

Pollen sterols are highly diverse but phylogenetically conserved

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Summary

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- Phytosterols regulate cell membrane fluidity and are precursors for plant hormones and secondary metabolites in plants. Insects are auxotrophic for sterols; therefore, they have to consume phytosterols and dealkylate them to cholesterol. Some insects, including bees, which rely on dietary sources, primarily pollen, for sterols, cannot modify them; therefore, they have adapted to use them unmodified as they occur in pollen.
- Here, using high resolution chemical analysis, we describe the distribution of 78 different pollen sterols from 295 UK wildflower taxa and use this data to develop an evolutionary rationale for the diversity of sterols in pollen compared to vegetative tissues.
- The sterol occurrence in pollen was a function of plant lineage and conserved in groups as high as subfamily. Most pollen in the present study contained high proportions of $\Delta 5$ sterols including β -sitosterol, 24-methylenecholesterol and isofucosterol, which are important sterols for bees. The sterols recorded in honeybees occurred in the pollen of only 68% of plant taxa; however, none matched these proportions exactly, suggesting they must forage pollen from multiple plant taxa to satisfy their sterol requirements.
- We conclude that there is evidence for pollen sterol composition being the result of diverse driving forces including plant lineage and potentially pollinator nutritional requirements.

Introduction

Sterols are lipids that serve as essential cell membrane components. In vertebrates, cholesterol is dominant, whereas fungi primarily use ergosterol. Plants, however, produce a wide range of sterols with over 250 reported (Nes, 2011; Valitova *et al.*, 2016). While the most common sterols in vegetative tissues are β -sitosterol and stigmasterol (Behmer & Nes, 2003; Dufourc, 2008) pollen contains a more diverse range of sterols (Zu *et al.*, 2021). Some pollen, for example, has no β -sitosterol or stigmasterol but instead contains other sterols such as 24-methylenecholesterol (Van der Planck *et al.*, 2014; Villette *et al.*, 2015; Moerman *et al.*, 2017; Vanderplanck *et al.*, 2020b; Zu *et al.*, 2021; Furse *et al.*, 2023). The extent to which the sterol profile (sterolome) of angiosperm pollen varies and the adaptive function for this variation remains largely unknown.

Pollen sterols facilitate fertilization of the ovule by providing substrates for pollen tube elongation (Rotsch *et al.*, 2017; Stroppa *et al.*, 2023). Sterols are especially important for rapid cell elongation and cell polarisation during pollen tube growth (Rotsch *et al.*, 2017). However, the sterols used for pollen tubes are not those occurring in mature pollen grains (Rotsch *et al.*, 2017) but are created *de novo* during pollen tube growth (Rotsch *et al.*, 2017; Stroppa *et al.*, 2023). Since sterols are also metabolically expensive (Nes, 2011) and in the absence of an established explanation of their function, it is surprising that they accumulate in pollen with such diversity.

Pollen nutrients including lipids may influence foraging behaviour of bees (Vaudo *et al.*, 2024). Like other insects, bees cannot synthesize sterols *de novo*, but instead acquire them along with protein, fats and micronutrients from their diets (Wright *et al.*, 2018). However, unlike other phytophagous insects, bees cannot convert dietary sterols to cholesterol (Behmer & Nes, 2003). Instead, they incorporate specific pollen sterols directly into their tissues without modifying them (Svoboda *et al.*, 1986). The sterol profile of the few bees that have been studied (honeybees, leaf-cutting bees) is mainly composed of 24-methylenecholesterol, isofucosterol, β -sitosterol, campesterol, desmosterol and cholesterol (see Svoboda & Feldlaufer, 1991; Moore *et al.*, 2025). The use of pollen-specific sterols in bee tissues may be an adaptation to palynivory (Svoboda *et al.*, 1994) but would be an evolutionary risk if pollen that bees collected did not contain all the required sterols.

Anthropogenic change to flowering landscapes throughout the world has significantly altered floral resource availability for pollinators (Howard *et al.*, 2003; Vanbergen and Insect Pollinators Initiative, 2013; Baude *et al.*, 2016), increasing the likelihood of nutritional deficiencies. An understanding of pollen sterol variation could inform predictions about nutritional stress in bees. To measure sterol production in pollen, we measured the diversity and abundance of sterols in 295 UK wildflower taxa using a sterol analysis. We found that sterol profiles were remarkably stable within taxonomic groups, including subfamilies of Asteraceae

and Rosaceae. Sterolomes did not vary at different sites and a significant phylogenetic signal for almost half of the sterols was recorded. However, none of the pollen sterolomes matched those reported from honeybees.

Materials and Methods

Species selection

In total, 700 pollen samples were collected for sterol analysis, representing 295 taxonomic units, 275 identified to species level and 125 of which were represented by a single sample. A full summary of samples collected, dates and location are provided in Supporting Information Table S1. Samples included pollen from 172 species selected from the species list compiled by (Baude *et al.*, 2016) which targeted plants from the Countryside Survey in Great Britain that were rewarding to pollinators and considered sufficiently common to contribute to landscape-level floral resources. During collection visits for the above target species, 88 plants that produced easily extractable pollen or would contribute to wider taxonomic coverage in the dataset were also opportunistically collected. Asteraceae flowers produce copious and easily collectible pollen, and previous work has suggested nutritional deficiencies within Asteraceae pollen (Vanderplanck *et al.*, 2020a). Therefore, Asteraceae flowers were also targeted opportunistically, adding a further 18 species, while 17 more species were targeted due to their importance as forage plants for wild bee species in the United Kingdom (such as *Bryonia cretica* L. for *Andrena florea* (Fabricius), *Reseda* spp. for *Hylaeus signatus* (Panzer) and *Anthyllis vulneraria* L. for bumble bees).

Pollen collection

Samples were collected from a range of sites in England, including nature reserves, botanic gardens, public land and private gardens from spring to early autumn from 2021 to 2022. In total, samples were collected from 15 counties. Collection months and counties for all samples are shown in Table S1. Flowering heads, or branch tips in the case of woody species, were cut from the plant and placed in sealed sandwich bags. These were then taken back to the laboratory and placed in a beaker of room temperature tap water to dehisce. The duration needed for anthers to dehisce ranged from a few hours to a few days, but most were left for 24 h. Once pollen had been produced, it was removed from the flower using a clean paintbrush and forceps under a magnifying glass. Pollen was collected onto an ethanol-wiped glass microscope slide and visually inspected for debris or insects, which were subsequently removed. The pollen sample was then scraped into a clean 1.5 ml Eppendorf tube or glass vial using an ethanol-wiped razor blade. The exact method of pollen extraction varied depending on species and floral morphology. For daisy-type Asteraceae (Asteroideae), pollen was brushed from the tops of disk florets in clumps. For thistle-type Asteraceae (Carduoideae), pollen was brushed from anther tube tips and tubes were squeezed gently to remove pollen further. For legume-type Fabaceae flowers, wing petals were removed and the tip of the

keel was lightly squeezed, as for *Lotus corniculatus*, or all petals were removed entirely, as for *Trifolium pratense* L. Pollen samples were then frozen at -20°C before being moved to -80°C in batches. Freezing pollen has been shown to prevent nutritional degradation (Hagedorn & Burger, 1968; Pernal & Currie, 2000).

To assess inter- and intra-species variation in sterol profile, a subset of species was collected from at least three counties in England. Plant identification was confirmed by photos shared on iNaturalist and with botanists at Royal Botanic Gardens (RBG), Kew. A total of 275 samples were identified to species and sub-species level and 20 were identified to genus level.

A minimum of 10 mg fresh weight pollen per sample was required due to the lower end of pollen total sterol content being 0.1%, that is, 10 μg of sterol in a 10 mg sample, a level within the detectable range of our analysis. At the end of the collection season, all samples were transferred to 1.5 ml glass vials and partitioned or combined by species, depending on whether the sample was over or under 10 mg. The final weight of each sample was between 9.5 and 16.5 mg.

Sample preparation

Alongside the hand-collected pollen samples, additional samples were used in the sample run to monitor performance. First, Quality Assurance (QA) samples contained a solution of reference materials compiled to assign names to signals in our samples. This allowed us to assess the consistency of analysis by the instrument across a large cohort of samples and calculate the coefficient of variance. Reference materials verified by multi-nuclear NMR were 24-methylenecholesterol, 24-methylenecycloartanol, anhelsterol (a sterol found in *C. anthelmintica*, ST(28:3)), that is active under 330 nm UV but whose structure has not been determined formally using NMR (Furse *et al.*, 2023), avenasterol, β -sitosterol, brassicasterol, campesterol, cholesterol, cycloartenol, cycloeucalenol, cyclo-laudenol, desmosterol, episterol, ergosterol, isofucosterol, schottenol, sitostanol, spinasterol and stigmasterol. The m/z and retention time for each sterol are shown in Table S2. Aliquots of 40 μl were used in the run. Second, a quality control (QC) stock solution was made by homogenizing a set of commercially available honeybee-collected pollens: 95 g of *Helianthus annuus* L. (sunflower) pollen, 5 g of *Nymphaea* sp. pollen and 20 g of *Fagopyrum esculentum* (Moench.) (buckwheat) pollen in 120 ml water. For this project, three QC concentrations were used: 100%, 50% and 25%, corresponding to 40, 20 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 runs began with a blank containing LC-MS quality methanol only. This was designed to enable identification and removal of background noise.

Sterol extraction

To each vial of hand-collected pollen or QC sample, a 20 μl aliquot of 0.1 mg ml^{-1} (5.08 nMoles per sample) d_7 -cholesterol as the internal standard (IS) was added with 300 μl methanolic

KOH (10 g : 90 ml) and the vial was vortexed for 20 s. Vials were then placed in drilled aluminium heating blocks at 75°C in a water bath for 2 h. Saponification releases conjugated sterols (Evyugin *et al.*, 2023), such as sterol esters, which are reported to be common in pollen (reviewed in Ferrer *et al.*, 2017). The saponified material was then transferred to a corresponding deep 96-well plate (96-well plate, Esslab Plate+™, 2.4 ml per well, glass-coated). Pollen samples were randomised within plates and interspersed randomly with QA, QC and blank (300 µl methanol and 20 µl IS) samples.

Extraction of the sterol fraction was carried out on the plate using a 96-channel pipette (liquid-handling robot, Integra Via-flow 96/384 channel pipette) with DMT (dichloromethane (DCM)) (3 parts), methanol (1 part) and triethylammonium chloride (0.0005 parts, that is 500 mg l⁻¹). To each well, 500 µl of DMT (250 × 2) was added, followed by a 500 µl aliquot of deionised water and the mixture was agitated using the 96-channel pipette. The plate was centrifuged for 2 min to separate layers (methanol + water, solid and DMT + sterol). A 50 µl sample of extract (DMT and sterol) was transferred to the 384-well plate and left for 30 min for the DMT to evaporate. After repeating this process four times, 150 µl of LC-MS grade methanol was added and the plate was sealed with foil. A total of three 384-well plates were run immediately in sequence, analysing 700 hand-collected pollen samples and 281 analytical samples. One pollen sample was later excluded because it had experienced prolonged exposure to room temperature conditions during collection.

Sample analysis and data processing

Mass spectra were recorded using a Thermo Scientific Orbitrap Fusion mass spectrometer, which was equipped with an Atmospheric Pressure Chemical Ionization (APCI) probe and integrated with a Thermo Scientific Vanquish liquid chromatography system (Thermo Scientific, Waltham, MA, USA). Metabolites were separated before analysis on a Hypersil GOLD LC-MS C18 column (50 × 2.1 mm with particle size 1.9 µm) (Thermo Scientific) which was maintained at 55°C for analyses. An aliquot of 1 µl was injected, with a mobile phase flow rate of 0.60 ml min⁻¹ comprising a gradient of 84% methanol at *T* = 0 to 100% methanol at *T* = 11 min and held at 100% till *T* = 16.4 min and then returned to 84% methanol from 16.5 to 19 min. Mass spectrometric data were acquired in positive ionisation mode via APCI, operating at a resolution of 120 000. The spray voltage was set to 2.86 kV, with sheath nitrogen gas flows of 45 (sheath), 5 (auxiliary) and 1 (sweep) arbitrary units. The ion transfer tube and vaporizer temperatures were maintained at 300°C and 350°C, respectively. The mass acquisition range was calibrated to *m/z* 197–500, with the lower limit adjusted to detect the fluoranthene cation (*m/z* 202.077), which served as the internal mass calibration reference. Under these conditions, the internal standard used for the samples, *d*₇-cholesterol, eluted at 5.6 min, with the primary ion species identified as [M – H₂O + H]⁺ at *m/z* 376.395 within a mass variance of < 1 ppm. Both samples and external standards were analysed in a

single sequence. The pollen samples were randomised among themselves, while the external standards were run sequentially, beginning with the least concentrated standard. Instrument output was processed after Furse *et al.* (2023). Data files were processed using Analyzer PRO XD (SpectralWorks Ltd, Cheshire, UK). Signals were extracted from 3 to 11 min in a mass range of 360–442 *m/z*, with an area threshold of 500 000, smoothing of 15 arbitrary units, a width of 0.01 min, mass accuracy of 3 dp and a retention time window of 0.1 min.

All compound signals were determined by their *m/z* and retention time (level 3 annotation) 78 of which were further distinguished using the format ST (27:1)A where ST represents 'sterol', the first number indicates the number of carbons, the second number indicates the number of double bonds and the letter distinguishes sterols that differed but otherwise had a similar number of carbons and double bonds (level 2 annotation). Compounds were assigned names (a level 1 annotation) where reference materials that had been verified using multi-nuclear NMR were available as reference samples. These included 24-methylenecholesterol, 24-methylenecycloartenol, agnosterol, anthelsterol, avenasterol, β-sitosterol, brassicasterol, campesterol, cholesterol, cycloartenol, cycloeucalenol, cyclolaudenol, desmosterol, episterol, ergosterol, isofucosterol, lophenol, schottenol, sitostanol, spinasterol and stigmaterol (Fig. 1). Anthelsterol is a trivial name for a sterol that has not been determined using NMR (Furse *et al.*, 2023).

Instrument output reported mass to charge ratio (*m/z*), signal size and retention time. Signal size output was first converted by dividing the signal area for each metabolite by the signal of internal standard (*d*₇-cholesterol). These relative abundance values were used for the majority of the data analysis. To generate a value of total sterol production in sterol/fresh weight of pollen in mg kg⁻¹, the following formula was used:

$$\frac{\text{Sterol (mg)}}{\text{Pollen (kg)}} = \frac{\text{molecular mass of sterol (g mol}^{-1}\text{)} \times (\text{signal} \times 5.08e^{-9} \text{ mols}) \times 1000 \text{ 000}}{\text{Starting weight (mg)} / 1000}$$

Data analysis

All data analysis was done in R v.4.4.0 (2024-04-24 ucrt) (R Core Team, 2024) and RSTUDIO (2024.9.1.394) (Posit Team, 2024). A total of 78 sterols and related compounds were identified in the sample set and a total of 295 taxonomic units were analysed including those identified to genus, species and subspecies level. Summary statistics were calculated using means of these 295 units, with some then removed for species level and phylogenetic analyses as detailed below.

Outliers were identified using *disana()* from the LABDSV package v.2.1-0 (Roberts, 2023) on a Bray–Curtis dissimilarity matrix of proportion data from all samples. Any data point with a minimum dissimilarity > 0.5 compared to all other points in the data was identified as an outlier, as per author guidance and so removed. This led to the removal of one *Campanula trachelium* L. sample. Simpsons diversity index was calculated using

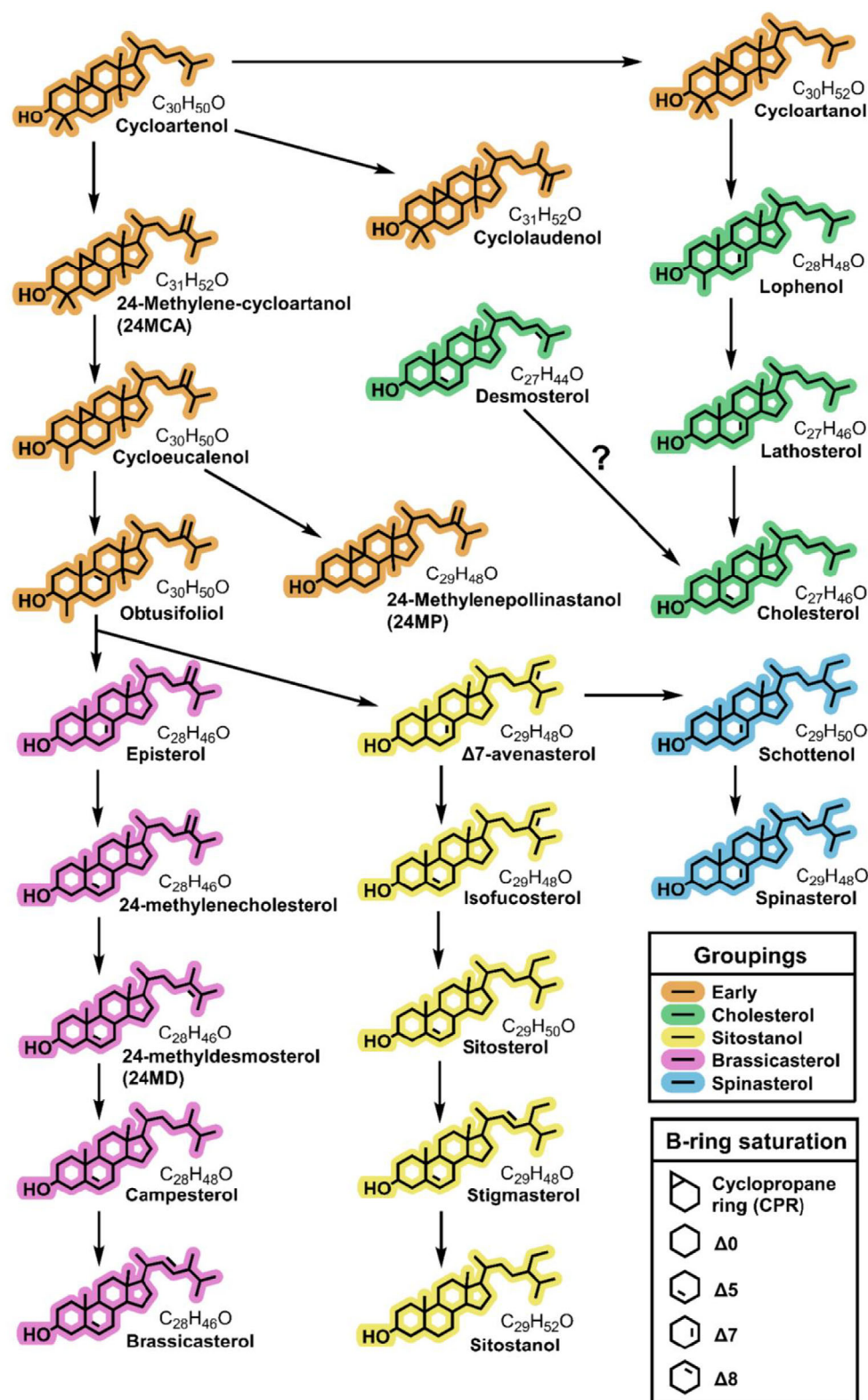


Fig. 1 Many of the sterols abundant in pollen, such as 24-methylenecholesterol, isofucosterol and β -sitosterol, are not the end products of these synthesis pathways. Sterol synthesis pathway in plants collated from (Sucrow & Radüchel, 1971; Schaller, 2003; Morikawa *et al.*, 2006; Desmond & Gribaldo, 2009; Villette *et al.*, 2015; Sonawane *et al.*, 2016). All sterols which could be named in this study using reference materials are shown, excluding ergosterol which is not synthesised by plants. Sterols which could only be identified to level two annotation (number of carbons and double bonds) are not shown. Sterols are coloured by section of the synthesis pathway to highlight structural similarities such as carbon count and emphasise branching points in the synthesis pathway.

diversity(index = 'simpson') from the VEGAN package v.2.6-6.1 (Oksanen *et al.*, 2024) for all 295 taxonomic units to better understand differences in sterol diversity between pollens.

NMDS were plotted using metaMDS(trymax = 200, $k = 2$, autotransform = FALSE). The lowest number of axes was selected where stress was ≤ 0.2 (established cut off

(Bakker, 2024)). Stress values reflect how well the ordination aligns with the original data. Proportion data (0–1) was used for analysis, where zero denotes a sterol is absent from a sample and one denotes it is completely dominant. Proportions were arcsine square root transformed and converted into a Bray–Curtis dissimilarity matrix using `vegdist(method='bray')`. Where NMDS stress value was < 0.2 a 2D biplot was made using `GGPLOT2` v.3.5.1 (Wickham, 2016) and `GGREPEL` v.0.9.5. (Slowikowski, 2024) to show significant ($P < 0.010$) and strong (> 0.7 absolute value) associations. PERMANOVA was calculated using `adonis2()` to test for significant differences between groups. `Betadisper()` and `anova()` were used to confirm non-significant difference in dispersion. `Adonis2()` was also used to test for pairwise comparisons between groups following Bakker (2024), using a Benjamini–Hochberg correction which corrects for multiple testing to reduce the chances of false positives. All functions were from `VEGAN` (Oksanen *et al.*, 2024) except `anova()` from the `STATS` package (R Core Team, 2024). The `STATS` package from R (R Core Team, 2024) was used to calculate summary statistics. Indicator Species Analysis (ISA) was carried out on arcsine square root transformed data to accompany NMDS plots using `multipatt()` from the `INDICESPECIES` package v.1.7.14. (De Cáceres & Legendre, 2009) to determine significant associations between groups and individual sterols. ISA was done at the level of individual groups only and not for combinations of groups.

Phylogeny construction and phylogenetic signal

A phylogenetic tree of the study species was constructed based on the date calibrated and rooted tree from Zanne *et al.* (2014) (Fig. 2). The tree was pruned for our study species using `keep.tip()` from the R package `APE` v.5.8. (Paradis & Schliep, 2019). Any synonyms present in the tree were manually renamed to match the POW taxonomy used in the project (19 species). In the case of species which did not have a corresponding match on the Zanne phylogeny, the following approach was taken: where a subspecies level match was not available, a species level branch was renamed instead (two species). Where a hybrid species was not available, a matching parent not already in the dataset was renamed (five species). Where a species match was not available, another species from the same genus was renamed instead (seven species), but only where there was not already a species correctly matched within that genus. Out of 295 taxonomic units, 34 could not be suitably placed on the tree and were removed from phylogenetic analysis.

Families were checked for monophyly using `AssessMonophyly()` from the R package `MONOPHY` v.1.3.1. (Schwery & O'Meara, 2016). Phylogeny was plotted with `ggtree()` from `GGTREE` v.3.12.0. (Yu *et al.*, 2017, 2018; Yu, 2020; Xu *et al.*, 2022). Heatmap and bar graph associated with phylogeny were plotted using `ggplot()` from `TIDYVERSE` v.2.0.0. (Wickham *et al.*, 2019). The composite graph was then plotted using `APLOT` v.0.2.2. (Yu, 2023).

Of the 78 sterol compounds detected, the 69 that had a maximum proportion $> 1\%$ were analysed for phylogenetic signal (Zu *et al.*, 2021). To determine if there was a phylogenetic signal in sterol production across the data, whereby closely related

species would share more similar profiles than expected by chance, Pagel's λ and Blomberg's K were calculated. Both metrics use a Brownian model of trait evolution; in both cases, a value of 0 indicates the trait has evolved independently of phylogeny; therefore, closely related species are not more similar to each other than distantly related ones. Low phylogenetic signal can mean high rates of evolution are causing big differences between closely related species, or perhaps convergent evolution is causing distant relatives to be more similar than close ones. High phylogenetic signal ($K, \lambda = 1$) can be caused by low rates of evolution or stabilising selection. Interpretation of high and low phylogenetic signal varies between studies (Kamilar & Cooper, 2013). `Phylo4d` objects were generated from species tree and proportion data using `phylo4d()` from `PHYLOBASE` v.0.8.12. (R Hackathon *et al.*, 2024) and `phyloSignal()` from the R package `PHYLOSIGNAL` v.1.3.1. (Keck *et al.*, 2016) used to calculate Pagel's λ and Blomberg's K . The number of iterations was set to 999. Quantitative values (mg kg^{-1}) were used for total sterol content and proportions (0–1) for all other sterol traits. To identify trends in different types of sterols, proportion data was also grouped according to carbon number and position of the B-ring double bond. Ergosterol was classed as NA for double bond position as it contains double bonds at positions 5 and 7 and sterols with a cyclopropane ring were classed as CPR. Un-named sterols were all classed as ΔNA ; full details are available in Table S3.

Family, genus and species level sterol diversity

Before analysis of interspecies differences, 17 samples identified to only genus level, where there was already a species level representative in the genus, were removed.

Species within the family Asteraceae were grouped according to subfamily: Asteroideae ($n = 21$, tubular and ray florets), Cichorioideae ($n = 17$, ray florets only), Carduoideae ($n = 14$, tubular florets only). Subfamily classifications were taken from the OPEN TREE OF LIFE v.14.7 (OpenTreeOfLife *et al.*, 2019) and (Mandel *et al.*, 2019). Mean sterol proportions were calculated for each species. An NMDS was plotted containing all Asteraceae species (stress = 0.166) to compare sterol profiles between the subfamilies and an ISA was carried out to test for associations between subfamilies and specific sterols.

For each species, sterols were then grouped by B-ring double bond saturation (CPR, $\Delta 0$, $\Delta 5$, $\Delta 7$, $\Delta 8$ or ΔNA) and their proportions summed (B-ring double bond position data is available in Table S3). Ergosterol was classed as NA for double bond position as it contains double bonds at C-5 and C-7. Unnamed sterols were all classed as ΔNA . Proportion data (0–1) was arcsine square root transformed and a Kruskal–Wallis rank sum test was carried out using `kruskal.test()`, for each double bond group, followed by a *post hoc* Dunn test using `dunnTest()` from the `FSA` package 0.9.5 (Ogle *et al.*, 2023) to test for differences between Asteraceae subfamilies and the rest of the dataset. Both analyses used a Benjamini–Hochberg correction for multiple testing.

Three families (Asteraceae, Fabaceae and Rosaceae) contained three genera of at least three species each and so were

used to analyse intra-family variation in sterolome at the genus level. An NMDS and PERMANOVA were computed separately for the three families using data from individual samples to test for significant differences in sterolome between genera. Species identity was also included in the models (Fig. 2).

A total of 48 species were collected from at least three counties in England. As 48 groups would be too many to separate on an NMDS, the species with the highest number of samples were chosen for statistical comparison. Species which were sampled from four different counties and had at least five samples overall were used. An NMDS and PERMANOVA were used to test for significant differences between these most well-sampled species (stress value = 0.096).

Ecological associations of sterol profiles

In order to establish how many plants could suitably meet the sterol requirements established for honeybees, data for 18 prepupal honeybee samples (15 prepupae per sample) was used from Svoboda *et al.* (1980) and compared to all hand collected pollen species in this dataset. In order to compare these datasets, only sterols detected in the Svoboda work were used (24-methylenecholesterol, isofucosterol, β -sitosterol, campesterol, cholesterol and desmosterol) and pollen sterol profiles were not recalculated following this removal. An NMDS ($k = 3$) and PERMANOVA were used to show differences in sterol profile composition and variation between the bees and pollens (stress = 0.162).

Total sterols

For families which contained at least three genera, species data were used to create a box plot showing the range in total sterol production (mg kg^{-1}) across families.

To compare the effect of collection county on total sterol production, data were subset as follows: Total sterol (mg kg^{-1}) was calculated for all samples; data were filtered to only include species which had been collected from at least three counties. Counties were then ranked by the number of these species they included, and those with ≥ 10 kept (Oxfordshire (33), Greater London (25), County Durham (21), North Yorkshire (20) and Sussex (10)). This led to a total of 35 species across five counties being included in the analysis. A linear mixed-effects model was created with total sterol production (mg kg^{-1}) as the response variable and collection county as a fixed effect. Species identity was included as a random effect. Where a species had been collected more than once from a county, the mean value was used for that county. Total sterol data was $\log_{10}$ transformed in order to reduce non-normality of the residuals. The model was run using `lme()` from the NLME package v.3.1.164. (Pinheiro *et al.*, 2023). The residuals of the fixed effects showed normal distribution (Shapiro–Wilk test: $w = 0.986$, $P > 0.100$) and homogeneity of variance (Levene test: $F_{4,104} = 0.685$, $P > 0.500$). Random effects also showed normal distribution (Shapiro–Wilk test: $w = 0.962$, $P > 0.100$).

Results

Quantity of pollen sterol production varies among plant species

We observed interspecies variation in total sterol content of greater than two orders of magnitude. For example, the dandelion, *Taraxacum sect. Taraxacum*, contained the highest amount of sterol ($75423.52 \text{ mg kg}^{-1}$ sterol per fresh weight pollen). The pollen with the lowest concentration of sterol was observed in the common mallow, *Malva sylvestris*, ($729.98 \text{ mg kg}^{-1}$, Table S4). Asteraceae accounted for some of the highest and lowest sterol amounts; specifically, Cichorioideae subfamily flowers showed a consistently higher total sterol production than the other Asteraceae flowers (Table S4). Brassicaceae showed the highest sterol content on average, as Asteraceae was still dominated by low sterol producing species (Fig. S2).

In general, the location of collection did not have a significant impact on total sterol production (mg kg^{-1}) ($F_{4,70} = 1.254$, $P > 0.100$). However, variation in total sterol production was still present, potentially caused by abiotic factors, such as temperature, affecting total fat production, or a result of handling and storage procedures working at the mg scale. Mean and SD for all species replicated at the county level are reported in Table S5.

Pollen shows a diverse but phylogenetically constrained sterol profile

Within the 295 taxonomic units we sampled (including genera, species and subspecies), we recorded 78 distinct metabolites based on their retention time and accurate mass, of which 24 were fully assigned by comparing them to reference compounds (level 1 annotation) and 51 were identified putatively, each determined to be structurally distinct based on retention time and accurate mass, from which the number of carbons and degree of saturation were determined (noted as ST(carbon number: degree of saturation) from here onwards) (level 2 annotation) (see Furse *et al.*, 2023). Where two level 2 annotated sterols had the same carbon number and the same degree of saturation, they were allocated a letter. Most sterols were present in small proportions even if they dominated in some pollens; for example, desmosterol (median 0.02%, max 83.50%) and cholesterol (median 0.01%, max 61.67%) (Table S6). Thirty-eight sterols were not recorded at higher than 5% in any species. The only sterols with a median of $> 2.5\%$ were β -sitosterol, isofucosterol and avenasterol (Table S6).

Only four sterols: campesterol, cycloartenol, ST(30:2)B and ST(30:2)C were detected in all taxa and varied in maximum abundance, from 30.23% in campesterol up to 77.92% in cycloartenol, both of which had a median of $> 1\%$ (Table S7). Cyclolaudenol, ST(30:2)A, ST(30:2)E and ST(31:2)A were in all but one taxonomic unit. Avenasterol, 24-methylenecholesterol and schottenol were in all but two.

Ergosterol, a sterol restricted to fungi (Weete, 1973), was detected in 253 taxonomic units, suggesting the near ubiquitous occurrence of fungi in pollen, indicating that fungi are

inadvertently likely to be a significant part of the diet of pollen-feeding animals such as bees. It was not recorded at > 1.33% in any species (maximum amount in honesty, *Lunaria*

annua L.). The maximum in an individual sample was recorded in meadow buttercup (*Ranunculus acris* L., 1.40%) and had a median proportion across the dataset of 0.01%. Of the 12

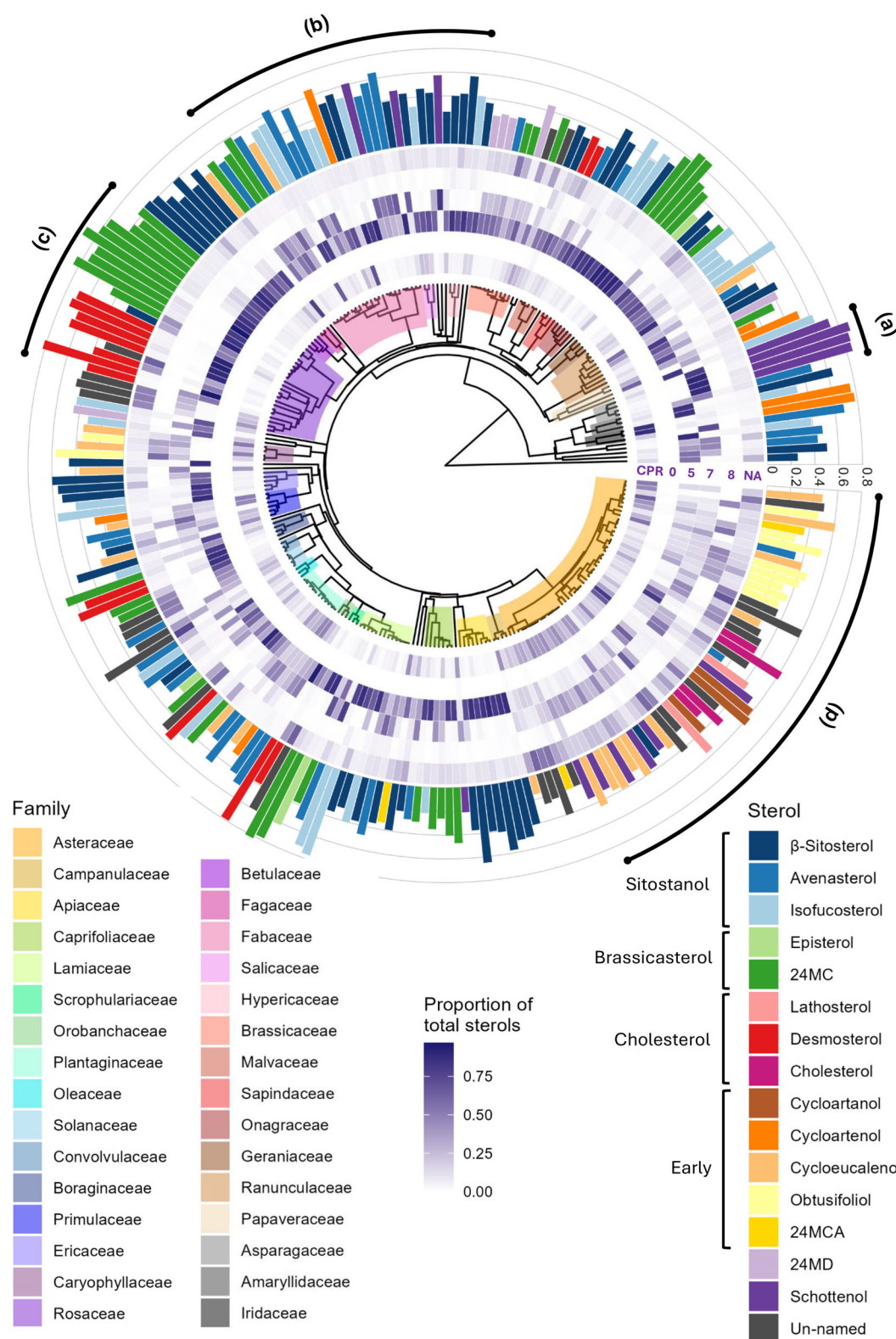


Fig. 2 Plant sterol profiles are diverse and variable, as revealed in a phylogenetic analysis illustrating the pollen sterol composition across 261 plant species. Phylogeny is adapted from (Zanne *et al.*, 2014) showing all families with three or more species are highlighted (inner circle), proportion of total sterols (middle circle) and dominant sterols (outer circle). Families can be read from the right of the phylogeny in a clockwise direction, following the legend top left to bottom right. The inner rings show a heatmap of sterol proportions grouped according to B-ring saturation (cyclopropane ring (CPR), $\Delta 0$, $\Delta 5$, $\Delta 7$, $\Delta 8$ and NA). All sterols which could not be assigned a name using reference materials were categorised as NA for B-ring saturation. In addition, ergosterol which is both $\Delta 5$ and $\Delta 7$ was classed as NA. This heatmap shows a dominance of $\Delta 5$ sterols across the dataset and very low proportions of $\Delta 0$ sterols (stanols). Outer bars show the proportion and identity of the most dominant sterol in each species, showcasing the diversity of sterol production of pollen. All named sterols are grouped according to their position in the sterol synthesis pathway. (a) Despite the diversity of the sterolome in many pollens, some species are strongly dominated by a single sterol. This can be seen in these Asparagaceae pollens, which mostly contain schottenol. (b) In some areas of the tree, sterols close together in the synthesis pathway are produced in high proportions by related species. In this case, β -sitosterol, avenasterol and isofucosterol are the dominant sterols in a range of Fabaceae, Salicaceae and Hypericaceae species. (c) There are clear switches in the dominant sterol between some taxa; within the two Rosaceae subfamilies plotted, Amygdaloideae is dominated by 24-methylenecholesterol, whilst Rosoideae is dominated by desmosterol. (d) Asteraceae represented our most sampled family and showed an interesting sterol profile compared to other families. Species contained higher proportions of sterols with a cyclopropane ring or where B-ring saturation could not be determined (NA) than other families.

samples where ergosterol occurred at $> 0.75\%$, nine were *Ranunculus* spp. On average, sampled taxa had 62 sterols in their pollen. The smallest range of sterols (35) was recorded in the pollen of creeping cinquefoil (*Potentilla reptans* L.), while the largest range was recorded in black alder (*Alnus glutinosa* Gaertn.), which contained 76 sterols. Species also showed a wide variation in sterol diversity as calculated by Simpson's diversity index. The most diverse species was *Papaver cambricum* L. (index = 0.913) and the least was *Torminalis glaberrima* (Gand.) Sennikov & Kurtto (index = 0.169) (Fig. S1; Table S8).

Typically, the pollen sterol profile was dominated by one to three major sterols (Fig. S3). β -Sitosterol and isofucosterol were the most common major sterol components (defined as ranking in top 3 by proportion of any given species) in over 100 taxonomic units (Table S9). Some plant species had a clear dominant sterol. Schottenol, 24-methylenecholesterol, desmosterol, cycloartenol and isofucosterol all occurred at $> 75\%$ in at least one taxonomic unit (Table S6). In total, 29 species of the 261 which could be placed on our phylogeny had a sterol that accounted for $\geq 65\%$ of their total. In 11 out of 29 species, this sterol was 24-methylenecholesterol.

Of the 69 sterols which occurred at $> 1\%$ in pollen, 31 showed a significant phylogenetic signal for both metrics tested (K and λ), along with total sterol content (mg kg^{-1}) (Table S11). Furthermore, when sterols were grouped according to number of carbons (CC: 27–31) and B-ring double bond substitutions (DB: CPR, 0, 5, 7, 8, NA (level 2 annotated sterol structures and ergosterol ($\Delta 5\&7$))), most groups showed significant phylogenetic signal for both metrics (K and λ) (27, 28, 29, 30 (CC) and CPR, 0, 5, 7, NA (DB)). Twenty-six additional sterols showed significant phylogenetic signal for a single metric (all Pagel's λ except salisterol). The 31 carbon group and $\Delta 8$ group were also significant for Pagel's λ .

Phylogeny predicts profile: conservation of sterol biosynthesis at subfamily, genus and species levels

There was a strong influence of phylogenetic group on the pollen sterolome, even at the subfamily level. The sterol profiles of all three Asteraceae subfamilies differed significantly (PERMANOVA: $F_{2,49} = 10.234$, $P = 0.001$, $R^2 = 0.295$, all pairwise

comparisons $P = 0.001$, comparison of variance: $F_{(2,49)} = 8.003$, $P < 0.001$) as shown in Fig. 3(a) (NMDS plot stress value: 0.166). Our analysis indicated that each subfamily was associated with at least six level 2 annotated sterols. For example, the Carduoideae had 15 of these putatively identified sterols that distinguished it from the Asteroideae and the Cichorioideae (Table S10) indicating there is still much we do not know about sterol production in plants and especially in the Asteraceae.

Overall and when compared to other families, Asteraceae pollen had a highly distinctive sterolome (Fig. 3b). There were also significant differences between Asteraceae subfamilies and other pollen in all sterol double bond classes (CPR: $H(3) = 49.6$, $P < 0.001$, $\Delta 0$: $H(3) = 78.6$, $P < 0.001$, $\Delta 5$: $H(3) = 55.8$, $P < 0.001$, $\Delta 7$: $H(3) = 17.0$, $P < 0.001$, $\Delta 8$: $H(3) = 14.2$, $P < 0.005$, ΔNA : $H(3) = 41.6$, $P < 0.001$). All Asteraceae subfamilies had significantly higher proportions of sterols with a B-ring cyclopropane substitution (CPR), undetermined B-ring structures (ΔNA – Level 2 annotated sterols) and lower proportions of $\Delta 5$ sterols than all other families (*post hoc* Dunn tests: CPR: $P < 0.005$, $\Delta 0$: $P < 0.001$, $\Delta 5$: $P < 0.005$, ΔNA : $P < 0.001$). Furthermore, Carduoideae had significantly higher $\Delta 7$ sterols than all non-Asteraceae families ($P < 0.001$) and significantly lower $\Delta 8$ than all other groups (other families: $P < 0.010$, Asteroideae: $P < 0.005$, Cichorioideae: $P < 0.005$).

Comparing between genera of the same family, we found significant differences in sterolome, demonstrating that species within a genus often display similar sterolomes (Fig. 4a). In Asteraceae, *Centaurea* and *Cirsium* both belong to the subfamily Carduoideae and have similar sterolomes, whilst *Crepis* belongs to the Cichorioideae. However, genera were still significantly different from each other (PERMANOVA: Genus: $F_{2,20} = 26.087$, $P = 0.001$, Species: $F_{9,20} = 2.311$, $P < 0.010$. Variance: Genus: $F_{2,29} = 0.810$, $P > 0.100$, Species: $F_{11,20} = 1.543$, $P > 0.100$). All pairwise differences between genera were significantly different (all $P = 0.001$) with genus and species identity together explaining a high proportion of the variance in the dataset (R^2 : genus = 0.561, species = 0.224). All genera were associated with at least five level two annotated sterols, affirming findings above about the undetermined sterol composition of Asteraceae flowers (Table S12). This pattern was repeated in the three Fabaceae genera *Trifolium*, *Ulex* and *Vicia* (PERMANOVA: Genus:

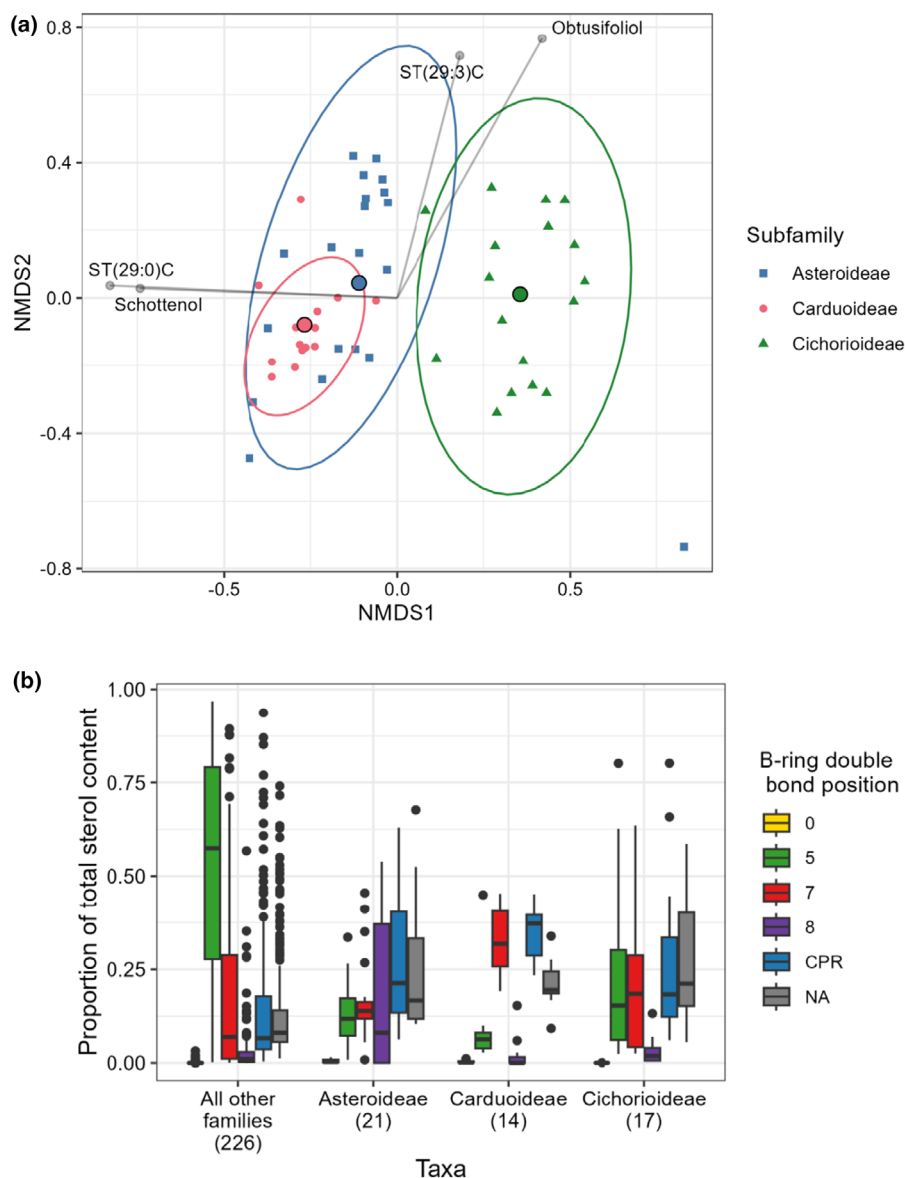


Fig. 3 Several sterols found in the Asteraceae are uncommon and unique to this plant family. (a) NMDS biplot showing differences in sterol profile between Asteraceae subfamilies Asteroidae, Carduoideae and Cichorioideae. All pairwise differences between groups were significant (PERMANOVA, $P < 0.005$) and each subfamily was associated with at least six level two annotated sterols which distinguished them from other Asteraceae species (ISA, Supporting Information Table S10). Plot stress value was 0.166 and groups showed significantly different variances with Carduoideae species showing the smallest variation in sterolome. Asteroidae and Carduoideae species also showed the greatest overlap in sterolome. (b) Comparison of sterol composition between Asteraceae subfamilies (Asteroidae (21 spp.), Carduoideae (14 spp.) and Cichorioideae (17 spp.)) and all other species (226 spp.) when sterols are grouped by B-ring saturation (left to right: $\Delta 0$, $\Delta 5$, $\Delta 7$, $\Delta 8$, cyclopropane ring (CPR), NA, example structures shown in Fig. 1). On average, non-Asteraceae species were dominated by $\Delta 5$ sterols. By contrast, Asteraceae flowers had higher levels of CPR, $\Delta 7$ and $\Delta 8$ sterols. Within Asteraceae, Carduoideae had a dominance of $\Delta 7$ sterols and Asteroidae showed a higher proportion of $\Delta 8$ sterols. The plot shows the first and third quartile, median and 1.5 $\times$ interquartile range.

$F_{2,22} = 28.756$, $P = 0.001$, Species: $F_{8,22} = 2.567$, $P = 0.001$. Variance: Genus: $F_{2,30} = 1.212$, $P > 0.100$, Species: $F_{10,22} = 2.423$, $P < 0.05$, Pairwise differences: all $P < 0.005$, R^2 : Genus = 0.575, Species = 0.205). The strongest associations between genera and sterols were *Trifolium* with schottenol and *Ulex* with sitostanol. In the Rosaceae, strong differences were recorded between genera, even within the same subfamily; however, species-level differences within genera were not statistically significant, despite *Potentilla* and *Rubus* both belonging to the same subfamily Rosoideae (PERMANOVA: Genus: $F_{2,17} = 16.695$, $P = 0.001$, $R^2 = 0.632$, Species: $F_{6,17} = 0.414$, $P > 0.500$, $R^2 = 0.047$. Variance: Genus: $F_{2,23} = 1.183$, $P > 0.100$, Species: $F_{8,17} = 0.417$, $P > 0.500$). Pairwise comparisons: *Prunus*: *Potentilla* ($P < 0.005$), *Prunus*: *Rubus* ($P < 0.005$), *Potentilla*: *Rubus* ($P < 0.050$). All three genera were associated with a high number of level two annotated sterols, but the strongest association was *Rubus* with desmosterol.

Furthermore, we examined variation in the sterol profile of individual species collected in different sites across the United Kingdom. Intraspecies variation between sites was not greater than the interspecies variation in the dataset. Species displayed significantly different sterol profiles (PERMANOVA, $F_{6,39} = 33.757$, $P = 0.001$, all pairwise comparisons between species $P < 0.050$), with species identity explaining a large proportion of the variance in the data ($R^2 = 0.839$). By contrast, the sterol profile of pollen collected from the same species in different locations across the United Kingdom exhibited relatively little variation (Fig. 4b,c (stress value = 0.096)). Interestingly, the amount of variation in the sterol profile within species was not significantly different ($F_{6,39} = 0.600$, $P > 0.500$). The sea aster, *Tripolium pannonicum* (Jacq.) Dobrocz, was significantly associated with the most individual sterols (14), including a very strong association with ST(27:1)B. The meadow buttercup, *Ranunculus acris*, had a strong association with ergosterol. Since this sterol is produced

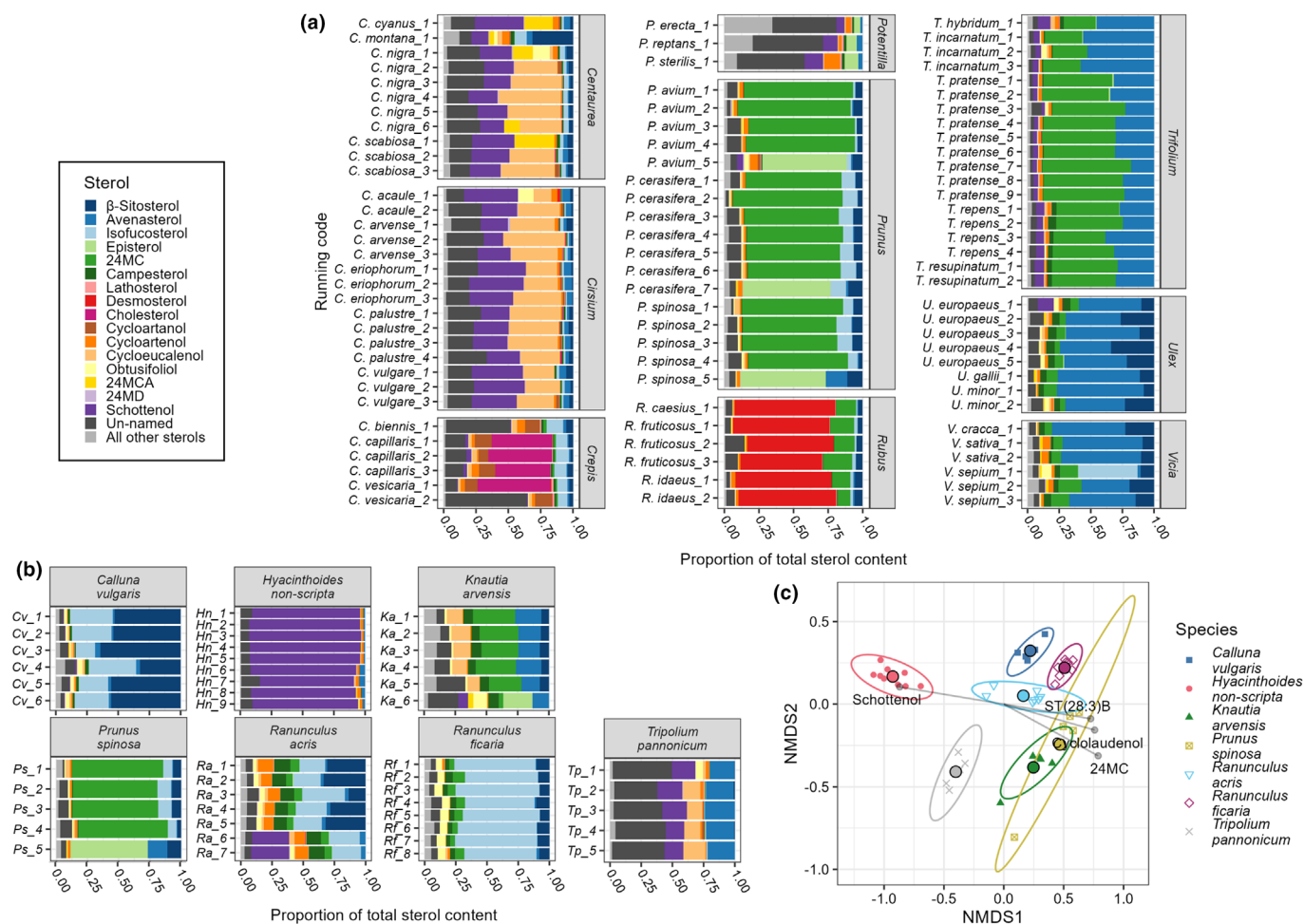


Fig. 4 Sterol profiles are phylogenetically conserved in several plant genera. (a) Sterol composition of species belonging to repeatedly sampled genera in the most sampled families; Asteraceae, Fabaceae and Rosaceae. The magnitude of intergenus differences varied widely; some confamilial genera share similar sterolomes (*Vicia* and *Ulex*, *Centaurea* and *Cirsium*) whilst others are dominated by different sterols (*Rubus* and *Prunus*, *Crepis* and *Cirsium*). Within genera, sterolomes appeared largely consistent between species. All non-major sterols have been collapsed into 'All other sterols' and all sterols annotated to level two (carbon count and double bond saturation) are included as 'Un-named'. Sterols are coloured left-to-right following figure legend bottom-to-top. (b) Sterol composition of species collected from at least four counties and with at least five samples total. *Tripolium pannonicum* shows the highest proportion of sterols which could only be annotated to level two (carbon count and double bond saturation) suggesting there is still much we do not know about sterol production in this species. Species generally show a strong consistency in sterolome, this is reflected in tight group distributions when plotted by NMDS (c). All non-major sterols have been collapsed into 'All other sterols' and all sterols annotated to level two are included as 'Un-named'. Sterols are coloured left-to-right following figure legend bottom-to-top. (c) NMDS plot of proportion data from samples shown in (b) arcsine square root transformed. Difference among species groups were significant ($P = 0.001$) and variance was not significantly different ($P = 0.729$). This suggests there is consistency in sterol profile between variable abiotic conditions which aligns with a strong genetic control of sterol production, as indicated by the phylogenetic signal across the data. Lines show associations with different sterols, only sterols with significant ($P < 0.0100$ and strong > 0.7) associations were used. Stress value of NMDS was 0.096.

exclusively by fungi, it suggests buttercups are more likely to be contaminated with fungi. Species that collect pollen from this species are therefore likely to be provisioning significant quantities of fungi to their brood. The common bluebell, *Hyacinthoides non-scripta* (L.) Chouard ex Rothm., was strongly associated with schottenol, the lesser celandine, *Ficaria verna* (Huds.) (formerly *Ranunculus ficaria*.) with isofucosterol, blackthorn (*Prunus spinosa* L.) with 24-methylenecholesterol and heather (*Calluna vulgaris* (L.) Hull) with sitostanol, β -sitosterol and stigmasterol. A full list of all 45 associations found can be seen in Table S13.

Comparison of sterols found in honeybees and pollen

The honeybee sterol profile from Svoboda *et al.* (1980) was compared to the sterol profile of all hand-collected pollen species in this dataset (Fig. 5). Bee sterols differed significantly from pollen in sterol profile (PERMANOVA: $F_{1,294} = 10.567$, $P = 0.001$, $R^2 = 0.035$) and in variance in sterol profile ($F_{1,294} = 98.678$, $P < 0.001$). The pollen sterolomes that were most like honeybees were replotted from the full NMDS (stress = 0.101) in Fig. 5(a) and their sterol profiles shown in Fig. 5(b).

We used data from Svoboda *et al.*'s (1980) analysis of prepupal honeybees to estimate the sterols required by bee species, although the sterol requirements of other bee species may differ from this. While we recognise that this may not be a universal

measure of honeybee sterol requirements, more recent work suggests this is close (Moore *et al.*, 2025). From these data, we estimated that bees required > 30% of 24-methylenecholesterol, > 19% of β -sitosterol, > 10% of isofucosterol, > 5% of

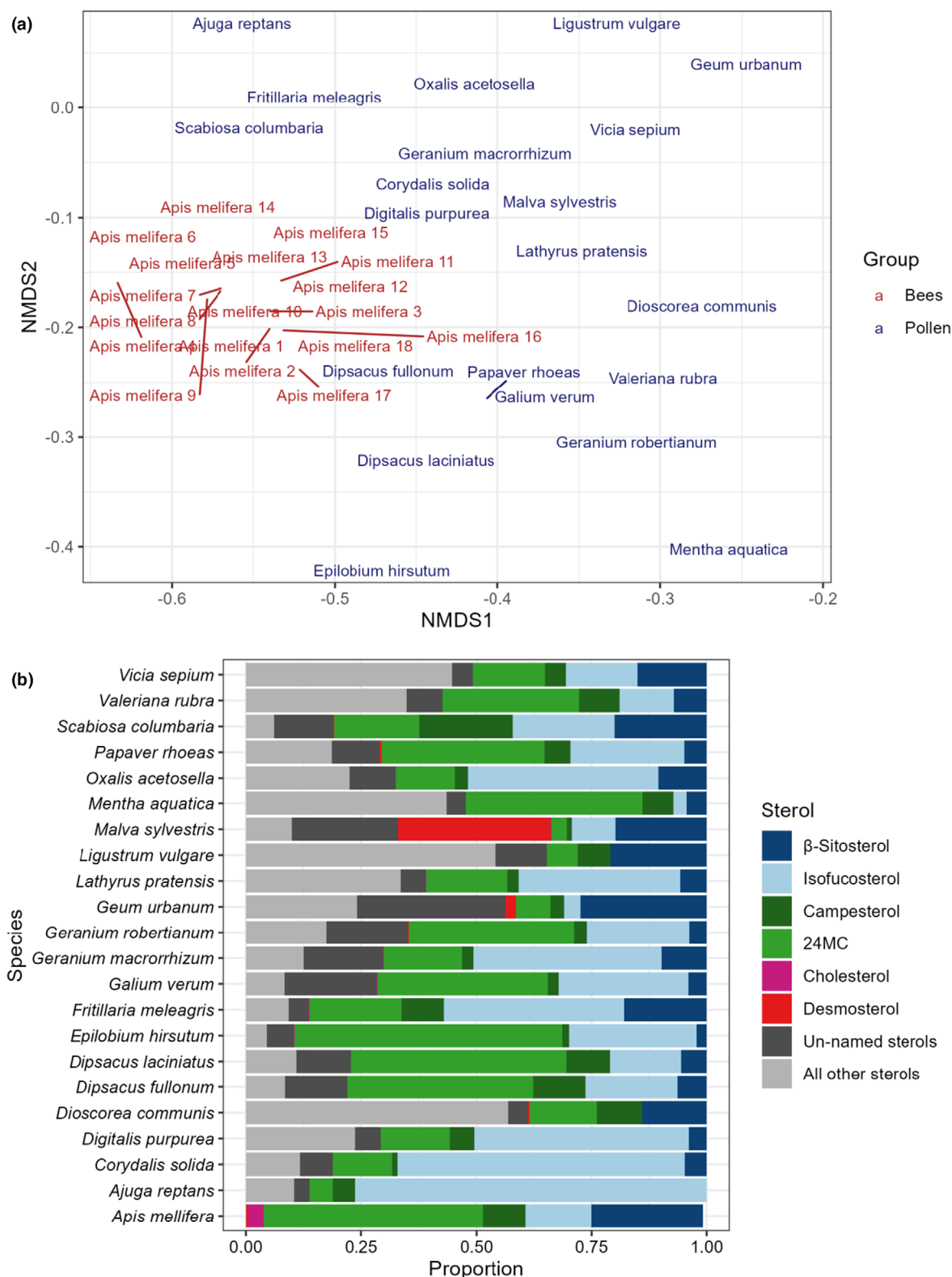


Fig. 5 Sterol profiles of bee pollinators are unlikely to match the sterol profiles found in pollen. (a) Subsection of an NMDS comparing 18 honeybee prepupae sterol samples from Svoboda *et al.* (1980) with all hand collected pollen units reported in this paper (278). Bees and pollen showed significantly different sterol profiles and the stress value of the 3D NMDS was 0.101. Only those pollens closest to the bees in NMDS space are plotted here in 2D for clarity. Many species that are popular with honeybees, for instance *Rubus fruticosus*, did not cluster closely with honeybees suggesting that similarity in sterol profile alone does not motivate foraging by honeybees. Pollens showed a much wider variation in sterolome than the bees though this is to be expected as they cover a much wider ecological and taxonomic range. Pollen also contains a much wider array of sterols compared to honeybees and so no pollen is likely to directly resemble honeybee tissues. (b) Proportions of honeybee-relevant sterols for all plant species shown in (a) plus a mean of the 18 honeybee prepupae sterol samples from (Svoboda *et al.*, 1980). All pollens contained high proportions of 24-methylenecholesterol, β -sitosterol and isofucosterol. However, many pollens contained more isofucosterol and less 24-methylenecholesterol and β -sitosterol than bees. In addition, honeybee prepupae contained higher proportions of cholesterol than any of the similar pollen. It is again clear that no individual pollen will provide all the sterols required by honeybees in their requisite proportions. Sterols in this figure are coloured left-to-right following the figure legend bottom-to-top.

campesterol, $> 0.5\%$ of cholesterol and $> 0.5\%$ of desmosterol. Using these thresholds, we found that 88 species did not meet any of the sterol requirements of honeybees (Fig. 6a) and no single species met the required proportions of all six sterols, even though 188 species contained all six sterols. Of these sterols, 24-methylenecholesterol and isofucosterol have been reported to be especially important (Svoboda *et al.*, 1980; Bogaert *et al.*, 2025; Moore *et al.*, 2025) yet only 35 out of 278 taxonomic units contained sufficient 24-methylenecholesterol to meet this threshold (Fig. 6a). Out of these 35, only 12 also contained enough isofucosterol and none of these 35 contained enough β -sitosterol. Other species were sufficient in individual bee-relevant sterols. For example, 82 species contained enough β -sitosterol and 90 contained enough isofucosterol; 48 contained enough of both compounds. Likewise, 62 contained enough campesterol. The fewest species contained sufficient cholesterol (21) or desmosterol (28).

The highest proportions of the honeybee relevant sterols were often found in specific plant families as shown in Fig. 6(b) (24-methylenecholesterol = Rosaceae, campesterol = Apiaceae, cholesterol = Asteraceae subfamily Cichorioideae). Interestingly, the highest content of desmosterol was from diverse flowers popular with honeybees and bumblebees such as *Rubus* spp., *Rosa* spp., *Borago officinalis* L. and *Symphytum* sp. β -sitosterol was not recorded more frequently by any family and three of the species in which this sterol was most abundant are wind pollinated trees (*Carpinus betulus* L., *Corylus avellana* L. and *Quercus robur* L.).

Discussion

This dataset provides a major contribution to our knowledge of pollinator nutrition at a scale never previously undertaken and informs our understanding of the evolution of pollen sterol pathways across angiosperms. We also show that abiotic factors do not significantly influence sterol profiles and that instead they are under evolutionary constraints. This concurs with earlier work that suggested mean annual temperature correlated with total pollen sterol content (Zu *et al.*, 2021) and may be an adaptation to mitigate membrane heat stress (Narayanan *et al.*, 2016).

Sterols are needed for pollen tube elongation. However, these sterols can be synthesised *de novo* from squalene during pollen tube growth (Villette *et al.*, 2015). It is possible that storage of sterols in pollen grains would make them a target for insect herbivores, which could have a dramatic negative effect on plant

fitness. The high diversity of uncommon sterols in pollen could therefore be a mechanism for the plant to escape palynivory, as a diverse sterolome could hinder herbivore adaptation to targeted efficient sterol conversion pathways. It is possible that such sterols could either be unusable or even toxic to palynivores. Considering the mixture of sterols we have found in pollen, it is worth noting that Behmer (2017) explores how the ratio of what they describe as good to bad sterols impacts insect performance, but this can be mitigated through diet mixing (Behmer & Elias, 2000). During their evolution, palynivores including bees may have adapted to be able to use the diverse range of pollen sterols to overcome this defence. Previous studies have hypothesized that bees had lost the genes for the dealkylation of longer chain sterols to cholesterol and instead adapted a specific subset of pollen sterols for their use (Svoboda *et al.*, 1983). This would also allow bees to use several different sterols flexibly rather than relying on obtaining a single sterol abundant enough for them to convert efficiently. Overcoming such a defence would also enable bees to use a valuable nutritional resource unavailable to other palynivores.

The sterols most abundant in honeybees (24-methylenecholesterol, β -sitosterol and isofucosterol) are also commonly abundant in pollen sterolomes. The structural properties of these sterols (C28: Δ 5, C29: Δ 5 and C29: Δ 5 respectively) may be useful in bee cell membranes, influencing properties such as fluidity (Furse *et al.*, 2023). The dominance of these nutrients in honeybees may represent an adaptation of bees to use the sterols available in the widest range of pollens. It is interesting that only 29 out of 261 species in the phylogeny had a sterol that accounted for $\geq 65\%$ of total sterols. Ten out of 29 of these species were Rosaceae, with the dominant sterol being 24-methylenecholesterol or desmosterol. In fact, 24-methylenecholesterol was the most common sterol that accounted for $\geq 65\%$ of total sterols (11 species).

It is conceivable that co-evolution of plants and bees has been driven in part by the specific sterols in pollen to encourage bee visitation and support bee populations; although this is more likely the case in specialist bees (Vanderplanck *et al.*, 2017). However, the six sterols required by honeybees (24-methylenecholesterol, β -sitosterol, isofucosterol, campesterol, cholesterol and desmosterol) (Moore *et al.*, 2025) were not recorded in the pollen of any one single plant species in the same proportions recorded in bees. While floral constancy presents benefits to plants of pollen being delivered more efficiently and

effectively to conspecifics (Grüter & Ratnieks, 2011), it is also conceivable that producing sterols in quantities that are inadequate for palynivores could potentially prevent overharvesting of

pollen from one source and instead encourage a generalist foraging strategy. The miss-match between bee nutritional needs and pollen chemistry (especially among crops such as *Malus* spp.)

(a)

Honeybee sterol requirements met	No. of species	Honeybee sterol requirements met	No. of species
None	88	Isofucosterol, Campesterol	5
Sitosterol, Isofucosterol, Campesterol	24	Isofucosterol, Cholesterol	4
Desmosterol	22	24MC, Desmosterol	3
Isofucosterol	21	24MC, Isofucosterol, Campesterol	3
Sitosterol, Isofucosterol	20	Sitosterol, Isofucosterol, Cholesterol	3
24MC	18	24MC, Campesterol	2
Sitosterol, Campesterol	16	Sitosterol, Cholesterol	2
Sitosterol	14	Sitosterol, Desmosterol	2
Campesterol	10	24MC, Isofucosterol, Cholesterol	1
Cholesterol	10	Campesterol, Desmosterol	1
24MC, Isofucosterol	8	Sitosterol, Isofucosterol, Campesterol, Cholesterol	1

(b)

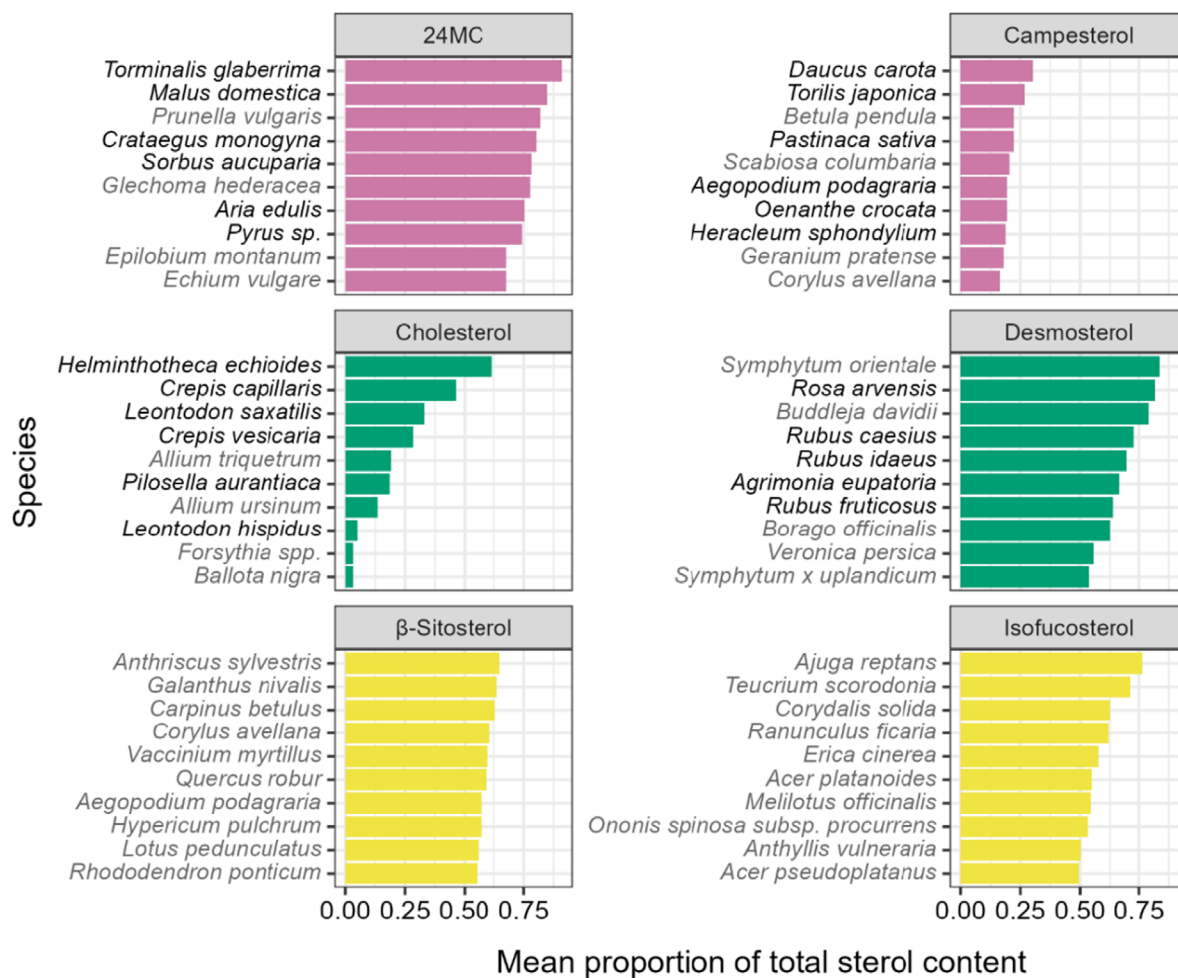


Fig. 6 Most plant sterol profiles do not supply all of the sterols required by honeybees. (a) Summary of how many pollen species met the sterol requirements identified from analysis of prepupal honeybee tissues (24-methylenecholesterol > 30%, β -sitosterol > 19%, isofucosterol > 10%, campesterol > 5%, cholesterol > 0.5%, desmosterol > 0.5% – Svoboda *et al.* (1980)). Out of 278 species, 88 met none of these requirements, only 32 contained three or more at the required levels. A single species, *Ballota nigra*, fulfilled four of the six required (β -sitosterol, isofucosterol, campesterol and cholesterol). (b) The 10 highest producing species for the six sterols detected in honeybee tissues (Svoboda *et al.*, 1980). Campesterol and cholesterol are only ever present in lower proportions and are never dominant in pollen, unlike 24-methylenecholesterol, desmosterol and isofucosterol, which are over 75% of total sterols in the highest producing species. Similarities in sterol production between related species mean many of the top species for a sterol belong to a single family. Dominant families are highlighted by species names in black. 24-Methylenecholesterol: Rosaceae, Campesterol: Apiaceae, Cholesterol: Asteraceae, Desmosterol: Rosaceae. Sterols are coloured according to their positioning in the plant sterol synthesis pathway in Fig. 1.

may help explain the effectiveness of agri-environmental floral plantings in improving crop pollination. It is possible that where sterol requirements are known, such plantings might be specifically selected for complementary nutritional properties.

It is plausible that sterol diversity in honeybees is a result of their nutritional flexibility and diet mixing achieved through foraging from a range of plant species. Honeybee colonies develop for longer if their diet is supplemented with key $\Delta 5$ sterols including 24-methylenecholesterol and isofucosterol (Bogaert *et al.*, 2025; Moore *et al.*, 2025) while the sterolome of larvae on sterol supplemented diets matched that of honeybees in naturally provisioned hives, indicating that honeybees selectively transfer only the most biologically important sterols to their young. However, the pollen sterolome also contains a range of sterols that are absent from the tissues of honeybees, such as obtusifoliol, schottenol and cycloeucalenol. While these sterols may serve key physiological functions for plants, unlike $\Delta 5$ sterols, they may be unsuitable as replacements for cholesterol in insect tissues (e.g., sterols with B-ring cyclopropane substitutions or $\Delta 8$ double bond which are generally not suitable for insects (Chen *et al.*, 2024)). The nutritional role of sterols in wild bees is under studied. However, red mason bees (*Osmia bicornis* L.) provided pollen that contained or was supplemented with sterols, including campesterol, had increased growth rates, and there is some evidence for selective uptake of specific sterols by larvae (Martel *et al.*, 2025).

The 'Asteraceae paradox' refers to the frequent use of Asteraceae pollen by specialists and its infrequent collection by generalists (Müller & Kuhlmann, 2008). It is thought to be the result of chemical defences and nutritional deficiencies (Praz *et al.*, 2008; Vanderplanck *et al.*, 2020a). Our results showed that Asteraceae pollen contained lower proportions of $\Delta 5$ sterols such as 24-methylenecholesterol, β -sitosterol, isofucosterol, desmosterol and campesterol – typically favoured by honeybees, instead producing higher quantities of $\Delta 7$, $\Delta 8$, un-named (L2) or those sterols with B-ring cyclopropane substitutions. This supports the hypothesis that Asteraceae pollen is nutritionally less suitable for species of bees that typically forage for pollen which contains high levels of $\Delta 5$ sterols such as honeybees. Furthermore, sterols with B-ring cyclopropane substitutions occur early in the sterol synthesis pathway and are therefore energetically less costly to produce than sterols which require additional metabolic steps. The Asteraceae sterolome may therefore represent a strategy to escape palynivory by producing un-useable sterols which further benefit the flower by acting as precursors for the synthesis of downstream sterols during pollen tube growth. Additionally, the low

proportions of $\Delta 5$ sterols in Asteraceae flowers suggest the sterol requirements of Asteraceae-specialist solitary bees may have evolved different phytosterol use to that of common eusocial species (e.g., *Apis* spp. and *Bombus* spp.) and provide more support to the nutritional deficit argument of the Asteraceae paradox.

The variation in both sterol profile and total sterol content between species leads to a stark difference in sterol availability between flowering plants. When considering the availability of pollen nutrients in floral seed mixes, it is important to consider their accessibility to different pollinators through floral morphology and phenology. The strong phylogenetic signal and within-genus consistency in sterol profile presented here invite further investigation into the mechanisms regulating sterol production in pollen from an evolutionary perspective. An assessment of stigma-ovule distance in the study species may prove enlightening for the trends in total sterol production.

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Competing interests

None declared.

Author contributions

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

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Data availability

All the source data used for this publication can be found in Tables S1–S13. Raw chemical data is available at Sterols from the pollen of British plant species, Knowledge Network for Biocomplexity doi: [10.5063/F14B2ZSS](https://doi.org/10.5063/F14B2ZSS).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Sterol compositions of the species with the highest (left) and lowest (right) Simpson's diversity index as shown in Table S8.

Fig. S2 Total sterol production ranged greatly within species sample; graph shows the range in total sterol content across families containing at least three genera.

Fig. S3 Median percentage and interquartile range of the most dominant 18 sterol components within each taxonomic unit, ranked by proportion in the sample.

Fig. S4 Labelled phylogeny shown in Fig. 2 of main text, covering 261 species adapted from (Zanne *et al.*, 2014) and families with three or more species are highlighted in a clockwise direction.

Table S1 Collection details for all pollen samples: multi-site samples show where multiple under-weight samples were combined to reach a minimum analysis weight of 10 mg.

Table S2 Retention time, mass to charge ratio and structure description of sterols: double bond equivalents used in Quality Assurance mixture.

Table S3 Summary of carbon number and double bond positions of sterols identified using reference materials (full structure and formulae for each sterol, except ergosterol, is shown in Fig. 1).

Table S4 Species with the highest and lowest total sterol content calculated as species means.

Table S5 Mean, SD, maximum and minimum total sterol production of species collected from at least three counties.

Table S6 Maximum, minimum, median and interquartile range percentage of sterols which occurred at > 40% in at least one species.

Table S7 Maximum, minimum, median and interquartile range of sterols which were present in all taxonomic units analysed.

Table S8 Highest and lowest Simpson's diversity index values across all pollen species. Highest values are shown on the left side of the table and lowest values are on the right.

Table S9 Sterols most commonly dominant in pollen, defined as occurring within the top 3 sterols by proportion.

Table S10 Summary of indicator species analysis (ISA) results comparing the sterol profiles of all three Asteraceae subfamilies sampled (Asteroideae, Cichorioideae and Carduoideae, Fig. 3).

Table S11 Results of phylogenetic signal analysis on the proportions of all detected sterols with a maximum proportion > 1% (69), total sterols (mg kg^{-1}), proportion of different carbon length sterols (light grey: 27, 28, 29, 30 and 31) and proportion of different B-ring saturated sterols (dark grey: cyclopropane ring (CPR), $\Delta 0$, $\Delta 5$, $\Delta 7$, $\Delta 8$ and ΔNA).

Table S12 Summary of indicator species analysis (ISA) results on pollen sterol data comparing genera within families (Fig. 4a).

Table S13 Summary of indicator species analysis (ISA) results on pollen sterol data for the interspecies comparison shown in Fig. 4 (b,c).

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