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All the source data used for this publication can be found in the Supporting Information Tables. Chemical data is available at

https://knb.ecoinformatics.org/view/doi:10.5063/F14B2ZSS â€˜Sterols from the pollen of British plant speciesâ€™

Pollen sterols are highly diverse but phylogenetically conserved.

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Summary

1. Phytosterols regulate cell membrane fluidity and are precursors for plant hormones and secondary metabolites in plants. Insects are auxotrophic for sterols so consume phytosterols and dealkylate them to cholesterol. Some insects including bees which rely on dietary sources, primarily pollen, for sterols cannot modify them therefore have adapted to use them unmodified as they occur in pollen.

2. 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.

3. 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 Δ5 sterols including β-sitosterol, 24-methylenecholesterol and isofucosterol, which are important sterols for bees. The sterols recorded in honey bees 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.

4. 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.

Key Words: phytosterol, plant lipids, phylogeny, pollen nutrients, pollinator guild.

Introduction

31 Sterols are lipids which serve as essential cell membrane components. In vertebrates, cholesterol is

32 dominant whereas fungi primarily use ergosterol. Plants, however, produce a wide range of sterols

33 with over 250 reported (Valitova, Sulkarnayeva and Minibayeva, 2016; Nes, 2011). While the most

34 common sterols in vegetative tissues are β-sitosterol and stigmasterol (Behmer and Nes, 2003;

35 Dufourc, 2008) pollen contains a more diverse range of sterols (Zu *et al.*, 2021). Some pollen, for

36 example, has no β-sitosterol or stigmasterol but instead contains other sterols such as 24-

37 methylenecholesterol (Vanderplanck *et al.*, 2014; Villette *et al.*, 2015; Moerman *et al.*, 2017;

38 Vanderplanck, Zerck, *et al.*, 2020; Zu *et al.*, 2021; Furse, Martel, *et al.*, 2023). The extent to which the

39 sterol profile (sterolome) of angiosperm pollen varies and the adaptive function for this variation

40 remains largely unknown.

41 Pollen sterols facilitate fertilization of the ovule by providing substrates for pollen tube elongation

42 (Rotsch *et al.*, 2017; Stroppa *et al.*, 2023). Sterols are especially important for rapid cell elongation

43 and cell polarisation during pollen tube growth (Rotsch *et al.*, 2017). However, the sterols used for

44 pollen tubes are not those occurring in mature pollen grains (Rotsch *et al.*, 2017) but are created *de*

45 *novo* during pollen tube growth (Rotsch *et al.*, 2017; Stroppa *et al.*, 2023). Since sterols are also

46 metabolically expensive (Nes, 2011) and in the absence of an established explanation of their

47 function, it is surprising that they accumulate in pollen with such diversity.

48 Pollen nutrients including lipids may influence foraging behaviour of bees (Vaudo, Dyer and Leonard,

49 2024). Like other insects bees cannot synthesize sterols *de novo* but instead acquire them along with

50 protein, fats and micronutrients from their diets (Wright, Nicolson and Shafir, 2018). However, unlike

51 other phytophagous insects, bees cannot convert dietary sterols to cholesterol (Behmer and Nes,

52 2003). Instead, they incorporate specific pollen sterols directly into their tissues without modifying

53 them (Svoboda *et al.*, 1986). The sterol profile of the few bees that have been studied (honeybees,

54 leaf-cutting bees) is mainly composed of 24-methylenecholesterol, isofucosterol, β-sitosterol,

55 campesterol, desmosterol and cholesterol (see Svoboda and Feldlaufer, 1991, Moore et al., 2025). The

56 use of pollen-specific sterols in bee tissues may be an adaptation to pollinivory (Svoboda, Feldlaufer

57 and Weirich, 1994) but would be an evolutionary risk if pollen that bees collected did not contain all

58 the required sterols.

59 Anthropogenic change to flowering landscapes throughout the world has significantly altered floral

60 resource availability for pollinators (Howard *et al.*, 2003; Vanbergen and Insect Pollinators Initiative,

61 2013; Baude *et al.*, 2016), increasing the likelihood of nutritional deficiencies. An understanding of

62 pollen sterol variation could inform predictions about nutritional stress in bees. To measure sterol

63 production in pollen, we measured the diversity and abundance of sterols in 295 UK wildflower taxa

64 using a sterol analysis. We found that sterol profiles were remarkably stable within taxonomic groups,

65 including subfamilies of Asteraceae and Rosaceae. Sterolomes did not vary at different sites and a

66 significant phylogenetic signal for almost half of the sterols was recorded. However, none of the

67 pollen sterolomes matched those reported from honeybees.

Methods

68

Species selection

69

70 In total, 700 pollen samples were collected for sterol analysis representing 295 taxonomic units, 275

71 identified to species level, and 125 of which were represented by a single sample. A full summary of

72 samples collected, dates and location are provided in Table S1. Samples included pollen from 172

73 species selected from the species list compiled by (Baude et al., 2016) which targeted plants from the

74 Countryside Survey in Great Britain that were rewarding to pollinators and considered sufficiently

75 common to contribute to landscape level floral resources. During collection visits for the above target

76 species, 88 plants which produced easily extractable pollen or would contribute to wider taxonomic

77 coverage in the dataset were also opportunistically collected. Asteraceae flowers produce copious and

78 easily collectable pollen and previous work has suggested nutritional deficiencies within Asteraceae

79 pollen (Vanderplanck, Gilles, *et al.*, 2020). Therefore, Asteraceae flowers were also targeted

80 opportunistically adding a further 18 species while 17 more species were targeted due to their

81 importance as forage plants for wild bee species in the UK (such as *Bryonia cretica* for *Andrena*

82 *florea*, *Reseda* spp. for *Hylaeus signatus* and *Anthyllis vulneraria* for bumble bees).

Pollen collection

83

84 Samples were collected from a range of sites in England including nature reserves, botanic gardens,

85 public land and private gardens from spring to early autumn from 2021-2022. In total, samples were

86 collected from 15 counties. Collection months and counties for all samples are shown in Table S1.

87 Flowering heads, or branch tips in the case of woody species, were cut from the plant and placed in

88 sealed sandwich bags. These were then taken back to the laboratory and placed in a beaker of room

89 temperature tap water to dehisce. The duration needed for anthers to dehisce ranged from a few hours

90 to a few days, but most were left for 24 hours. Once pollen had been produced it was removed from

91 the flower using a clean paintbrush and forceps under a magnifying glass. Pollen was collected onto

92 an ethanol-wiped glass microscope slide and visually inspected for debris or insects which were

93 subsequently removed. The pollen sample was then scraped into a clean 1.5ml Eppendorf tube or

94 glass vial using an ethanol-wiped razor blade. The exact method of pollen extraction varied depending

95 on species and floral morphology. For daisy-type Asteraceae (Asteroideae), pollen was brushed from

96 the tops of disk florets in clumps. For thistle-type Asteraceae (Carduoideae), pollen was brushed from

97   anther tube tips and tubes squeezed gently to remove pollen further. For legume-type Fabaceae

98   flowers, wing petals were removed and the tip of the keel lightly squeezed, as for *Lotus corniculatus,*

99   or all petals removed entirely, as for *Trifolium pratense*. Pollen samples were then frozen at -20°C

100 before being moved to -80°C in batches. Freezing pollen has been shown to prevent nutritional

101 degradation (Hagedorn and Burger, 1968; Pernal and Currie, 2000).

102 To assess inter- and intra-species variation in sterol profile, a subset of species was collected from at

103 least three counties in England. Plant identification was confirmed by photos shared on iNaturalist and

104 with botanists at Royal Botanic Gardens (RBG), Kew. A total of 275 samples were identified to

105 species and subspecies level and 20 identified to genus level.

106 A minimum of 10 mg fresh weight pollen per sample was required due to the lower end of pollen total

107 sterol content being 0.1%, i.e., 10 µg of sterol in a 10 mg sample, a level within the detectable range

108 of our analysis. At the end of the collection season all samples were transferred to 1.5 ml glass vials

109 and partitioned or combined by species, depending on whether the sample was over or under 10 mg.

110 The final weight of each sample was between 9.5-16.5 mg.

111 ***Sample preparation***

112 Alongside the hand collected pollen samples, additional samples were used in the sample run to

113 monitor performance. Firstly, Quality Assurance (QA) samples contained a solution of reference

114 materials compiled to assign names to signals in our samples. This allowed us to assess the

115 consistency of analysis by the instrument across a large cohort of samples and calculate the coefficient

116 of variance. Reference materials verified by multi-nuclear NMR were 24-methylenecholesterol, 24-

117 methylenecycloartanol, anthelsterol (a sterol found in *C. anthelmintica*, ST(28:3), that is active under

118 330 nm UV but whose structure has not been determined formally using NMR (Furse, Martel, *et al.*,

119 2023), avenasterol, β-sitosterol, brassicasterol, campesterol, cholesterol, cycloartenol, cycloeucalenol,

120 cyclolaudenol, desmosterol, episterol, ergosterol, isofucosterol, schottenol, sitostanol, spinasterol and

121 stigmasterol. The *m/z* and retention time for each sterol is shown in Table S2. Aliquots of 40 µl were

122 used in the run. Secondly, a Quality Control (QC) stock solution was made by homogenizing a set of

123 commercially available honey bee collected pollens: 95 g of *Helianthus annus* (sunflower) pollen, 5 g

124 of *Nymphaea* sp. pollen, 20 g of *Fagopyrum esculentum* (buckwheat) pollen in 120 ml water. For this

125 project three QC concentrations were used: 100%, 50%, 25%, corresponding to 40 µl, 20 µl and 10 µl

126 aliquots of the QC stock solution. These were analysed in the same way as the samples to calculate

127 the correlation between analyte concentration and signal size. Variables whose ratio was <0.75 were

128 discarded. All runs began with a blank containing LCMS quality methanol only. This was designed to

129 enable identification and removal of background noise.

130 *Sterol extraction*

131 To each vial of hand collected pollen or QC sample a 20 µl aliquot of 0.1 mg/ml (5.08

132 nMoles/sample) d_7-cholesterol as the internal standard (IS) was added with 300 µl methanolic KOH

133 (10 g:90 ml) and the vial vortexed for 20 seconds. Vials were then placed in drilled aluminium

134 heating blocks at 75°C in a water bath for two hours. Saponification releases conjugated sterols

135 (Evtyugin *et al.*, 2023), such as sterol esters which are reported to be common in pollen (reviewed in

136 Ferrer *et al.*, 2017). The saponified material was then transferred to a corresponding deep 96-well

137 plate (96-well plate, Esslab Plate+™, 2.4 ml/well, glass-coated). Pollen samples were randomised

138 within plates and interspersed randomly with QA, QC and blank (300 µl methanol and 20 µl IS)

139 samples.

140 Extraction of the sterol fraction was carried out on the plate using a 96-channel pipette (liquid-

141 handling robot, Integra Viaflow 96/384 channel pipette) using DMT (dichloromethane (DCM) (3

142 parts), methanol (1 part) and triethylammonium chloride (0.0005 parts, i.e. 500 mg/l). To each well

143 500 µl of DMT (250x2) was added followed by a 500 µl aliquot of deionised water and the mixture

144 agitated using the 96-channel pipette. The plate was centrifuged for 2 min to separate layers

145 (methanol + water, solid, DMT + sterol). A 50 µl sample of extract (DMT & sterol) was transferred to

146 the 384-well plate and left for 30 min for the DMT to evaporate. After repeating this process four

147 times, 150 µl of LCMS grade methanol was added and the plate sealed with foil. A total of three 384-

148 well plates were run immediately in sequence, analysing 700 hand collected pollen samples and 281

149 analytical samples. One pollen sample was later excluded because it had experienced prolonged

150 exposure to room temperature conditions during collection.

151 *Sample analysis and data processing*

152 Mass spectra were recorded using a Thermo Scientific Orbitrap Fusion mass spectrometer, which was

153 equipped with an Atmospheric Pressure Chemical Ionization (APCI) probe and integrated with a

154 Thermo Scientific Vanquish liquid chromatography system (Thermo Scientific, UK). Metabolites

155 were separated prior to analysis on a Hypersil GOLD LCMS C18 column (50 × 2.1 mm with particle

156 size 1.9 µm) (Thermo Scientific, UK) which was maintained at 55 °C for analyses. An aliquot of 1 µL

157 was injected, with a mobile phase flow rate of 0.60 mL/min comprising a gradient of 84% methanol at

158 T = 0 to 100% methanol at T = 11 min and held at 100% till T = 16.4 min and then returned to 84%

159 methanol from 16.5 to 19 minutes. Mass spectrometric data were acquired in positive ionisation mode

160 via APCI, operating at a resolution of 120,000. The spray voltage was set to 2.86 kV, with sheath

161 nitrogen gas flows of 45 (sheath), 5 (auxiliary), and 1 (sweep) arbitrary units. The ion transfer tube

162 and vaporizer temperatures were maintained at 300 °C and 350 °C, respectively. The mass acquisition

163 range was calibrated to *m/z* 197–500, with the lower limit adjusted to detect the fluoranthene cation

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164   (*m/z* 202.077), which served as the internal mass calibration reference. Under these conditions, the

165   internal standard used for the samples, d_7-cholesterol, eluted at 5.6 minutes, with the primary ion

166   species identified as [M - H_2O + H] + at *m/z* 376.395 within a mass variance of less than 1 ppm. Both

167   samples and external standards were analysed in a single sequence. The pollen samples were

168   randomised among themselves, while the external standards were run sequentially, beginning with the

169   least concentrated standard. Instrument output was processed after Furse, Martel, *et al.* (2023). Data

170   files were processed using Analyzer PRO XD (SpectralWorks Ltd, Cheshire, UK). Signals were

171   extracted from 3 to 11 min in a mass range of 360–442 *m/z*, with an area threshold of 500 000,

172   smoothing of 15 arbitrary units, a width of 0.01 min, mass accuracy of 3 d. p. and a retention time

173   window of 0.1 min.

174   All compound signals were determined by their *m/z* and retention time (level 3 annotation) 78 of

175   which were further distinguished using the format ST (27:1)A where ST represents 'sterol', the first

176   number indicates number of carbons, the second number indicates number of double bonds and the

177   letter distinguishes sterols that differed but otherwise had a similar number of carbons and double

178   bonds (level 2 annotation). Compounds were assigned names (a level 1 annotation) where reference

179   materials that had been verified using multi-nuclear NMR were available as reference samples. These

180   included 24-methylenecholesterol, 24-methylenecycloartenol, agnosterol, anthelsterol, avenasterol, β-

181   sitosterol, brassicasterol, campesterol, cholesterol, cycloartenol, cycloeucalenol, cyclolaudenol,

182   desmosterol, episterol, ergosterol, isofucosterol, lophenol, schottenol, sitostanol, spinasterol, and

183   stigmasterol. Anthelsterol is a trivial name for a sterol that has not been determined using NMR

184   (Furse et al., 2023).

185   Instrument output reported mass to charge ratio (*m/z*), signal size and retention time. Signal size

186   output was first converted by dividing the signal area for each metabolite by the signal of internal

187   standard (d_7-cholesterol). These relative abundance values were used for the majority of the data

188   analysis. To generate a value of total sterol production in sterol/fresh weight of pollen in mg/kg the

189   following formula was used:

190 $$\frac{Sterol\ (mg)}{Pollen\ (kg)} = \frac{molecular\ mass\ of\ sterol\left(\frac{g}{mol}\right) \times \left(signal \times 5.08e^{-9}\ mols\right) \times 1,000,000}{Starting\ weight\ (mg)/1,000}$$

191   ***Data analysis***

192   All data analysis was done in R version 4.4.0 (2024-04-24 ucrt) (R Core Team, 2024) and R studio

193   (2024.9.1.394) (Posit team, 2024). A total of 78 sterols and related compounds were identified in the

194   sample set, and a total of 295 taxonomic units were analysed including those identified to genus,

195   species and subspecies level. Summary statistics were calculated using means of these 295 units with

196   some then removed for species level and phylogenetic analyses as detailed below.

197 Outliers were identified using disana() from the LABDSV package v.2.1-0 (Roberts, 2023) on a Bray-
198 Curtis dissimilarity matrix of proportion data from all samples. Any data point with a minimum
199 dissimilarity >0.5 compared to all other points in the data was identified as an outlier, as per author
200 guidance, and so removed. This led to the removal of one *Campanula trachelium* sample. Simpsons
201 diversity index was calculated using diversity(index="simpson") from the VEGAN package v.2.6-6.1
202 (Oksanen *et al.*, 2024) for all 295 taxonomic units to better understand differences in sterol diversity
203 between pollens.

204 NMDS were plotted using metaMDS(trymax=200, k=2, autotransform=FALSE). The lowest number
205 of axes was selected where stress was ≤0.2 (established cut off, (Bakker, 2024)). Stress values reflect
206 how well the ordination aligns with the original data. Proportion data (0-1) was used for analysis,
207 where zero denotes a sterol is absent from a sample, and one denotes it is completely dominant.
208 Proportions were arcsine square root transformed and converted into a Bray-Curtis dissimilarity
209 matrix using vegdist(method="bray"). Where NMDS stress value was <0.2 a 2D biplot as made using
210 GGPLOT2 v.3.5.1 (Wickham, 2016) and GGREPEL v.0.9.5. (Slowikowski, 2024) to show significant
211 ($p<0.010$) and strong (>0.7 absolute value) associations. PERMANOVA was calculated using
212 adonis2() to test for significant differences between groups. Betadisper() and anova() were used to
213 confirm non-significant difference in dispersion. Adonis2() was also used to test for pairwise
214 comparisons between groups following Bakker (2024), using a Benjamini-Hochberg correction which
215 corrects for multiple testing to reduce the chances of false positives. All functions were from VEGAN
216 (Oksanen *et al.*, 2024) except anova() from the STATS package (R Core Team, 2024). The STATS
217 package from R (R Core Team, 2024) was used to calculate summary statistics. Indicator Species
218 Analysis (ISA) was carried out on arcsine square root transformed data to accompany NMDS plots
219 using multipatt() from the INDICSPECIES package v.1.7.14. (De Cáceres and Legendre, 2009) to
220 determine significant associations between groups and individual sterols. ISA was done at the level of
221 individual groups only and not for combinations of groups.

222 ***Phylogeny construction and phylogenetic signal***

223 A phylogenetic tree of the study species was constructed based on the date calibrated and rooted tree
224 from Zanne *et al.* (2014). The tree was pruned for our study species using keep.tip() from the R
225 package APE v.5.8.(Paradis and Schliep, 2019). Any synonyms present in the tree were manually
226 renamed to match the POW taxonomy used in the project (19 species). In the case of species which
227 did not have a corresponding match on the Zanne phylogeny the following approach was taken:
228 Where a subspecies level match was not available, a species level branch was renamed instead (2
229 species). Where a hybrid species was not available, a matching parent not already in the dataset was
230 renamed (5 species). Where a species match was not available, another species from the same genus
231 was renamed instead (7 species), but only where there was not already a species correctly matched

232 within that genus. Out of 295 taxonomic units, 34 could not be suitably placed on the tree and were

233 removed from phylogenetic analysis.

234 Families were checked for monophyly using AssessMonophyly() from the R package MONOPHY

235 v.1.3.1.(Schwery and O'Meara, 2016). Phylogeny was plotted with ggtree() from GGTREE

236 v.3.12.0.(Yu *et al.*, 2017, 2018; Yu, 2020; Xu *et al.*, 2022). Heatmap and bar graph associated with

237 phylogeny were plotted using ggplot() from TIDYVERSE v.2.0.0.(Wickham *et al.*, 2019). The

238 composite graph was then plotted using APLOT v.0.2.2. (Yu, 2023).

239 Of the 78 sterol compounds detected, the 69 that had a maximum proportion >1% were analysed for

240 phylogenetic signal (Zu *et al.*, 2021). To determine if there was a phylogenetic signal in sterol

241 production across the data, whereby closer related species would share more similar profiles than

242 expected by chance, Pagel's λ and Blomberg's K were calculated. Both metrics use a Brownian model

243 of trait evolution and in both cases a value of 0 indicates the trait has evolved independently of

244 phylogeny, therefore closely related species are not more similar to each other than distantly related

245 ones. Low phylogenetic signal can mean high rates of evolution are causing big differences between

246 closely related species or perhaps convergent evolution is causing distant relatives to be more similar

247 than close ones. High phylogenetic signal (K, λ=1) can be caused by low rates of evolution or

248 stabilising selection. Interpretation of high and low phylogenetic signal varies between studies

249 (Kamilar and Cooper, 2013). Phylo4d objects were generated from species tree and proportion data

250 using phylo4d() from PHYLOBASE v.0.8.12.(R Hackathon et al., 2024) and phyloSignal() from the

251 R package PHYLOSIGNAL v.1.3.1.(Keck *et al.*, 2016) used to calculate Pagel's λ and Blomberg's K.

252 The number of iterations was set to 999. Quantitative values (mg/kg) were used for total sterol content

253 and proportions (0-1) for all other sterol traits. To identify trends in different types of sterols,

254 proportion data was also grouped according to carbon count and position of the B-ring double bond.

255 Ergosterol was classed as NA for double bond position as it contains double bonds at positions 5 and

256 7 and sterols with a cyclopropane ring were classed as CPR. Un-named sterols were all classed as

257 ΔNA, full details are available in Table S3.

258 ***Family, genus and species level sterol diversity***

259 Prior to analysis of inter-species differences, 17 samples identified to only genus level where there

260 was already a species level representative in the genus were removed.

261 Species within the family Asteraceae were grouped according to subfamily: Asteroideae (n=21,

262 tubular and ray florets), Cichorioideae (n=17, ray florets only), Carduoideae (n=14, tubular florets

263 only). Subfamily classifications were taken from the Open Tree of Life version 14.7 (OpenTreeOfLife

264 *et al.*, 2019) and (Mandel *et al.*, 2019). Mean sterol proportions were calculated for each species. An

265 NMDS was plotted containing all Asteraceae species (stress = 0.166) to compare sterol profile

266 between the subfamilies and an ISA carried out to test for associations between subfamilies and

267 specific sterols.

268 For each species, sterols were then grouped by B-ring double bond saturation (CPR, $\Delta 0$, $\Delta 5$, $\Delta 7$, $\Delta 8$

269 or ΔNA) and their proportions summed (double bond position data is available in Table S3).

270 Ergosterol was classed as NA for double bond position as it contains double bonds at positions 5 and

271 7. Un-named sterols were all classed as ΔNA. Proportion data (0-1) was arcsine square root

272 transformed and a Kruskal-Wallis rank sum test carried out using kruskal.test(),for each double bond

273 group, followed by a post-hoc Dunn test using dunnTest() from the FSA package 0.9.5. (Ogle *et al.*,

274 2023) to test for differences between Asteraceae subfamilies and the rest of the dataset. Both analyses

275 used a Benjamini-Hochberg correction for multiple testing.

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

277 each and so were used to analyse intra-family variation in sterolome at the genus level. An NMDS

278 and PERMANOVA were computed separately for the three families using data from individual

279 samples to test for significant differences in sterolome between genera. Species identity was also

280 included in the models.

281 A total of 48 species were collected from at least three counties in England. As 48 groups would be

282 too many to separate on an NMDS, the species with the highest number of samples were chosen for

283 statistical comparison. Species which were sampled from four different counties and had a least five

284 samples overall were used. An NMDS and PERMANOVA were used to test for significant

285 differences between these most well sampled species (stress value=0.096).

286 ***Ecological associations of sterol profiles***

287 In order to establish how many plants could suitably meet the sterol requirements established for

288 honey bees, data for 18 prepupal honeybee samples (15 prepupae per sample) was used from Svoboda

289 *et al.*, (1980) and compared to all hand collected pollen species in this dataset. In order to compare

290 these datasets, only sterols detected in the Svoboda work were used (24-methylenecholesterol,

291 isofucosterol, β-sitosterol, campesterol, cholesterol and desmosterol) and pollen sterol profiles were

292 not recalculated following this removal. An NMDS (k=3) and PERMANOVA were used to show

293 differences in sterol profile composition and variation between the bees and pollens (stress = 0.162).

294 ***Total sterols***

295 For families which contained at least three genera, species data were used to create a box plot

296 showing range in total sterol production (mg/kg) across families.

297   To compare the effect of collection county on total sterol production, data was subset as follows:

298   Total sterol (mg/kg) was calculated for all samples, data was filtered to only include species which

299   had been collected from at least three counties. Counties were then ranked by the number of these

300   species they included and those with ≥10 kept (Oxfordshire (33), Greater London (25), County

301   Durham (21), North Yorkshire (20), Sussex (10)). This led to a total of 35 species across five counties

302   being included in the analysis. A linear mixed-effects model was created with total sterol production

303   (mg/kg) as the response variable and collection county as a fixed effect. Species identity was included

304   as a random effect. Where a species had been collected more than once from a county the mean value

305   was used for that county. Total sterol data was $\log_{10}$ transformed in order to reduce non-normality of

306   the residuals. Model was run using lme() from the NLME package v.3.1.164. (Pinheiro, Bates and R

307   Core Team, 2023). The residuals of the fixed effects showed normal distribution (Shapiro-Wilk test:

308   w=0.986, $p>0.100$) and homogeneity of variance (Levene test: $F_{4,104}=0.685$, $p>0.500$). Random

309   effects also showed normal distribution (Shapiro-Wilk test: w=0.962, $p>0.100$).

310

Results

Quantity of pollen sterol production varies among plant species

313   We observed interspecies variation in total sterol content of greater than two orders of magnitude. For

314   example, the dandelion, *Taraxacum sect. Taraxacum,* contained the highest amount of sterol

315   (75,423.52 mg/kg sterol/fresh weight pollen). The pollen with the lowest concentration of sterol was

316   observed in the common mallow, *Malva sylvestris,* (729.98 mg/kg, Table S4). Asteraceae accounted

317   for some of the highest and lowest sterol amounts, specifically, Cichorioideae subfamily flowers

318   showed a consistently higher total sterol production than the other Asteraceae flowers (Table S4).

319   Brassicaceae showed the highest sterol content on average, as Asteraceae was still dominated by low

320   sterol producing species (Figure S2).

321   In general, location of collection did not have a significant impact on total sterol production (mg/kg)

322   ($F_{4,70} = 1.254$, $p>0.100$). However, variation in total sterol production was still present, potentially

323   caused by abiotic factors, such as temperature, affecting total fat production, or a result of handling

324   and storage procedures working at mg scale. Mean and standard deviation for all species replicated at

325   the county level are reported in Table S5.

Pollen shows a diverse but phylogenetically constrained sterol profile

327   Within the 295 taxonomic units we sampled (including genera, species and subspecies), we recorded

328   78 distinct metabolites based on their retention time and accurate mass of which 24 were fully

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329   assigned by comparing them to reference compounds (level 3 annotation) and 51 were identified

330   putatively and each determined to be structurally distinct based on retention time and accurate mass

331   from which number of carbons and degree of saturation were determined (noted as ST(carbon

332   number: degree of saturation) from here onwards) (level 2 annotation) (see Furse at al., 2023). Where

333   two level 2 annotated sterols had the same carbon number and the same degree of saturation they were

334   allocated a letter. Most sterols were present in small proportions even if they dominated in some

335   pollens e.g., desmosterol (median 0.02%, max 83.50%) and cholesterol (median 0.01%, max 61.67%)

336   (Table S6). Thirty-eight sterols were not recorded at higher than 5% in any species. The only sterols

337   with a median of >2.5% were β-sitosterol, isofucosterol and avenasterol (Table S6).

338   Only four sterols: campesterol, cycloartenol, ST(30:2)B and ST(30:2)C were detected in all taxa and

339   varied in maximum abundance, from 30.23% in campesterol up to 77.92% in cycloartenol, both of

340   which had a median of >1% (Table S7). Cyclolaudenol, ST(30:2)A, ST(30:2)E and ST(31:2)A were

341   in all but one taxonomic unit. Avenasterol, 24-methylenecholesterol and schottenol were in all but

342   two.

343   Ergosterol, a sterol restricted to fungi (Weete, 1973), was detected in 253 taxonomic units suggesting

344   the near ubiquitous occurrence of fungi in pollen indicating that fungi are inadvertently likely to be a

345   significant part of the diet of pollen feeding animals such as bees. It was not recorded at >1.33% in

346   any species (maximum amount in honesty, *Lunaria annua*). The maximum in an individual sample

347   was recorded in meadow buttercup (*Ranunculus acris,* 1.40%) and had a median proportion across the

348   dataset of 0.01%. Of the 12 samples where ergosterol occurred at >0.75%, nine were *Ranunculus* spp.

349   On average, sampled taxa had 62 sterols in their pollen. The smallest range of sterols (35) was

350   recorded in the pollen of creeping cinquefoil (*Potentilla reptans),* while the largest range was

351   recorded in black alder (*Alnus glutinosa),* which contained 76 sterols. Species also showed a wide

352   variation in sterol diversity as calculated by Simpson's diversity index. The most diverse species was

353   *Papaver cambricum* (index = 0.913) and the least was *Torminalis glaberrima* (index = 0.169) (Figure

354   S1; Table S8).

355   Typically, the pollen sterol profile was dominated by 1-3 major sterols (Figure S3). β-sitosterol and

356   isofucosterol were the most common major sterol components (defined as ranking in top three by

357   proportion of any given species), in over 100 taxonomic units (Table S9). Some plant species had a

358   clear dominant sterol. Schottenol, 24-methylenecholesterol, desmosterol, cycloartenol and

359   isofucosterol all occurred at >75% in at least one taxonomic unit (Table S6). In total, 29 species of the

360   261 which could be placed on our phylogeny had a sterol which accounted for ≥65% of their total. In

361   11 out of 29 species this sterol was 24-methylenecholesterol.

362   Of the 69 sterols which occurred at >1% in pollen, 31 showed a significant phylogenetic signal for

363   both metrics tested (K and λ), along with total sterol content (mg/kg) (Table S11). Furthermore, when

364   sterols were grouped according to number of carbons (CC: 27-31) and B-ring double bond

365   substitutions (DB: CPR, 0, 5, 7, 8, NA (*level 2 annotated sterol structures and ergosterol (Δ5&7)*)),

366   most groups showed significant phylogenetic signal for both metrics (K and λ) (27, 28, 29, 30 (CC)

367   and CPR, 0, 5, 7, NA (DB)). Twenty-six additional sterols showed significant phylogenetic signal for

368   a single metric (all Pagel's λ except salisterol). The 31 carbon group and Δ8 group were also

369   significant for Pagel's λ.

370   Figure 1 presents the phylogenetic tree covering 261 of the 295 taxa analysed representing 54 families

371   (a labelled species tree is provided in Figure S4). The dominant sterol produced in pollen was plotted

372   across taxa. In this figure, for example, the four Asparagaceae species were dominated by a single

373   sterol, schottenol, at proportions exceeding 70% (Figure 1A). Many species across Fabaceae,

374   Salicaceae and Hypericaceae produced high levels of one of either isofucosterol, β-sitosterol or

375   avenasterol (Figure 1B). These sterols arise from the same biosynthetic pathway and therefore might

376   be expected to co-occur in related species (Figure 2). By contrast, in the closely related subfamilies of

377   the Rosaceae, each exhibited a different sterol profile. The subfamily Rosoideae, was dominated by

378   desmosterol whereas its sister subfamily, the Amygdaloideae, was dominated by 24-

379   methylenecholesterol (Figure 1C). The sterolome for the Asteraceae was different from all other

380   families in the tree. This family exhibited a consistently low proportion of Δ5 sterols, and instead

381   produced pollen with high proportions of obtusifoliol or cycloeucalenol; sterols occurring early in the

382   biosynthetic pathway (Figure 1D).

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

384   ***levels***

385   There was a strong influence of phylogenetic group on the pollen sterolome, even at subfamily level.

386   The sterol profiles of all three Asteraceae subfamilies differed significantly (PERMANOVA: $F_{(2,49)}$

387   =10.234, p=0.001, R^2=0.295, all pairwise comparisons p=0.001, comparison of variance: $F_{(2,49)}$

388   =8.003, p<0.001) as shown in Figure 3A (NMDS plot stress value: 0.166). Our analysis indicated that

389   each subfamily was associated with *at least* six level 2 annotated sterols. For example, the Carduoidae

390   had 15 of these putatively identified sterols that distinguished it from the Asteroideae and the

391   Cichorioideae (Table S10) indicating there is still much we do not know about sterol production in

392   plants and especially in the Asteraceae.

393   Overall and when compared to other families, Asteraceae pollen had a highly distinctive sterolome

394   (Figure 3B). There were also significant differences between Asteraceae subfamilies and other pollen

395   in all sterol double bond classes (CPR: H(3)=49.6, p<0.001, Δ0: H(3)=78.6, p<0.001, Δ5: H(3)=55.8,

396 $p<0.001$, $\Delta 7$: 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

397 Asteraceae subfamilies had significantly higher proportions of sterols with a B-ring cyclopropane

398 substitution (CPR), undetermined B-ring structures (ΔNA – Level 2 annotated sterols), and lower

399 proportions of $\Delta 5$ sterols than all other families (*post-hoc* Dunn tests: CPR: $p<0.005$, $\Delta 0$: $p<0.001$,

400 $\Delta 5$: $p<0.005$, ΔNA: $p<0.001$). Furthermore, Carduoideae had significantly higher $\Delta 7$ sterols than all

401 non-Asteraceae families ($p<0.001$) and significantly lower $\Delta 8$ than all other groups (other families:

402 $p<0.010$, Asteroideae: $p<0.005$, Cichorioideae: $p<0.005$).

403 Comparing between genera of the same family we found significant differences in sterolome,

404 demonstrating that species within a genus often display similar sterolomes (Figure 4A). In Asteraceae,

405 *Centaurea* and *Cirsium* both belong to the subfamily Carduoideae and show more similar sterolomes

406 whilst *Crepis* belongs to the Cichorioideae. However, genera were still significantly different from

407 each other (PERMANOVA: Genus: $F_{2,20}=26.087$, $p=0.001$, Species: $F_{9,20} = 2.311$, $p<0.010$. Variance:

408 Genus: $F_{2,29}=0.810$, $p >0.100$, Species: $F_{11,20}=1.543$, $p>0.100$). All pairwise differences between

409 genera were significantly different (all $p=0.001$) with genus and species identity together explaining a

410 high proportion of the variance in the dataset (R^2: genus = 0.561, species= 0.224). All genera were

411 associated with at least five level two annotated sterols, affirming findings above about the

412 undetermined sterol composition of Asteraceae flowers (Table S12). This pattern was repeated in the

413 three Fabaceae genera *Trifolium*, *Ulex* and *Vicia* (PERMANOVA: Genus: $F_{2,22}=28.756$, $p=0.001$,

414 Species: $F_{8,22} = 2.567$, $p=0.001$. Variance: Genus: $F_{2,30}=1.212$, $p>0.100$, Species: $F_{10,22}=2.423$, $p<0.05$,

415 Pairwise differences: all $p<0.005$, R^2: Genus = 0.575, Species = 0.205). The strongest associations

416 between genera and sterols were *Trifolium* with schottenol and *Ulex* with sitostanol. In the Rosaceae,

417 strong differences were recorded between genera, even within the same subfamily, however, species-

418 level differences within genera were not statistically significant, despite *Potentilla* and *Rubus* both

419 belonging to the same subfamily Rosoideae (PERMANOVA: Genus: $F_{2,17}=16.695$, $p=0.001$, $R^2 =$

420 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:

421 $F_{8,17}=0.417$, $p>0.500$). Pairwise comparisons: *Prunus*: *Potentilla* ($p<0.005$), *Prunus*: *Rubus* ($p<0.005$),

422 *Potentilla*: *Rubus* ($p<0.050$). All three genera were associated with a high number of level two

423 annotated sterols but the strongest association was *Rubus* with desmosterol.

424 Furthermore, we examined variation in the sterol profile of individual species collected in different

425 sites across the UK. Intraspecies variation between sites was not greater than the interspecies variation

426 in the dataset. Species displayed significantly different sterol profiles (PERMANOVA, $F_{6,39} = 33.757$,

427 $p=0.001$, all pairwise comparisons between species $p<0.050$), with species identity explaining a large

428 proportion of the variance in the data ($R^2=0.839$). In contrast, the sterol profile of pollen collected

429 from the same species in different locations across the UK exhibited relatively little variation (Figure

430 4B, C (stress value = 0.096)). Interestingly, the amount of variation in the sterol profile within species

431 was not significantly different ($F_{6,39} = 0.600$, $p>0.500$). The sea aster, *Tripolium pannonicum,* was

432 significantly associated with the most individual sterols (14), including a very strong association with

433 ST(27:1)B. The meadow buttercup, *Ranunculus acris,* had a strong association with ergosterol. Since

434 this sterol is produced exclusively by fungi it suggests buttercups are more likely to be contaminated

435 with fungi. Species that collect pollen from this species are therefore likely to be provisioning

436 significant quantities of fungi to their brood. The common bluebell, *Hyacinthoides non-scripta,* was

437 strongly associated with schottenol, the lesser celandine, *Ranunculus ficaria,* with isofucosterol,

438 blackthorn (*Prunus spinosa)* with 24-methylenecholesterol and heather (*Calluna vulgaris)* with

439 sitostanol, β-sitosterol and stigmasterol. A full list of all 45 associations found can be seen in Table

440 S13.

441 ***Comparison of sterols found in honeybees and pollen***

442 The honeybee sterol profile from Svoboda *et al.*, (1980) was compared to the sterol profile of all hand

443 collected pollen species in this dataset (Figure 5). Bee sterols differed significantly from pollen in

444 sterol profile (PERMANOVA: $F_{(1,294)} = 10.567$, $p=0.001$, $R^2=0.035$) and in variance in sterol profile

445 ($F_{(1,294)} = 98.678$, $p<0.001$). The pollen sterolomes that were most like honeybees were replotted from

446 the full NMDS (stress=0.101) in Figure 5A and their sterol profiles shown in Figure 5B.

447 We used data from Svoboda *et al.*, (1980)'s analysis of prepupal honeybees to estimate the sterols

448 required by bee species although the sterol requirements of other bee species may differ from this.

449 While we recognise that this may not be a universal measure of honey bee sterol requirements more

450 recent work suggests this is close (Moore et al., 2025). From these data, we estimated that bees

451 required >30% of 24-methylenecholesterol, >19% of β-sitosterol, >10% of isofucosterol, >5% of

452 campesterol, >0.5% of cholesterol and > 0.5% of desmosterol. Using these thresholds, we found that

453 88 species did not meet any of the sterol requirements of honeybees (Figure 6A) and no single species

454 met the required proportions of all six sterols, even though 188 species contained all six sterols. Of

455 these sterols, 24-methylenecholesterol and isofucosterol have been reported to be especially important

456 (Svoboda et al. 1980; Bogaert et al., 2025; Moore et al., 2025) yet only 35 out of 278 taxonomic units

457 contained sufficient 24-methylenecholesterol to meet this threshold (Figure 6A). Out of these 35, only

458 12 also contained enough isofucosterol and none of these 35 contained enough β-sitosterol. Other

459 species were sufficient in individual bee-relevant sterols. For example, 82 species contained enough

460 β-sitosterol and 90 contained enough isofucosterol: 48 contained enough of both compounds.

461 Likewise, 62 contained enough campesterol. The fewest species contained sufficient cholesterol (21)

462 or desmosterol (28).

463 The highest proportions of the honeybee relevant sterols were often found in specific plant families as

464 shown in Figure 6B (24-methylenecholesterol = Rosaceae, campesterol = Apiaceae, cholesterol =

465   Asteraceae subfamily Cichorioideae). Interestingly, the highest content of desmosterol was from

466   diverse flowers popular with honeybees and bumblebees such as *Rubus* spp., *Rosa* spp., *Borago*

467   *officinalis* and *Symphytum* sp. β-sitosterol was not recorded more frequently by any family and three

468   of the species in which this sterol was most abundant are wind pollinated trees (*Carpinus betulus*,

469   *Corylus avellana*, *Quercus robur*).

Discussion

471   This dataset provides a major contribution to our knowledge of pollinator nutrition at a scale never

472   previously undertaken and informs our understanding of the evolution of pollen sterol pathways

473   across angiosperms. We also show that abiotic factors do not significantly influence sterol profiles

474   and that instead they are under evolutionary constraints. This concurs with earlier work that suggested

475   mean annual temperature correlated with total pollen sterol content (Zu et al., 2021) and may be an

476   adaptation to mitigate membrane heat stress (Narayanan et al., 2016).

477   Sterols are needed for pollen tube elongation. However, these sterols can be synthesised *de novo* from

478   squalene during pollen tube growth (Villette *et al.*, 2015). It is possible that storage of sterols in pollen

479   grains would make them a target for insect herbivores, which could have a dramatic negative effect on

480   plant fitness. The high diversity of uncommon sterols in pollen could therefore be a mechanism for

481   the plant to escape pollinivory, as a diverse sterolome could hinder herbivore adaptation to targeted

482   efficient sterol conversion pathways. It is possible that such sterols could either be unusable or even

483   toxic to palynivores. Considering the mixture of sterols we have found in pollen it is worth noting that

484   Behmer (2017) explores how the ratio of what they describe as good to bad sterols impacts insect

485   performance, but this can be mitigated through diet mixing (Behmer and Elias, 2000). During their

486   evolution, palynivores including bees may have adapted to be able to use the diverse range of pollen

487   sterols to overcome this defence. Previous studies have hypothesized that bees had lost the genes for

488   the dealkylation of longer chain sterols to cholesterol and instead adapted a specific subset of pollen

489   sterols for their use (Svoboda, Herbert and Thompson, 1983). This would also allow bees to use

490   several different sterols flexibly rather than relying on obtaining a single sterol abundant enough for

491   them to convert efficiently. Overcoming such a defence would also enable bees to use a valuable

492   nutritional resource unavailable to other palynivores.

493   The sterols most abundant in honeybees (24-methylenecholesterol, β-sitosterol and isofucosterol) are

494   also commonly abundant in pollen sterolomes. The structural properties of these sterols (C28:Δ5,

495   C29:Δ5 and C29:Δ5 respectively) may be useful in bee cell membranes, influencing properties such

496   as fluidity (Furse, Koch, *et al.*, 2023). The dominance of these nutrients in honeybees may represent

497   an adaptation of bees to use the sterols available in the widest range of pollens. It is interesting that

498   only 29 out of 261 species in the phylogeny had a sterol which accounted for ≥65% of total sterols.

499   Ten out of 29 of these species were Rosaceae with the dominant sterol being 24-methylenecholesterol

500   or desmosterol. In fact, 24-methylenecholesterol was the most common sterol that accounted for

501   ≥65% of total sterols (11 species).

502   It is conceivable that co-evolution of plants and bees has been driven in part by the specific sterols in

503   pollen to encourage bee visitation and support bee populations although this is more likely the case in

504   specialist bees (Vanderplanck et al., 2017). However, the six sterols required by honey bees (24-

505   methylenecholesterol, β-sitosterol, isofucosterol, campesterol, cholesterol, desmosterol) were not

506   recorded in the pollen of any one single plant species in the same proportions recorded in bees.

507   While floral constancy presents benefits to plants of pollen being delivered more efficiently and

508   effectively to conspecifics (Grüter and Ratnieks, 2011), it is also conceivable that producing sterols in

509   quantities that are inadequate for palynivores could potentially prevent overharvesting of pollen from

510   one source and instead encourage a generalist foraging strategy. The miss-match between bee

511   nutritional needs and pollen chemistry (especially among crops such as *Malus* spp.) may help explain

512   the effectiveness of agri-environmental floral plantings in improving crop pollination. It is possible

513   that where sterol requirements are known that such plantings might be specifically selected for

514   complementary nutritional properties.

515   It is plausible that sterol diversity in honeybees is a result of their nutritional flexibility and diet

516   mixing achieved through foraging from a range of plant species. Honey bee colonies develop for

517   longer if their diet is supplemented with key Δ5 sterols including 24-methylenecholesterol and

518   isofucosterol (Bogaert et al., 2025, Moore et al., 2025) while the sterolome of larvae on sterol

519   supplemented diets matched that of honey bees in naturally provisioned hives indicating that honey

520   bees selectively transfer only the most biologically important sterols to their young. However, the

521   pollen sterolome also contains a range of sterols that are absent from the tissues of honeybees such as

522   obtusifoliol, schottenol and cycloeucalenol. While these sterols may serve key physiological functions

523   for plants unlike Δ5 sterols they may be unsuitable as replacements for cholesterol in insect tissues

524   (e.g., sterols with B-ring cyclopropane substitutions or Δ8 double bond which are generally not

525   suitable for insects Chen et al., 2024). The nutritional role of sterols in wild bees is under studied.

526   However, red mason bees (*Osmia bicornis*) provided pollen that contained or was supplemented with

527   sterols including campesterol had increased growth rate and there is some evidence for selective

528   uptake of specific sterols by larvae.

529   The 'Asteraceae paradox' refers to the frequent use of Asteraceae pollen by specialists and its

530   infrequent collection by generalists (Müller and Kuhlmann, 2008). It is thought to be the result of

531   chemical defences and nutritional deficiencies (Praz, Müller and Dorn, 2008; Vanderplanck, Gilles, *et

532   al.*, 2020). Our results showed that Asteraceae pollen contained lower proportions of Δ5 sterols such

533   as 24-methylenecholesterol, β-sitosterol, isofucosterol desmosterol and campesterol – typically

favoured by honeybees, instead producing higher quantities of Δ7, Δ8, 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 Δ5 sterols such as honey bees. 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 Δ5 sterols in Asteraceae flowers suggests the sterol requirements of Asteraceae-specialist solitary bees may have evolved different phytosterol use to that of common eusocial species (*Apis* spp. and *Bombus* spp.) and provides 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 invites 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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Author Contributions

571   EB, GAW, PCS and WEK designed the study, performed the analyses, and drafted the first version of

572   the manuscript. EB, EL, JQ, SK, AS and EM undertook sampling and prepared samples for analysis.

573   All authors contributed to analyses, interpretation of the results, and the final form of the manuscript.

574

Data availability

576   All the source data used for this publication can be found in the Supporting Information Tables.

577   Chemical data is available at

578   https://url.uk.m.mimecastprotect.com/s/KrjdCj0PYi3925KIRhLhmXzq_ 'Sterols from the pollen of

579   British plant species'.

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Figure 1.

Figure 2.

Figure 3.

A

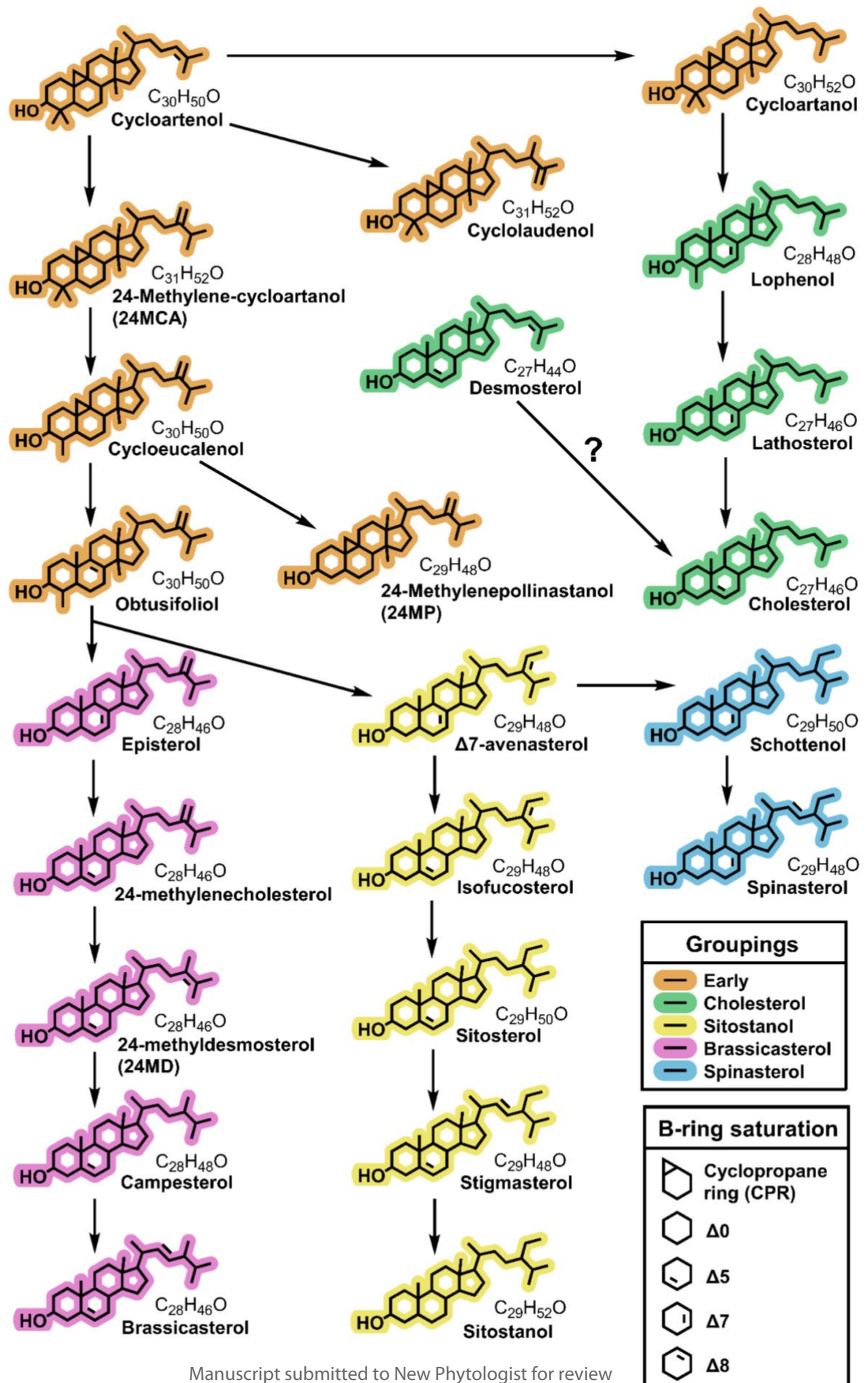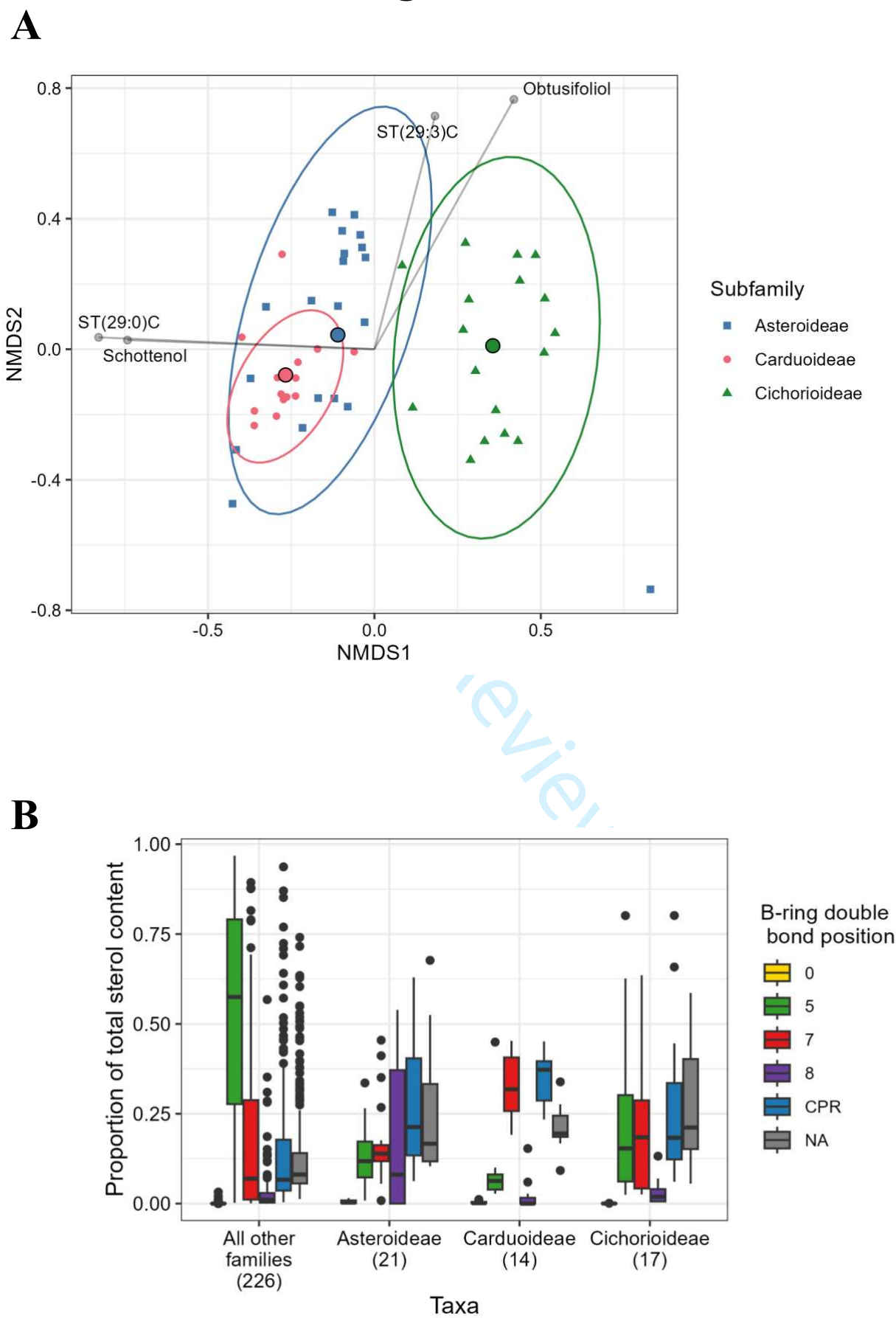

B

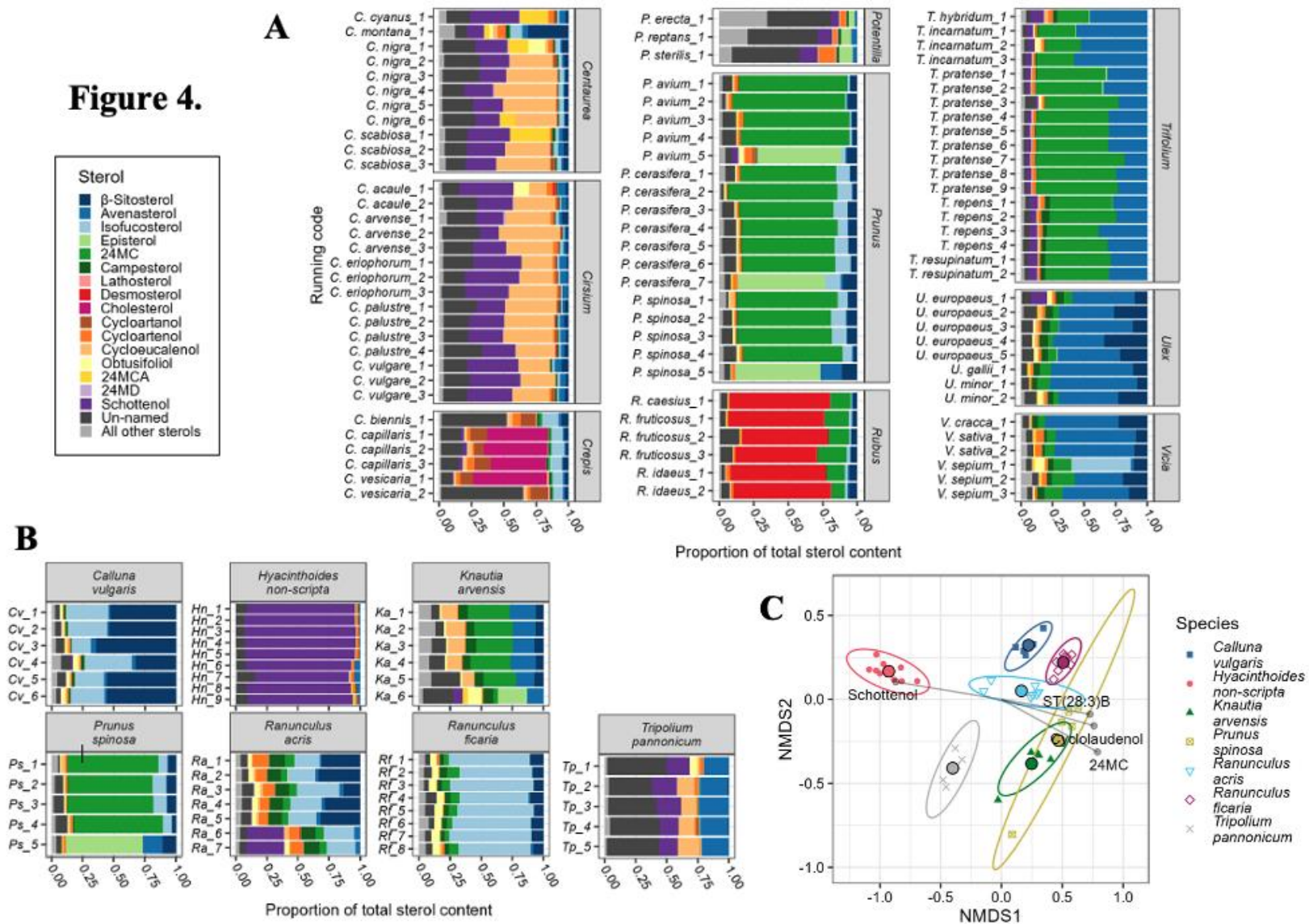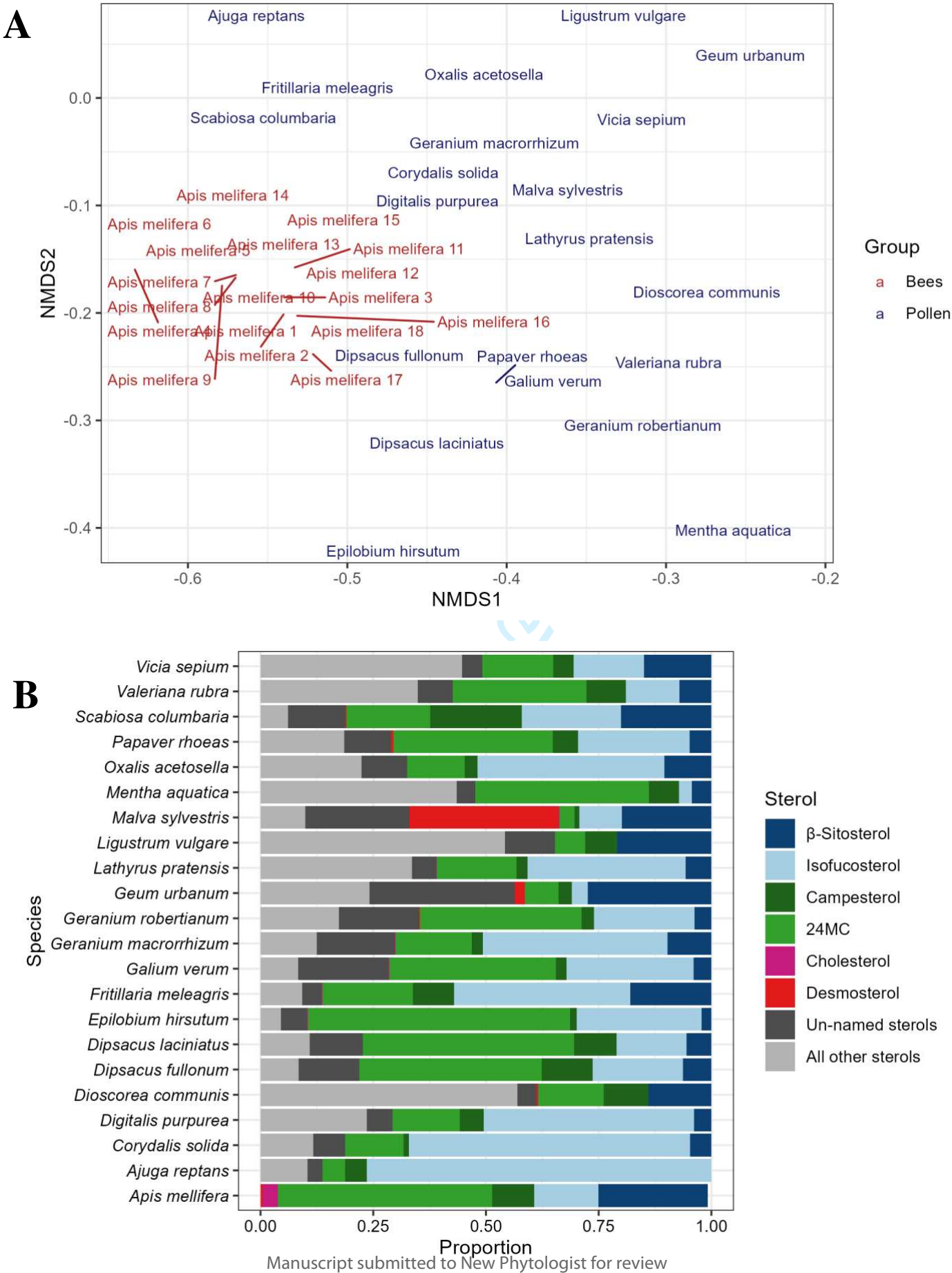**Figure 4.**

Figure 5.

A

B
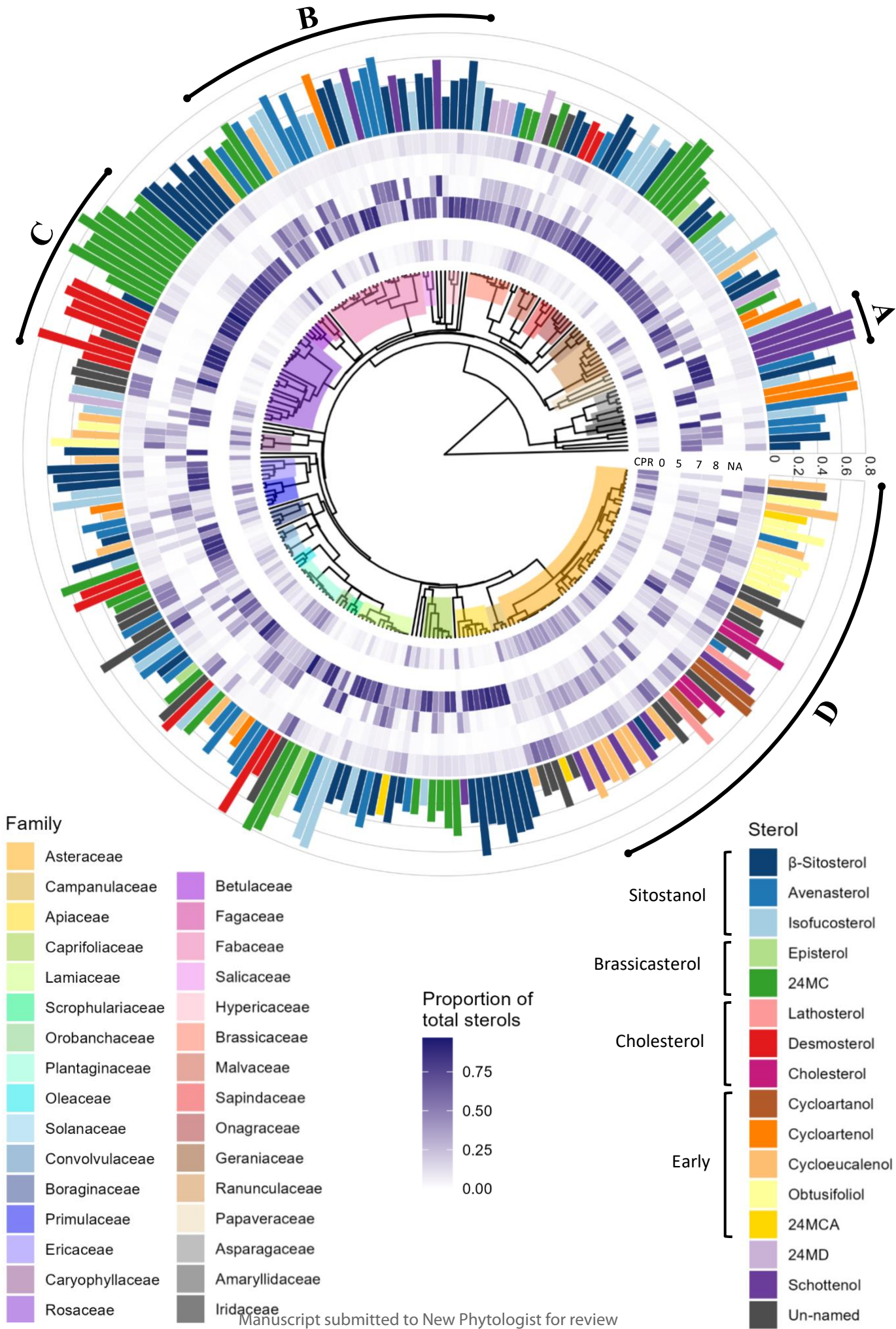
Figure 6.

Honeybee sterol requirements met	Number of species	Honeybee sterol requirements met	Number 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