was selected where stress was ≤0.2 (established cut off, [56]. The stress value reflects how well the ordination represents the original data. Adonis2() was used to carry out a PERMANOVA testing for significant differences between groups. Differences in variance between groups which could invalidate comparisons were tested using anova() and betadisper(). Pairwise comparisons between groups were carried out following Bakker (2024) [56], using adonis2() with a Benjamini-Hochberg correction. All functions were from VEGAN [57] except anova() from the STATS package [58]. A 2D biplot was created from the NMDS with a stress value of <0.2. Strong (>0.7 absolute value) and significant (p<0.010) sterol associations were plotted over the datapoints using ggplot() and ggrepel() from GGPLOT2 [49] and GGREPEL [59].

The INDICSPECIES package [60] was used to carry out Indicator Species Analysis (ISA) using multipatt() to determine significant associations between and individual sterols. ISA was done at the level of individual groups only and not for combinations of groups. Hereafter IV refers to indicator value in the results section.

Differences in sterol profile among UK bumblebee species

The six species with the highest number of samples, including male and female specimens, were used in an NMDS (trymax=200, k=3) to compare sterol profile between species. The first two dimensions of the NMDS are plotted in Figure S2. All available whole-body samples were used in an ISA to test for associations between individual sterols and species.

A total of 40 large pollen baskets were collected from females to analyse for their individual sterol content. An NMDS was calculated using this data (n=40), means of all bumblebee species (n=18) and all 295 pollen taxa detailed in 8Baker et al., (2025)[8] (n=295) (trymax = 200, k=2). ISA was then carried out to test for associations between sterols and each of these groups. As the pollen analysis had produced a wider range of sterols, only those present in both analyses were used to calculate total sterols and then proportions for the pollen data. These sterols were 24MC, β-Sitosterol, campesterol, cholesterol, cycloartenol, cyclolaudenol, desmosterol, ergosterol and isofucosterol.

To determine whether there were significant intra-species differences driven by habitat, female workers of a set of species that are common in a range of habitats were collected from an urban garden environment (Royal Botanic Gardens, Kew), a semi-natural, dry, calcareous grassland site (sites near Rollestone Camp and Bulford Camp, Salisbury Plain Training Area, Wiltshire) [61], and a semi-natural grass moor and heather moorland site (Harthope Valley, Cheviot hills, Northumberland National Park, Northumberland) [62]. These species were *Bombus terrestris/lucorum*, *B. pascuorum*, and *B. lapidarius*. Between site comparisons were plotted for *Bombus pascuorum*, *B. lapidarius* and *B. terrestris/lucorum* individually using NMDS (trymax = 200, k = 2) and sites were compared statistically using PERMANOVA.

Tagmata were analysed by NMDS (trymax = 200, k=2) and PERMANOVA to test for significant differences in sterol profile between head, thorax and abdomen. The strata argument was used to control for the effect of tagma being from the same individual. Species identity was also included as a fixed effect in the model. Additionally, ISA was carried out on the data to test for associations between tagmata and individual sterols.

Sex differences in sterol profiles in bumblebees

Where collection of females was not possible, male bumblebees were collected instead. For each species which had at least one male and female specimen (*B. barbutellus*, *B. hortorum/ruderatus*, *B. lapidarius*, *B. monticola*, *B. terrestris/lucorum*, *B. rupestris*, *B. sylvestris*, *B. vestalis*) an NMDS and PERMANOVA was calculated using means for each species-sex combination to prevent the often-higher female sample sizes affecting the results (trymax=200, k=2). Sex and species identity were included as fixed effects. *Bombus vestalis* had highest number of male specimens (11) and a comparable number of female specimens (8) and was therefore used for a separate NMDS and PERMANOVA (trymax=200, k=2).

Differences in sterol profile across wild solitary bee species

Species which were represented by at least 10 samples in the dataset were used for an NMDS comparison of inter-species differences. This included nine species from seven genera. An NMDS was calculated from proportion data using all available samples for these species (trymax=200, k=2).

To compare sterol of generalist and specialist wild solitary bees we looked at *Andrena* because this was the most speciose genus in the dataset and contained a similar number of oligolectic/monolectic (6) and polylectic (7) species which were not in monophyletic groups. *Andrena* species were therefore used in a NMDS (trymax=200, k=2) to calculate if the two lecty groups had significantly different sterol profiles.

Colletes hederae and *C. succinctus* were both sampled from >3 sites with at least 10 replicates and have different foraging preferences. They were therefore used in an NMDS (trymax=200, k=2) to compare sterolome between these two closely related species that had different pollen hosts.
The dataset contained four monolectic bee species and two species which are narrowly specialised in the UK (*Colletes halophilus* and *Colletes hederae*). Comparisons were made between the bee and pollen samples and between the bee-pollen groups via NMDS (trymax=200, k=2) and PERMANOVA to determine whether specialist bees grouped closer with their pollen host or other bee species.

An NMDS (trymax=200, k=2) was calculated to compare the sterol profiles of solitary bees specialising on Asteraceae, non-Asteraceae specialists, polylectic bees and pollen from three tribes of Asteraceae (Carduoideae, Cichorioideae, Asteroideae). When comparing pollen and solitary bee sterol profiles, only sterols that had been detected in both datasets were used and proportions re-calculated using the remaining sterols. This led to a total of 17 sterols with ST(30:1)A and ST(30:1)B being removed from the bee dataset. Aligning data in this way will have altered the proportions of the remaining sterols in both datasets. However, to retain them would have made it impossible to fairly compare in an analysis.

Sterol data were then also grouped by B-ring double bond saturation (cyclopropane ring (CPR), Δ0, Δ5, Δ7, Δ8, NA) with ergosterol and all unidentified sterols classed as NA (ergosterol is a Δ5 and Δ7 sterol) after Baker et al., (2025)[8]. In order to compare the proportions of different B-ring double bond groups between Asteraceae and non-Asteraceae specialist bees a Kruskal-Wallis rank sum test was carried out using kruskal.test() followed by post-hoc Dunn tests using dunnTest(),from the STATS package [54] and FSA package [63] respectively. Both analyses used a Benjamini-Hochberg correction for multiple testing.

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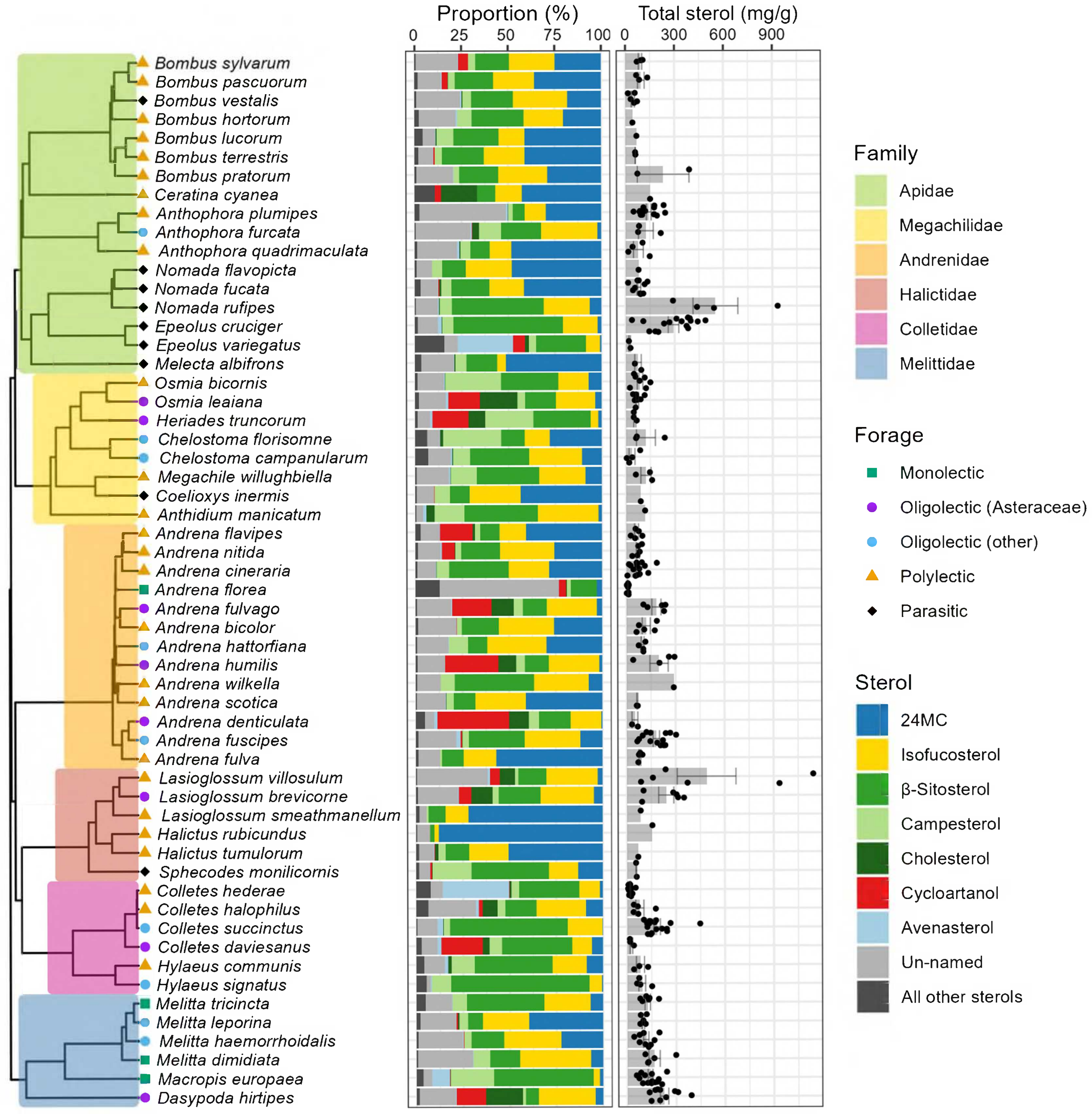
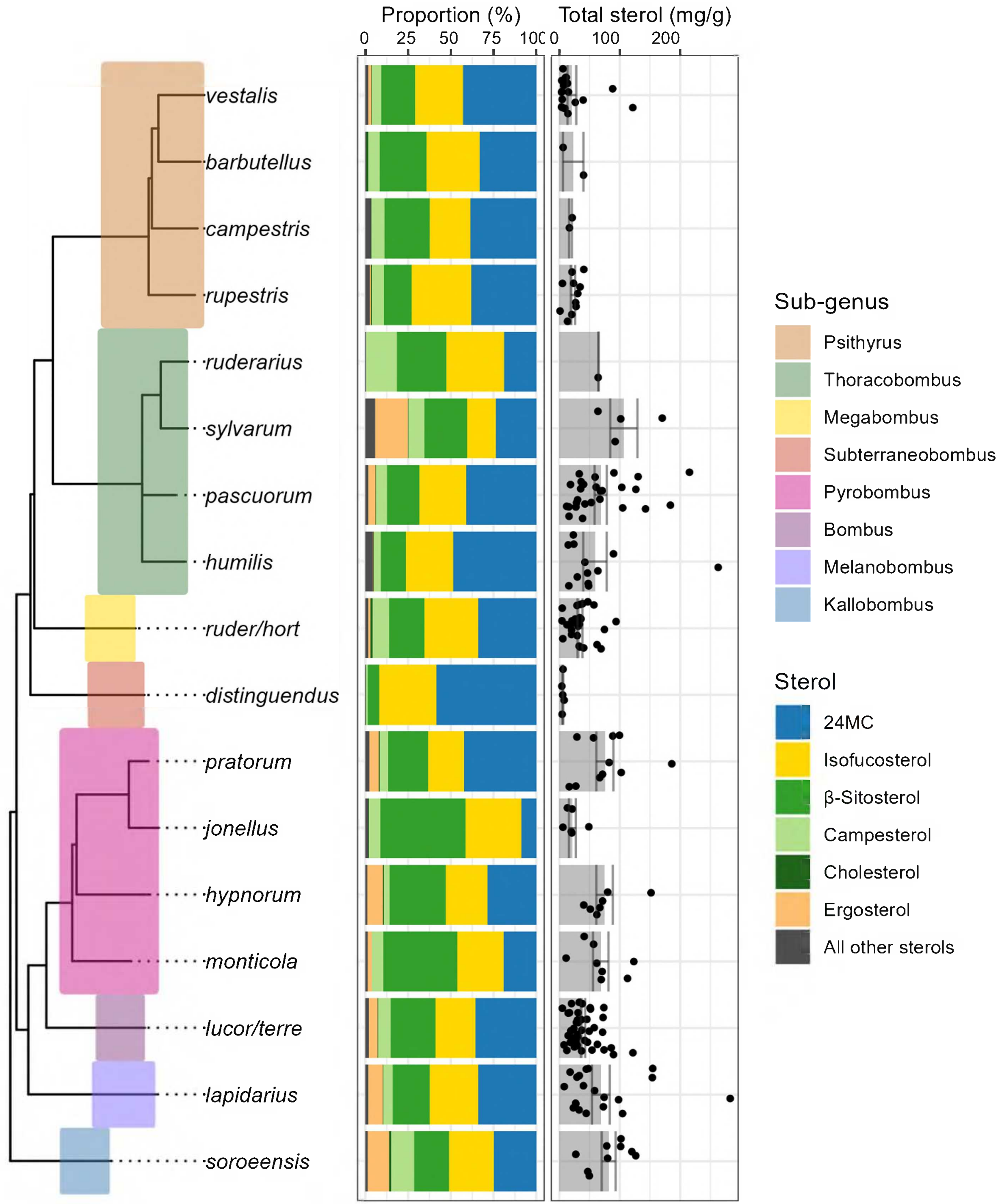
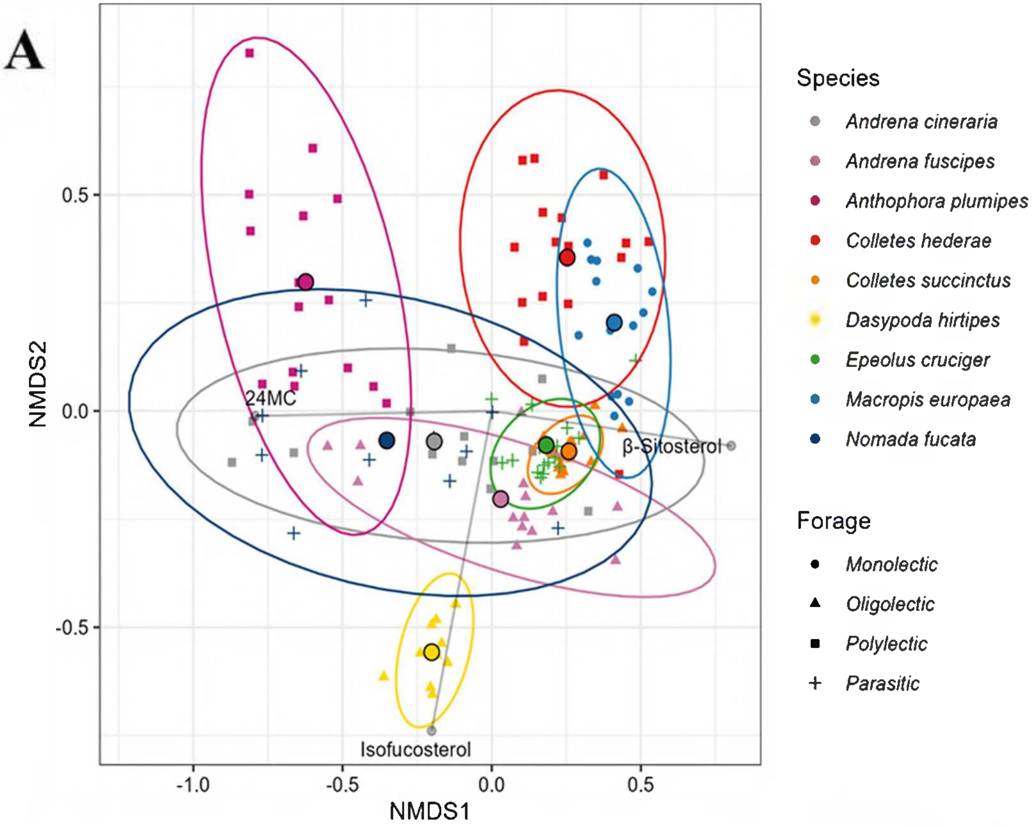
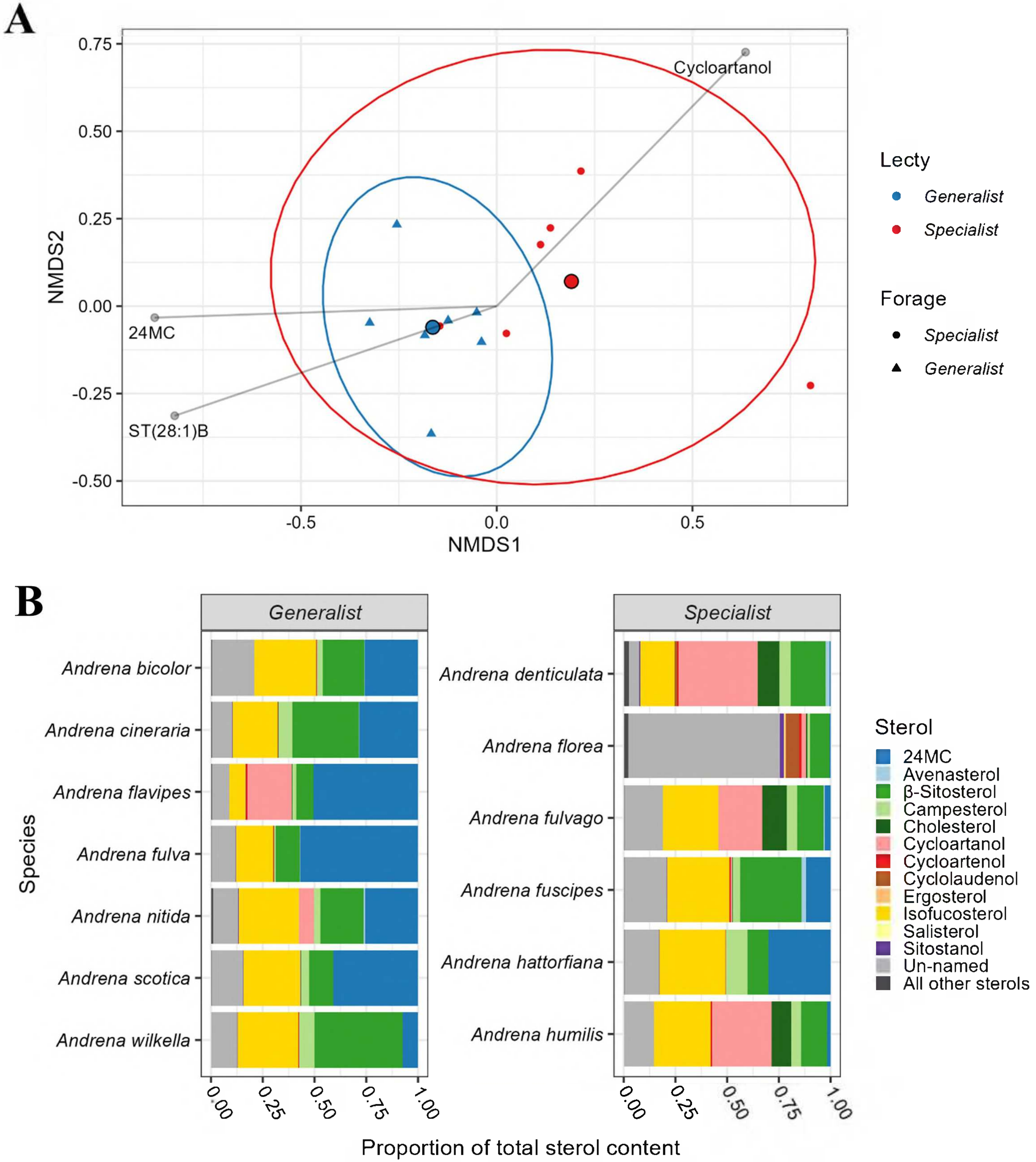

Proportion (%)
0
25
50
75
100
Total sterol (mg/g)
0
300
600
900
Bombus sylvarum
Bombus pascuorum
Bombus vestalis
Bombus hortorum
Bombus lucorum
Bombus terrestris
Bombus pratorum
Ceratina cyanea
Anthophora plumipes
Anthophora furcata
Anthophora quadrimaculata
Nomada flavopicta
Nomada fucata
Nomada rufipes
Epeolus cruciger
Epeolus variegatus
Melecta albifrons
Osmia bicornis
Osmia leaiana
Heriades truncorum
Chelostoma florisomne
Chelostoma campanularum
Megachile willughbiella
Coelioxys inermis
Anthidium manicatum
Andrena flavipes
Andrena nitida
Andrena cineraria
Andrena florea
Andrena fulvago
Andrena bicolor
Andrena hattorfiana
Andrena humilis
Andrena wilkella
Andrena scotica
Andrena denticulata
Andrena fuscipes
Andrena fulva
Lasioglossum villosulum
Lasioglossum brevicorne
Lasioglossum smeathmanellum
Halictus rubicundus
Halictus tumulorum
Sphecodes monilicornis
Colletes hederae
Colletes halophilus
Colletes succinctus
Colletes daviesanus
Hylaeus communis
Hylaeus signatus
Melitta tricincta
Melitta leporina
Melitta haemorrhoidalis
Melitta dimidiata
Macropis europaea
Dasypoda hirtipes
Family
Apidae
Megachilidae
Andrenidae
Halictidae
Colletidae
Melittidae
Forage
■ Monolectic
● Oligolectic (Asteraceae)
● Oligolectic (other)
▲ Polylectic
◆ Parasitic
Sterol
24MC
Isofucosterol
β-Sitosterol
Campesterol
Cholesterol
Cycloartanol
Avenasterol
Un-named
All other sterols

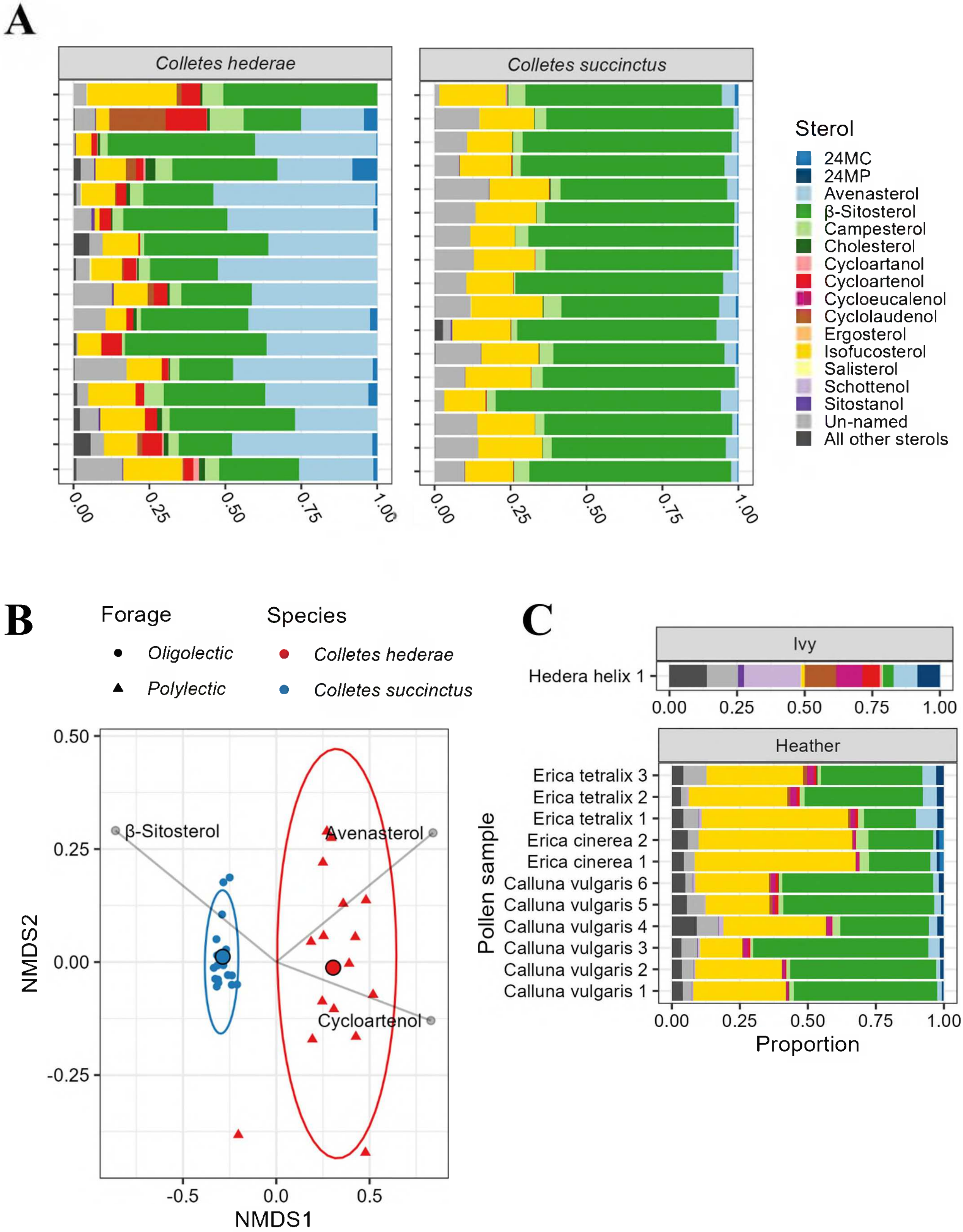

Proportion (%)
0
25
50
75
100
Total sterol (mg/g)
0
100
200
vestalis
barbutellus
campestris
rupestris
ruderarius
sylvarum
pascuorum
humilis
ruder/hort
distinguendus
pratorum
jonellus
hypnorum
monticola
lucor/terre
lapidarius
soroeensis
Sub-genus
Psithyrus
Thoracobombus
Megabombus
Subterraneobombus
Pyrobombus
Bombus
Melanobombus
Kallobombus
Sterol
24MC
Isofucosterol
β-Sitosterol
Campesterol
Cholesterol
Ergosterol
All other sterols

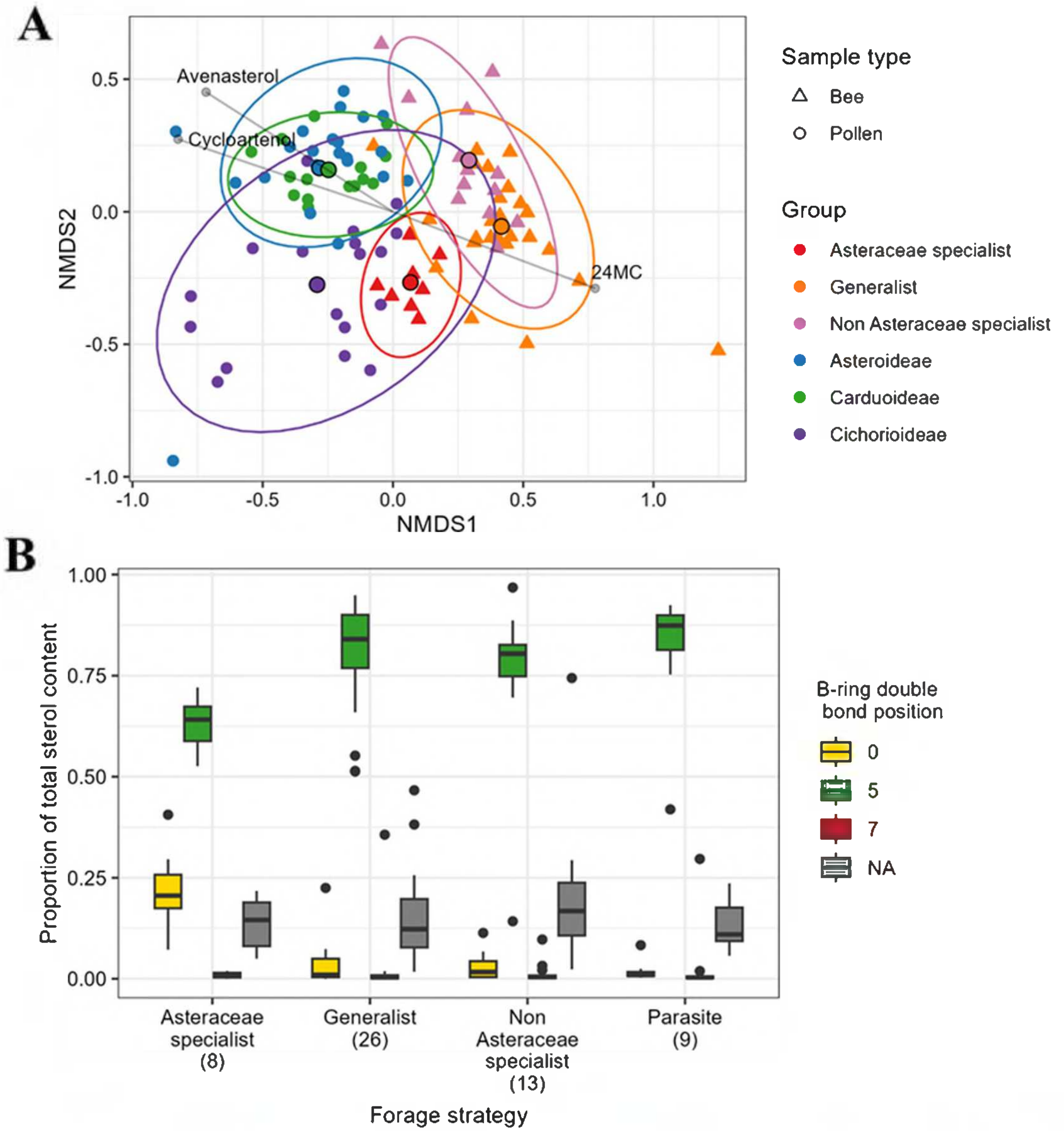


B

Sterol	IV	P-value	Sterol	IV	P-value
Andrena cineraria			***Dasypoda hirtipes***		
ST(28:1)A	0.523	0.005	Cycloartanol	0.803	0.005
Andrena fuscipes			Cholesterol	0.744	0.005
ST(29:1)A	0.419	0.020	Ergosterol	0.589	0.005
Anthophora plumipes			ST(29:2)A	0.416	0.020
ST(28:1)B	0.830	0.005	Isofucosterol	0.394	0.005
Sitostanol	0.477	0.005	***Macropis europaea***		
Colletes hederae			Campesterol	0.497	0.005
Avenasterol	0.611	0.005	***Nomada fucata***		
Cycloartenol	0.581	0.005	24MC	0.515	0.005
ST(30:1)B	0.477	0.005			
Colletes succinctus					
β-Sitosterol	0.416	0.005			


A
0.75
0.50
0.25
0.00
-0.25
-0.50
NMDS2
-0.5
0.0
0.5
NMDS1
Cycloartanol
24MC
ST(28:1)B
Lecty
Generalist
Specialist
Forage
Specialist
Generalist
B
Generalist
Specialist
Species
Andrena bicolor
Andrena cineraria
Andrena flavipes
Andrena fulva
Andrena nitida
Andrena scotica
Andrena wilkella
Andrena denticulata
Andrena florea
Andrena fulvago
Andrena fuscipes
Andrena hattorfiana
Andrena humilis
0.00
0.25
0.50
0.75
1.00
Proportion of total sterol content
Sterol
24MC
Avenasterol
β-Sitosterol
Campesterol
Cholesterol
Cycloartanol
Cycloartenol
Cyclolaudenol
Ergosterol
Isofucosterol
Salisterol
Sitostanol
Un-named
All other sterols


A
Colletes hederae
Colletes succinctus
0.00
0.25
0.50
0.75
1.00
Sterol
24MC
24MP
Avenasterol
β-Sitosterol
Campesterol
Cholesterol
Cycloartanol
Cycloartenol
Cycloeucalenol
Cyclolaudenol
Ergosterol
Isofucosterol
Salisterol
Schottenol
Sitostanol
Un-named
All other sterols
B
Forage
Oligolectic
Polylectic
Species
Colletes hederae
Colletes succinctus
β-Sitosterol
Avenasterol
Cycloartenol
NMDS2
NMDS1
0.50
0.25
0.00
-0.25
-0.5
0.0
0.5
C
Ivy
Hedera helix 1
Heather
Erica tetralix 3
Erica tetralix 2
Erica tetralix 1
Erica cinerea 2
Erica cinerea 1
Calluna vulgaris 6
Calluna vulgaris 5
Calluna vulgaris 4
Calluna vulgaris 3
Calluna vulgaris 2
Calluna vulgaris 1
Pollen sample
Proportion
0.00
0.25
0.50
0.75
1.00



C

Sterol	IV	P-value	Sterol	IV	P-value	Sterol	IV	P-value
Asteraceae specialist			**Asteroideae**			**Carduoideae**		
Cholesterol	0.614	0.005	Cycloartenol	0.564	0.005	Avenasterol	0.609	0.005
Non-Asteraceae specialist			**Cichorioideae**			Salisterol	0.590	0.005
β-sitosterol	0.477	0.005	Cycloartanol	0.576	0.010	Cyclolaudenol	0.568	0.005
Generalist						Sitostanol	0.555	0.010
24MC	0.632	0.005						