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Ecology and evolution of sterols in

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pollen and wild bees

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10

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12 **Declaration**

13 I declare that this thesis was composed by myself and that the work contained herein is my
14 own except where explicitly stated in the text. The work has not been submitted for any
15 degree or professional qualification except as specified.

16 Ellen Baker, Trinity 2024

17

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Abstract

93

94 Bees are reliant on flowers for their dietary intake of protein and fats. This includes sterols which
95 act as essential membrane components and hormone precursors in insects. The sterol composition
96 of pollen has been shown to vary widely between plant species and bees are unable to develop with
97 insufficient or unsuitable sterols. However, we know little about the sterol requirements of most
98 bee species. My research addresses the current deficit of information on pollen sterol resources and
99 wild bee sterol requirements by analysing a taxonomically and ecologically wide range of species
100 using a novel and highly sensitive LCMS method developed at RBG Kew.

101 My analysis discovered constraints on the diversity and quantity of pollen sterols, reflected in the
102 phylogenetically conserved profile shown by related species. Flowers belonging to Asteraceae
103 showed a lower proportion of sterols with a double bond at C-5 of the B-ring in comparison to
104 other plant species, a result mirrored in the tissue profiles of bee species specialising on Asteraceae
105 pollen. All *Bombus* species, including cuckoos (subgenus *Psithyrus*), were dominated by 24-
106 methylenecholesterol, isofucosterol and β -sitosterol. This trend was mirrored in solitary bee
107 species; however there was greater inter-species variation and significant differences between
108 generalists and specialist species.

109 In conclusion, bees acquire a specific suite of sterols from pollen and achieve this by foraging
110 across a wide range of plant species. The sterol profile variation among bee species may be due to
111 differing physiological requirements or foraging preferences. Pollen was in general more diverse in
112 sterol composition and expressed high proportions of sterols that were not abundant in bees. This
113 suggests bees do not passively uptake all sterols found in pollen and instead selectively acquire
114 sterols. It also indicates that plants are not tailoring pollen nutritional content to attract bees.

Chapter 1: General introduction

115

116 Bees are the cornerstone of the global insect pollination service (Ollerton, Winfree and Tarrant,
117 2011) but are declining in abundance and diversity in the UK and elsewhere due to a suite of
118 interacting stressors, including increased pesticide input levels, disease transmission, loss of floral
119 resources due to land use change and phenological shifts (Potts *et al.*, 2010; Goulson *et al.*, 2015;
120 van Klink *et al.*, 2020). A key link between these drivers is nutrition; poor diet reduces immune
121 resilience and a lack of available nutritional options drives foraging on pesticide treated areas
122 (Brown, Loosli and Schmid-Hempel, 2000; Goulson *et al.*, 2015). The complete reliance of bees
123 on floral rewards for their nutrition means the availability of these resources in the landscape has
124 direct consequences for the survival of bee populations (Pelletier and McNeil, 2003) as well as the
125 health and fecundity of individual colonies. In the UK, decline of floral resources has been caused
126 by historic loss of flower-rich grasslands; a ~97% loss in the 20th century (Howard *et al.*, 2003).
127 Being able to quantify the nutritional changes resulting from these losses is a recent, but key,
128 addition to our understanding of landscape resources (Baude *et al.*, 2016). The nutritional
129 complexity of pollen, however, makes this quantification more challenging than for nectar. Only
130 by cataloguing the nutritional composition of a wide range of plant species can we predict what
131 might be present in the landscape. Sterols are a recent addition to this catalogue and represent an
132 interesting avenue of study because of their essential function in bee growth and development and
133 poor understanding of what governs their composition in pollen. The aims of this thesis were
134 therefore to uncover trends in pollen and bee sterol composition from a phylogenetic and
135 ecological perspective and to look for nutritional relationships between pollinators and their hosts.
136 This was done by creating a novel dataset of sterol data from a wide range of UK plant and bee
137 species.

138 Sterols play an important physiological role in pollen function and their composition is likely a
139 result of many influences. The first section of this introduction will therefore cover pollen
140 biochemistry and current understanding of pollen sterol composition drivers. I will then discuss the
141 links between pollen foraging and insects, highlighting the different physiological requirements of
142 bees compared with other insects regarding sterol consumption. Dietary sterol intake is essential
143 for bees and there have been several experimental studies addressing the potential link between
144 preferred pollens and sterol content. Current understanding of sterol requirements is more
145 advanced for bumble bees than for solitary bees, an ecologically and phylogenetically more diverse
146 group, and will therefore be discussed separately. The overall relevance of this project is then
147 highlighted by discussing the importance of floral resources at the landscape scale, including a
148 brief review of nutritional data on agri-environment seed mixes.

149 **Pollen structure and function**

150 In angiosperms the function of pollen, a vegetative cell containing two sperm cells, is to serve as
151 the male gametophyte, and to travel from its anther to a receptive stigma. The grain then
152 germinates and grows a pollen tube to transport the sperm cells for fertilisation of the central and
153 egg cells. The biochemical composition of pollen is therefore adapted for the following functions:
154 transport between anther and stigma, persistence in the environment, receptive stigma recognition
155 and rapid pollen tube growth. In animal-pollinated plants, pollen may also contain compounds to
156 encourage or discourage consumption and aid adhesion. As a result of these selection pressures,
157 pollen contains a highly variable range of nutritional compounds including protein, fatty acids,
158 sterols, other lipids, amino acids, vitamins, minerals and secondary metabolites such as phenolics
159 and alkaloids (Roulston and Cane, 2000).

160 Pollen has a conserved structure of three layers: the pollenkitt (pollen coat), exine and intine. The
161 pollenkitt contains lipids, proteins, and pigments amongst other components. It creates a

162 hydrophobic outer layer, protecting against desiccation during transport, and includes lipid-rich
163 material for stigma recognition and adhesion as well as volatile lipids for pollinator attraction
164 (Pacini and Hesse, 2005). It is formed at the end of pollen development when tapetal cells inside
165 the stamen rupture and deposit lipids and proteins to cover the exine alongside ones produced by
166 the haploid vegetative cells (Lau *et al.*, 2022). The exine is a tough 3D structure designed to resist
167 environmental stressors. It is built from sporopollenin, an inert biological polymer made of
168 phenolics and lipids, such as fatty acids, and carotenoids. The intine is an inner wall made of
169 celluloses, pectin and protein which surrounds the cytoplasm. The cytoplasm contains the
170 resources for pollen tube growth and fertilisation and so is lipid rich by necessity (Edlund,
171 Swanson and Preuss, 2004). While all pollens share this basic structure there is variation in the
172 number of apertures (openings) in the pollen grain, which creates potential variation in the ease of
173 extracting the internal nutritional contents for both pollinators and researchers.

174 Carbohydrates, including simple sugars like glucose, starch and polysaccharides are all present in
175 pollen. Monosaccharides, such as glucose, can be used as respiratory fuel and for synthesising
176 other proteins and tissues (Chapman, 2013). Polysaccharides function for structural support and
177 maintaining viability during desiccation (Franchi *et al.*, 1996). Protein is a key macronutrient of
178 pollen and is selectively targeted by bees during foraging; however, there is debate whether
179 selection from insect pollination positively drives pollen protein content (Roulston and Cane, 2002;
180 Hanley *et al.*, 2008). Buzz-pollinated flowers, which require vibration for pollen release, tend
181 towards higher protein content pollen, however, this may be a result of their smaller pollen grains
182 (Roulston, Cane and Buchmann, 2000). The protein-bound and free amino acids found in pollen
183 are highly conserved between floral hosts and include those essential for insect development
184 (Kriesell, Hilpert and Leonhardt, 2017; Vanderplanck *et al.*, 2017).

185 **Pollen lipids**

186 Pollen has evolved a unique lipid composition distinct from other vegetative tissues while
187 containing the same lipid classes (Ischebeck, 2016). It contains a diverse lipidome of triglycerols,
188 galactolipids, glycerolipids, sphingolipids, fatty acids and sterols, as reviewed in Ischebeck (2016).
189 Lipids in different layers of the pollen grain serve different functions for its survival and it is still
190 unknown how pollen lipid composition changes during development. Pollen tube production
191 elongates by polar tip growth, spanning centimetres in some species, and requires a high level of
192 membrane lipid synthesis and fatty acids. Un-germinated pollen does not store enough lipids for
193 pollen tube growth by weight therefore synthesis of new lipids must occur, likely by taking up and
194 metabolising sugars from the female apoplast (Mellema *et al.*, 2002). The products of this
195 synthesis are expected to vary depending on the stigma length which must be covered, which
196 ranges from <1mm in watermeal (genus *Wolffia*), estimated using the size of the flower from
197 Bernard *et al.*, (1990) data by Ischebeck (2016), to 50 cm in maize (Mascarenhas, 1993).
198 Galactolipids are present in low amounts in mature pollen, synthesised during pollen tube growth,
199 but likely not essential. Glycerophospholipids serve functions in membrane signalling and are
200 important during pollen tube growth. Synthesis of triacylglycerols is essential for pollen
201 development, and there is continuous synthesis during tube growth. Sphingolipids are also essential
202 in pollen development and pollen tube growth (Ischebeck, 2016).

203 **Pollen sterols**

204 *'Sterols play a role in cell polarization, endocytosis and exocytosis in vegetative tissues*
205 *[116–118], all very pronounced processes also in pollen tubes. Therefore, it seems*
206 *possible that changes in the sterol composition evolved under the high selective pressure*
207 *to drive pollen tube elongation to the maximum.'* - (Ischebeck, 2016)

208 Sterols are a group of lipids defined by a hydroxyl group, four aliphatic rings (known as the steroid
209 nucleus) and an alkane side chain (Figure 1.1). Because of this structure, sterols are amphipathic in
210 nature; containing polar and non-polar parts. All sterols are derived from squalene, a triterpene,
211 and are differentiated by side chain length, side chain number and position of double bonds. The
212 hydroxyl group in sterols can be covalently bonded to fatty acids, sugars or sugar esters to form
213 conjugated sterols; sterol esters, steryl glycosides, and acyl steryl glycosides respectively
214 (Valitova, Sulkarnayeva and Minibayeva, 2016; Ferrer *et al.*, 2017). These modified sterols are
215 usually in lower proportion than free sterols. Stanols (saturated at C5) are a type of steroid similar
216 to sterols, found in low quantities in plants (Lau *et al.*, 2022).

217 Sterols are essential in all eukaryotes; they allow the cell to better regulate movement of polar
218 molecules across the membrane and the high sterol content in eukaryotic cells is believed to affect
219 permeability, fluidity and microdomain formation. Quantity and composition of sterols therefore
220 affects membrane properties such as fluidity. Subdomains ('rafts') of lipids can also form in
221 membranes and potentially function in cell signalling.

222 Animals and fungi both contain a dominant sterol, cholesterol and ergosterol respectively (Weete,
223 1973). Conversely, plant sterol profiles are composed of a range of phytosterols. Over 200
224 different sterols have been found in plants, with sitosterol, campesterol and stigmasterol being the
225 most common phytosterols (Valitova, Sulkarnayeva and Minibayeva, 2016). Vegetative and
226 reproductive tissues in plants show distinct sterol profiles. For example, β -sitosterol (also known as
227 sitosterol) is the major sterol in *Arabidopsis* leaves compared with 24-methylenecholesterol in its
228 pollen (Ischebeck, 2016). Pollen sterols are often intermediate products of the sterol production
229 pathway occurring in leaves suggesting their synthesis is not by a separate pathway but a truncated
230 version of the pathway present in vegetative tissues (Villette *et al.*, 2015). One proposed reason for
231 unique pollen sterol composition is that the demand for membrane components during pollen
232 growth requires faster sterol production and so steps in the synthesis chain are dropped.

233 The sterol composition of pollen is largely dominated by C27 and C28 length sterols. It is also
234 common for there to be differences in pollen sterol content even between closely related species.
235 Previous work looking for patterns in pollen sterol composition found a dominance of $\Delta 5$ sterols
236 such as 24-methylenecholesterol, isofucoesterol and β -sitosterol with high inter-taxonomic variation
237 but strong phylogenetic clustering (Villette *et al.*, 2015; Zu *et al.*, 2021). The dominance and
238 commonality of 24-methylenecholesterol (C28) in pollen phytosterols is evidently key to bee diets
239 as it is also the primary sterol present in honey bee larvae and adults (Svoboda *et al.*, 1980).
240 However, recent work has shown a lack of correlation between sterol composition and pollinator
241 guild (Zu *et al.*, 2021). Furthermore, pollen does not exhibit differences in sterol composition for
242 pollen-rewarding versus nectar-rewarding plant species (Furse, Martel, *et al.*, 2023), implying
243 pollinators do not apply a selective pressure to pollen sterol content. This is corroborated by wind
244 pollinated species which also have pollen sterol profiles distinct from their vegetative tissues
245 (Furse, Martel, *et al.*, 2023). Finally, pollen sterol content has been observed to be higher in
246 tropical-like climates, warm and wet, showing sterol production may be influenced by
247 environmental factors (Zu *et al.*, 2021).

248 **Pollinivory**

249 Most angiosperms (flowering plants) rely on animals to transport their pollen to a receptive stigma
250 and have co-evolved a system of mutual benefit whereby nutritionally valuable rewards are
251 exchanged for this service. These rewards are key sources of nutrition in the diets of many insect
252 pollinators including bees, moths, flies and beetles. Nectar provides a solution of different sugar
253 groups, such as sucrose, glucose, and fructose, which is metabolised to support energy intensive
254 flight. Pollen complements this by providing macronutrients such as fat and protein as well as a
255 source of protein-bound and free amino acids which act as the building blocks of enzymatic,
256 receptor and storage proteins and neurotransmitters in insects. The amino acids which insects are

257 unable to synthesise and must be obtained through diet, are termed ‘essential’ and a single absence
258 will prevent proper development even with high total protein availability. Pollen also provides a
259 source of lipids, key for energy storage, hormone production and membrane function, as well as
260 vitamins and minerals, which maintain certain metabolic functions (Behmer, 2005).

261 The exploitation of flowers by bees is a relationship more than 100 million years old that has led to
262 the majority of bees being fully dependent on both pollen and nectar at all lifecycle stages
263 (Nowakowski and Pywell, 2016). The higher nutritional complexity and variability in pollen,
264 alongside its importance for larval development, means bees are more selective of pollen hosts
265 than nectar hosts (Goulson and Darvill, 2004).

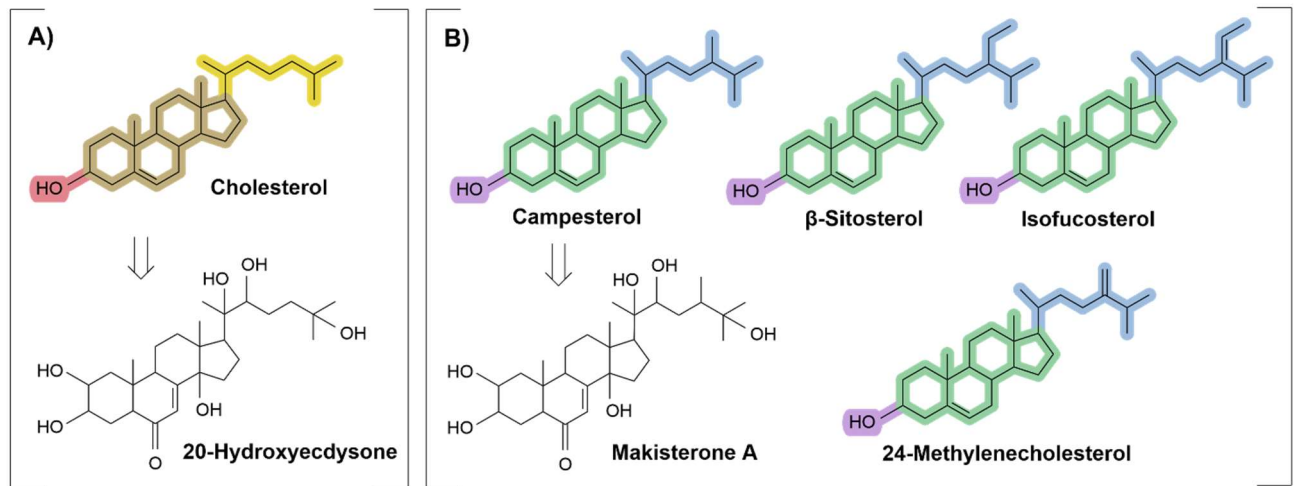
266 In eusocial bee species, colony development necessitates the provision of large quantities of high-
267 quality nutritional resources strengthening the need for this selectivity in pollen foraging by
268 workers. The developmental importance of pollen for colonies has already been established as
269 limiting brood production and colony size of honey bees (Requier *et al.*, 2017) and bumble bees
270 (Rotheray, Osborne and Goulson, 2017). It also has proven impacts on parasite and disease
271 prevention (Brown, Loosli and Schmid-Hempel, 2000; Didaras *et al.*, 2020; Fowler *et al.*, 2020).
272 Ongoing feedback between feeding behaviour and post-ingestive processing enables bees to
273 maintain a nutritional optimum when pollen foraging (Chapman, 2013).

274 **Insect sterol physiology**

275 As well as their essential functions in plants, pollen sterols are also important for the diets of
276 phytophagous insects (Behmer and Nes, 2003). In animals, lipids act as energy stores, membrane
277 components and the building blocks of hormones and some key nutrients e.g., vitamins. In insects,
278 the fat body serves as their main energy store and acts as a reserve for lipids during times of low

279 food consumption. In bees, this reserve is used to survive the low-resource availability of winter
280 and to enable growth.

281 As mentioned earlier, cholesterol ($C_{27}H_{46}O$) is the most important sterol in animals, functioning as
282 a precursor to other signalling compounds, including hormones, and is essential for the
283 maintenance of cell membrane fluidity. In insects, sterols are the precursors of several hormones
284 that are important signalling molecules for orchestrating development, such as ecdysone which
285 controls moulting (Behmer and Nes, 2003). Moulting is an essential processing in insects, enabling
286 them to shed their exoskeleton and grow between developmental stages (Figure 1.1). However,
287 unlike other animals, insects are unable to synthesise squalene, the structural precursor of sterols,
288 and consequently cannot synthesise sterols, so they can only obtain them through their diet and
289 then convert them into cholesterol through dealkylation pathways. Sterols are therefore acquired
290 through two routes: diet and maternal loading during oogenesis. Dealkylation is necessary as
291 phytosterols contain more carbons (C_{28} or C_{29}) than cholesterol (C_{27}). In some insect orders such
292 as Hymenoptera, Hemiptera and Diptera the ability to convert dietary sterols through these
293 pathways has been lost, constraining them even further (Behmer and Nes, 2003). As a result, bees
294 (Hymenoptera, Anthophila) must directly utilise these longer chain sterols and ensure their
295 physiological needs are met through optimum pollen foraging.



296
297

Figure 1.1

298 **A)** Structure of the major sterol in animals, cholesterol, and the associated moulting hormone for
299 cholesterol-metabolising insects, 20-hydroxyecdysone.

300 **B)** Structure of major pollen phytosterols also common in honey bee tissues and the moulting
301 hormone honey bees produce from dietary campesterol, makisterone A. Highlighted sections
302 across all diagrams show conserved structure of sterols: tetracyclic ring, alkane side chain and
303 hydroxyl group.

304 Figure was produced using ChemDraw from Revvity Signals Software.

305 Dietary sterols in insects

306 All insects require sterols in their diet for successful development from larvae (Behmer and Nes,
307 2003). This includes a bulk quantity for membrane development which is relatively low compared
308 to protein and carbohydrate intake requirements. Ugine *et al.* (2019) found that the seven-spot
309 ladybird (*Coccinella septempunctata*) showed normal growth and development on a low sterol diet
310 but that sterol feeding was necessary to prevent spermatogenic failure. During periods of reduced
311 dietary sterol availability, *Drosophila* larvae can reversibly pause their growth and development. In
312 this paused state, membrane sterol levels are up to six times lower (Carvalho *et al.*, 2010). Some
313 insects require very specific dietary sterols for instance *Lobesia botrana* requires ergosterol from a
314 mutualistic phyto-pathogenic fungus (Mondy and Corio-Costet, 2000).

315 Sterols may also have tissue-specific functions in insects, for instance, sterols have been shown to
316 be lost less readily from central nervous system neurons and the gut apical membrane (Carvalho *et*
317 *al.*, 2010) than from membranes where they can be substituted by other lipids. In *Drosophila*, the
318 phytosterols, stigmasterol and sitosterol, were able to fulfil the structural function of cholesterol in
319 the membrane and act as substrate for synthesising signalling molecules (Carvalho *et al.*, 2010).

320 *'Drosophila growth and development require a small amount of sterol that is suitable as*
321 *an ecdysone precursor, and larger amounts of sterols with more flexible structural*
322 *requirements, again suggesting that sterols might have important functions as bulk*
323 *components of the membrane'* - (Carvalho *et al.*, 2010)

324 During insect steroidogenesis cholesterol is biosynthesised into C27 ecdysteroids, such as
325 ecdysone, which function as moulting hormones. Ecdysone using insects must therefore always
326 have cholesterol available for producing this hormone. Insects unable to generate cholesterol use
327 campesterol to make C28 ecdysteroid makisterone A (24-methyl-20-hydroxyecdysone) (Svoboda
328 and Feldlaufer, 1991) (Figure 1.1) or β -sitosterol into C29 makisterone C.

329 **Dietary sterols in bees**

330 The inability of honey bees to de-alkylate phytosterols into cholesterol means they must instead
331 selectively uptake and sequester longer chain phytosterols for their physiological requirements
332 (Jing and Behmer, 2020). Honey bee prepupae contain mostly 24-methylenecholesterol and
333 significant levels of sitosterol, isofucosterol and campesterol and <1% of desmosterol (Svoboda *et*
334 *al.*, 1980). However, workers fed on a single-sterol diet produced pre-pupae with increased levels
335 of that sterol; for instance, cholesterol increased from 2.2% to 17.2% on a cholesterol diet.
336 Stigmasterol though, never increased above 6.3% suggesting different sterols vary in their wider
337 usability. Worker honey bees fed a diet supplemented with 24-methylenecholesterol produced

338 almost the same amount of sealed brood as cholesterol (Herbert *et al.*, 1980), the assumed ancestral
339 state of insects. Even in dietary absence 24-methylenecholesterol, β -sitosterol and isofucoesterol
340 were present in all prepupae (Svoboda *et al.*, 1980). Secretions from the hypopharyngeal and
341 mandibular glands and/or honey stomach transfer sterols from endogenous nurse bee pools to
342 larval food (Svoboda, Herbert and Thompson, 1986).

343 **Solitary bees and sterols**

344 The loss of dealkylation pathways in honey bees is thought to extend to solitary bees as they have
345 been shown to contain mostly phytosterols rather than cholesterol in their tissues (Vanderplanck,
346 Zerck, *et al.*, 2020a). The variation in pollen sterol content may therefore make some species
347 nutritionally unsuitable for different bee species. For instance, the high proportion of $\Delta 7$ sterols in
348 Asteraceae pollen has been identified as a potential chemical deterrent for generalist bees as they
349 are less desirable than other common sterols (Vanderplanck, Gilles, *et al.*, 2020). Conversely, if
350 unrelated plants share a similar pollen sterol profile, bees can visit a wide range of species while
351 still obtaining the same sterols. This would allow bees to appear generalist foragers, but still be
352 specialised in their sterol needs (Vanderplanck, Zerck, *et al.*, 2020a).

353 It has been noted that specialist bee species utilise pollens with higher levels of uncommon sterols,
354 including C29 and C27 sterols, such as $\Delta 7$ -stigmasterol and cholestenone (Vanderplanck, Zerck, *et*
355 *al.*, 2020b). This is in contrast to those used by generalists which had higher proportions of C28
356 24-methylenecholesterol (Vanderplanck, Zerck, *et al.*, 2020b). It has been proposed that some
357 solitary bees can add sterols to their pollen loads during foraging using secretions from Dufour's
358 gland, potentially as a way to compensate for these nutritional deficiencies (Vanderplanck, Zerck,
359 *et al.*, 2020b). However, the increases in cholesterol in bee versus hand collected pollen supporting
360 this idea were only found when one plant species, out of 48, was collected by two of the nine bee

361 species studied. This behaviour would therefore require further investigation of the physiological
362 mechanism to prove its existence.

363 Sterols have also been proposed as a constraint on pollen foraging choice (Vanderplanck, Zerck, *et*
364 *al.*, 2020a). Two generalist species, *Anthophora plumipes* and *Osmia cornuta* had high levels of
365 24-methylenecholesterol in their larval tissues but comparatively lower levels in un-emerged
366 females suggesting this sterol is being used as a precursor to makisterone A. The commonality of
367 24-methylenecholesterol in pollens would enable these species to visit a wide range of plants to
368 fulfil this physiological need. Two other species, *Colletes cunicularius* (generalist) and *Andrena*
369 *vaga* (specialist), contained higher levels of cholestenone (C27) in their larval tissues compared to
370 un-emerged females, so are hypothesised to use this sterol as the basis for synthesising a C27
371 moulting hormone, such as 20-hydroxyecdysone. Cholestenone was uncommon in the composition
372 of host pollens analysed in the study and so while *C. cunicularius* may forage as a generalist, it
373 could be fulfilling specialist sterol requirements.

374 Large pollen host shifts can occur within clades of solitary bees creating different specialists which
375 are still closely related. A proposed mechanism for facilitating these shifts is shared nutritional
376 content. In one study, the host plants of one solitary bee clade (*Colletes succinctus* group) had
377 dissimilar colour and scent profiles but conserved polypeptide and sterol content, facilitating host
378 shifts (Vanderplanck *et al.*, 2017). Members of another clade (*Melitta leporina* group) were
379 proposed to have greater physiological plasticity from their host plant compositions. Authors
380 suggest this plasticity could be mediated by Dufour's gland secretions as mentioned previously.

381 **Bumble bees and sterols**

382 The sterol requirements of bumble bees have been assessed experimentally using microcolonies,
383 small groups of unfertilised workers that reflect colony level responses. Vanderplanck *et al.* (2014)

384 fed *Bombus terrestris* microcolonies five different monofloral diets and found variation in resulting
385 larval weights but not pollen consumption, confirming the nutritional suitability of some pollens
386 over others. Further feeding experiments have shown that the nutritional content of pollen, sterol
387 and essential amino acid concentration, better predicts larval success than taxonomic diversity does
388 when mixed and monofloral diets are fed to *Bombus terrestris* (Moerman *et al.*, 2017). However,
389 most pollens used in this study contained similar dominant sterol components: 24-
390 methylencholesterol/ campesterol, β -sitosterol, $\Delta 5$ -avenasterol, limiting the nutritional variation
391 tested.

392 Bumble bees are generalist foragers and have been shown to collect pollens of both high and low
393 nutritional quality in relation to essential amino acid and sterol content (Somme *et al.*, 2015).

394 *B. lapidarius* and *B. pascuorum* have been shown to visit fewer but higher quality pollen hosts than
395 *B. terrestris*, however, the commonality of *B. terrestris* in many environments means flower visits
396 are recorded more often and therefore its foraging diversity is likely higher due to sampling bias
397 (Somme *et al.*, 2015).

398 **Bumble bee ecology**

399 The UK has 27 recorded bumble bee (*Bombus* spp.) species, three of which are declared extinct
400 and six of which are cuckoos (subgenus *Psithyrus*), making up 11% of the global total (Comont,
401 2017). Bumble bees serve key ecological and economic roles in pollinating UK plants, in part due
402 to their ability to forage at low temperatures and buzz pollinate flowers including potato, tomato,
403 borage, blueberry and cranberry (Falk and Lewington, 2015). Bumble bees visit a wide range of
404 plant species to ensure the fulfilment of nutritional requirements in a heterogeneous environment
405 and individuals display low floral constancy during foraging trips (Yourstone, Varadarajan and
406 Olsson, 2023). All UK bumble bee species display a generalist foraging habit but some species
407 display distinct floral preferences, and foraging is constrained by differences in tongue length and

408 colony cycle duration between species. Proboscis length is a phylogenetically conserved trait in
409 bumble bees, which impacts floral visitation (Arbetman *et al.*, 2017) and is independent of body
410 size. Bumble bee species nest in a wide range of habitats and have received the greatest amount of
411 study of any wild bee clades (Goulson, Lye and Darvill, 2008). Combined with variation in
412 population trend and distribution among species, this group is ideal for the exploration of questions
413 regarding nutritional constraint from many ecologically interesting angles.

414 Bumble bees, like honey bees, are progressive provisioners meaning they do not seal cells once
415 eggs are laid but continually feed them until the larvae are grown. This contrasts with solitary
416 species that ‘mass provision’ their young, sealing their egg in with its food. Unlike honey bees,
417 bumble bees do not have nurse bees to act as a filter between foraged resources and larvae. Bumble
418 bees are also unable to store food overwinter like honey bees.

419 The four most abundant UK bumble bees, *Bombus terrestris*, *B. lucorum*, *B. lapidarius* and
420 *B. pascorum*, all emerge in early spring. These species have 16-25 week colony cycles, requiring
421 access to a long period of floral resources and necessitating a flexible foraging strategy. In the UK
422 and Ireland, *B. terrestris* is even capable of foraging all year in urban microclimates, where non-
423 native shrubs flower over winter, making it facultatively trivoltine (three colony cycles per year)
424 (Falk and Lewington, 2015). Species which emerge later in the season (e.g., *B. humilis* and
425 *B. hortorum* in May), have a shorter window to ensure colony development, potentially
426 necessitating the prioritisation of higher quality food (Goulson and Darvill, 2004). In the UK there
427 are six cuckoo bumble bee species that vary in host specificity, parasitising either a single host,
428 such as *B. rupestris*, or a related group, such as *B. sylvestris*. These species will invade the nest of
429 their host and overpower or kill the queen. Her young are then reared by the host workers (Falk
430 and Lewington, 2015). As such they do not have hind legs adapted for packing pollen and so are
431 not included in analyses of pollen baskets. Their presence is regarded as a positive indicator for bee

432 populations as their host must be present at a sufficiently high density for them to survive
433 (Nowakowski and Pywell, 2016).

434 To best support bee populations, the temporal availability of nutritional resources must coincide
435 with the key points in their life cycle. The spring period (March-end of May) of early solitary bees'
436 emergence and bumble bees nest founding (Nowakowski and Pywell, 2016) in many areas
437 coincides with a shortage of resources, due to an absence of features such as hedgerows, trees and
438 weeds. By mid-summer (June-mid August) eusocial colonies are foraging and solitary species will
439 be producing their second brood. Late summer (late August-end of September) is often also a
440 period of resource shortage as fewer plants are flowering but mated bumble bee queens are trying
441 to build up the resources necessary for overwintering.

442 Bumble bees are flexible foragers and are able to utilise a range of host plants (Vanderplanck *et al.*,
443 2014), however, common bumble bee species are often over represented in visitation observations
444 leading to the reporting of more host plants due to sampling bias (Scheper *et al.*, 2014). Despite
445 this, certain species still maintain obvious foraging preferences, for instance *B. monticola* is highly
446 dependent on spring flowering bilberry, but will still utilise other species outside this period (Falk
447 and Lewington, 2015). The impact of constantly changing floral landscapes also means preferences
448 must be dynamic; the invasive species *Impatiens glandulifera* was not present in pollen loads
449 before 1950 but significantly contributed to the pollen loads of four of the five stable species
450 included in a study in 2004 and 2005 (Kleijn and Raemakers, 2008).

451 Goulson *et al.*, (2005) split the non-cuckoo UK bumble bees into three loosely defined dietary
452 groups. The first group includes long-tongued, late-emerging species such as *B. ruderatus*,
453 *B. humilis* and *B. subterraneus* which specialise in gathering from Fabaceae. This group of bees are
454 at the edge of their geographic ranges and have all declined. The second group comprises
455 abundant, mostly short-tongued, bumble bees which emerge early in the season and have less

456 specialised diets. These are common in suburban gardens where they can exploit a range of
457 resources. A third group of *Bombus* spp. is associated with Ericaceae in moorland and heath
458 environments, restricting their distribution. *B. soroeensis* and *B. ruderarius* are not dietary
459 specialists nor close to the limit of their geographic range but still declining.

460 The most comprehensive database to date on bumble bee pollen diets was assembled from 4132
461 pollen baskets covering 58 bumble bee species taken from museum specimens (Wood *et al.*, 2021).

462 When comparing dietary breadth and dissimilarity between species, breadth showed no
463 phylogenetic signal and each species collected from 9.9 ± 0.6 botanical families in 15 analysed
464 pollen loads. Dietary dissimilarity (dissimilarity to other bumble bee species) had a phylogenetic
465 signal, and a number of subgenera were defined by having high Fabaceae content:

466 *Mendacibombus*, *Bombias* and *Thoracobombus*. Fabaceae explained the greatest amount of
467 variation in diet between species (39.7%), >30% more than any other family. Percentage of
468 Fabaceae pollen collected also had a phylogenetic signal. Other families which also showed a
469 phylogenetic signal, Lamiaceae, Rosaceae and Hypericaceae, explained very little variation in the
470 data. More dietary restricted subgenera like *Thoracobombus* (all 13 species in study favour
471 Fabaceae) are seen to be declining, 64% of species declining, compared to less restricted subgenera
472 like *Pyrobombus* (14 species in study, 7 botanical families favoured), 6% of species declining
473 (Arbetman *et al.*, 2017 as cited in Wood *et al.*, 2021).

474 The zygomorphic Papilionoideae (Fabaceae), which are reliant on animal pollination, are a major
475 pollen source for bumble bees and the decline of many UK bumble bee species has been attributed
476 to the loss of unimproved flower-rich grasslands, which are rich in Fabaceae (Goulson *et al.*,
477 2005). In particular, *Trifolium pratense* and *Lotus corniculatus* are both important in the pollen
478 diets of many *Bombus* species in the UK (Brian, 1951; Carvell, 2002; Kleijn and Raemakers,
479 2008). The importance of *Trifolium* spp. at the landscape scale was shown by Rundlöf *et al.*, 2014
480 where five times more queens and 71% more males were found in landscapes with clover fields

481 compared to control landscapes, despite these fields constituting less than 0.2% of the landscape
482 area.

483 **Landscape loss of pollen resources**

484 Numerous studies have attempted to establish a link between the loss of forage plants and bee
485 species. Kleijn and Raemakers (2008) was the first quantitative study which used pollen from
486 museum bee specimens to correlate historic bee declines to their foraging preferences. By
487 comparing pre-1950 and modern specimens of five stable species and five declining species, the
488 authors found that the stable species maintained a similar diversity but altered composition of
489 forage plants i.e., were able to be flexible in their foraging and adapt to the changes in available
490 plant community. The declining species did not alter their foraging and mirrored the decline of
491 their host plants. These declining species also showed more pronounced host preferences than
492 stable species.

493 The hypothesis that bee species foraging on declining plant species also decline has since been
494 supported by more recent museum based studies (Scheper *et al.* 2014; Wood *et al.* 2019). These
495 studies also showed that declining species collected fewer pollen types than stable species. This
496 serves to convey, not only the importance of pollen as a limiting factor for population survival in
497 different landscapes, but the importance of quantifying the nutritional needs of different bee
498 species. Looking exclusively at solitary species, Biesmeijer *et al.* (2006) found an overall strong
499 decline in bee species richness, most frequent in habitat and flower specialists, univoltine (one
500 colony cycle per year) species (GB) and/or non-immigrants (non-migratory). Species which had
501 the reverse traits (i.e., generalist, fast developing and more mobile), were observed to be doing
502 better. More abundant species have also been recorded to have broader diets than rare ones
503 (Goulson and Darvill, 2004). More generally, the resilience of different bee species to future
504 abiotic changes including temperature and rainfall depends on life history traits such as nesting

505 habit, overwintering state and body size. In response to increasing temperature, smaller soil-nesting
506 bees are likely to increase while larger or comb-building cavity nesters will decline (Pardee *et al.*,
507 2022).

508 **Agri-environment mixes**

509 Agriculturally intense areas of mass-flowering crops present numerous nutritional challenges to
510 bees; boom-bust cycles of resource availability, monocultural nutritional landscapes and an
511 absence of non-treated forage alternatives. This can expose bees to agricultural pesticides and other
512 contaminants with lethal and sublethal effects such as altering their foraging behaviour (Raine and
513 Rundlöf, 2024). Further, areas dominated by arable grasses such as wheat are essentially nutritional
514 deserts to bees. Agri-environment measures such as seed mixes and the enhancement of natural
515 features, including hedgerows and trees, attempt to alleviate some of these issues by providing a
516 more continuous, heterogeneous and untreated source of nutrition.

517 Greater understanding of bee nutritional needs is a prerequisite for successful design of these
518 mixes; however, work to date has largely focussed only on nectar production. Timberlake,
519 Vaughan and Memmott (2019) found “hunger gaps” for bumble bees in farmlands during March
520 and much of August-September when nectar supply is unlikely to meet demand. Other studies have
521 similarly noted this bottleneck in resources during spring and late summer (Fitzpatrick *et al.*,
522 2007). Linear features such as hedgerows and field margins have been shown to provide the
523 greatest volume of nectar per unit area (Timberlake, Vaughan and Memmott, 2019). This
524 underlines the importance of diversity within forage areas; apparently unrewarding or unattractive
525 flowers can provide resources at key points in the season and native perennial weeds often make
526 important contributions (Hicks *et al.*, 2016).

527 **Thesis structure**

528 My thesis will seek to add to the body of work detailed above in the following ways:

529 **Pollen**

530 For the majority of UK wildflowers there is still an absence of pollen sterol composition and
531 quantity data. My first chapter reports the sterolomics data from over 250 species using a new high
532 throughput method of analysis and uses them to address the following questions:

- 533 • Is the phylogenetic signal in sterol composition demonstrated by Zu *et al.* (2021) replicable
534 and at what taxonomic level do we see phylogenetic trends in sterol production?
- 535 • Does the sterolome of pollen collected from the same plant species at different sites vary?
536 ○ Little has been done to measure between-site variation in pollen sterols which is key to
537 fully understanding the landscape level importance of dietary sterols from pollen.
- 538 • Is there variation in sterols which are important in the diets of eusocial bees, honey bees and
539 bumble bees, and does this variation align with species which are preferred by these bees?
540 ○ Pollen biochemistry is thought to be largely driven by plant physiological requirements
541 despite its importance in pollinator diets, however, the direct utilisation of phytosterols
542 by bees may prove to be a strong selective force for foraging choice.

543 These data will broaden our understanding of pollen nutrition and provide a valuable resource for
544 the compilation of whole-nutrition profiles of UK wildflowers. The long-term application of such
545 data would be in the design of agri-environment seed mixes and conservation management.

546 **Bumble bees**

547 My second chapter will focus on bumble bee sterols, reporting the whole body and tagmata level
548 sterol composition for a wide range of UK species. Many sterol-focussed studies have focussed on

549 *Bombus terrestris* and it is therefore still unknown the extent of within-genus variation in bumble
550 bees and whether this aligns with any ecological differences. This chapter will therefore address
551 the following questions:

- 552 • Do the sterol profiles of *Bombus* species show significant inter-species differences?
 - 553 ○ Despite bumble bees all showing a generalist foraging habit, differences in habitat and
 - 554 forage preference may result in visibly different sterol profiles.
- 555 • Do bumble bees of the same species collected from floristically distinct sites show different
556 sterol profiles?
 - 557 ○ If species showed different tissue sterol profiles in response to the differing available
 - 558 forage it would suggest bumble bees are physiologically flexible with regards to sterol
 - 559 usage and may explain their success in inhabiting much of the UK landscape. By
 - 560 sampling common species from a range of florally distinct sites I hope to address this
 - 561 question.
- 562 • Do the three body regions of bees; head, thorax and abdomen, have different sterol profiles?
 - 563 ○ Each tagma has distinct functions and therefore may show different membrane
 - 564 compositions and therefore different sterol profiles.
- 565 • Are the pollens collected by bumble bees similar in sterol profile to their own tissues?
 - 566 ○ By collecting pollen from leg baskets, corbiculae, in bumble bees we can make a direct
 - 567 comparison between what they are foraging for and what they are using in their
 - 568 membranes. However, it is worth noting that the pollen collected by the bees would
 - 569 mainly be intended to feed the next generation of larvae and the forager herself would
 - 570 likely have been raised on different pollens.
- 571 • Do cuckoo bumble bee species have sterol profiles similar to their host species?

572 ○ As there are multiple cuckoo species of bumble bee in the UK, it would also be
573 expected that cuckoo species show a sterol profile very similar to that of their host,
574 facilitating larval rearing by host workers.

575 These data will be the first comprehensive sterol survey of a bee genus and consequently will
576 address new questions on the impact of phylogeny and foraging within bumble bees. If there are
577 differences in sterol profile among species it will also demonstrate that feeding experiments carried
578 out on *Bombus terrestris* are not necessarily applicable to other UK bumble bees. Understanding
579 the variation in sterol profile among bumble bees will also contextualise the pollen sterol data and
580 reveal whether they are only targeting nutritionally desirable species.

581 **Solitary bees**

582 My final chapter will analyse the sterolome of solitary bee species in the UK alongside a subset of
583 bumble bee species (*Bombus* spp.). Solitary bees are distinct from bumble bees in a number of
584 ways relevant to nutrition including; mass provisioning their young, shorter foraging distances and
585 higher occurrence of a specialist foraging habit. As a result, they present a unique comparator for
586 the bumble bee data, focussing on broad trends across species rather than variation within a single
587 genus. By collecting sterol profile data from a wide taxonomic range of species, covering
588 polylectic, oligolectic, monolectic and kleptoparasitic species, I aim to address the following
589 questions:

- 590 ● Do closely related species with different foraging strategies, e.g., generalist vs specialist, have
591 distinct sterol profiles, that are more similar to distantly related species that share similar
592 foraging preferences?
- 593 ○ For highly specialised species, it has been shown they are adapted to their available
594 sterols and therefore body sterol composition resembles that of their pollen host.

595 Where shared specialism has evolved across different clades it then follows that these
596 species should share a similar profile.

- 597 • Is the sterol profile of monoleptic bee species very similar to that of their single host plant?
- 598 ○ Species which rely on a single pollen source for all of their sterol nutrition will only be
599 able to utilise those available sterols but may present them in different proportions if
600 they have mechanisms to selectively uptake and sequester sterols.
- 601 • Do kleptoparasites, such as *Nomada* spp., have a sterol profile similar to the species they
602 target?
- 603 ○ These brood parasites rely on their larvae consuming the food resources provisioned
604 by host species and therefore must be able to synthesise moulting hormones and
605 complete development using the sterols present.

606 Solitary bees are understudied in terms of nutrition and foraging because of the challenges in
607 identification and aversion to lab rearing. By analysing samples of solitary bees from a wide range
608 of families, alongside a set of bumble bee (*Bombus* spp.) samples, this data will provide a
609 comprehensive overview of sterol nutrition in UK solitary bees which may be used in combination
610 with other nutritional data to assess the effectiveness of planting schemes to support them.

Chapter 2: Characterising the diversity of sterols

in the pollen of wildflowers in the UK

Contributors: Ellen Baker (sample collection, sample processing, data analysis, writing), Samuel Furse (sterolomics analysis and signal processing), Hauke Koch (sample collection), Ellen Lamborn (sample collection), Julia Quiñonez (sample collection), Stefania Karlsdottir (sample collection), Abigail Sheppard (sample collection), Elynor Moore (sample processing), Owen Lewis (study design), Phil Stevenson (study design), Geraldine Wright (study design).

Abstract

The UK is home to over 270 bee species all of which rely on floral pollen as a source of lipid and protein nutrients. One important group of lipids is sterols which are required for development, hormone synthesis and cell membrane integrity in insects. However, the profile of sterols in plant pollens has been shown to vary widely between taxa, creating a heterogeneous foraging environment for bees with respect to sterol intake. To understand the full range of this nutritional environment and how it varies between related taxa, a full-scale survey of pollen sterol contents was undertaken. This study reports the pollen sterol composition of over 250 UK wildflower species. Sterol profile showed a strong phylogenetic signal and there was high within-genus similarity across species. Results also showed intra-species consistency in the sterol profiles across different environments. Asteraceae flower pollens were found to have a lower proportion of Δ^5 sterols than the rest of the dataset, a potential cause of their specialist bee associations. No differences were recorded between the sterol profiles of pollens specifically collected by honey and bumble bees compared to other pollens. This suggests pollen sterol composition is driven by plant physiology needs rather than pollinator identity as has been previously suggested for some other

633 pollen nutrient classes. These data will inform understanding of nutritional resource availability for
634 wild bees in the UK and enable future study of geographic trends in pollen sterol availability.

635 **Introduction**

636 Pollen is the male gametophyte of flowering plants but also an important dietary component for
637 many phytophagous insects, including the pollinators which transfer pollen to the stigma of
638 conspecific flowers to facilitate sexual reproduction. It contains a range of proteins and fats which
639 are influenced by floral morphology, pollen transport method and abiotic factors (Roulston, Cane
640 and Buchmann, 2000; Pacini and Hesse, 2005; Russo *et al.*, 2020). There is a conflict between
641 creating pollen which is ‘desirable’ to pollinators therefore ensuring its transportation, albeit at a
642 cost of consumption, but also fulfils the biochemical requirements of maintaining pollen integrity,
643 stigma recognition and pollen tube growth. As a result, pollen has a highly variable biochemical
644 composition, distinct from other, non-reproductive, plant tissues.

645 Lipids are a diverse class of molecules and include fatty acids, phospholipids and sterols. They
646 perform crucial physiological functions in energy storage, membrane maintenance and cell
647 signalling. In pollen, lipids are essential to the rapid cell elongation required during pollen tube
648 growth and can be found both inside and coating the pollen grain.

649 Sterols are lipids distinguished by a tetracyclic ring, hydroxyl group at C-3 and hydrocarbon chain
650 typically at C-24. Variation primarily occurs in the position of double bonds in the B-ring and the
651 hydrocarbon chain length (Figure 2.1). In animals and fungi there is a dominant sterol common
652 across the kingdom, cholesterol and ergosterol respectively. Plants instead produce a wide range of
653 sterols, with over 200 reported so far, which vary quantitatively and qualitatively across taxa
654 (Valitova, Sulkarnayeva and Minibayeva, 2016). These phytosterols are characterised by longer
655 carbon chains (C28 or C29) compared to cholesterol (C27). In Eukaryota, the sterol synthesis
656 pathway begins with squalene and branches into multiple enzymatic pathways (Desmond and
657 Gribaldo, 2009). This pathway is considered to be truncated in pollen, creating a sterol

658 composition different from that of vegetative tissues analysed from the same species (Villette *et*
659 *al.*, 2015; Furse, Martel, *et al.*, 2023).

660 The composition and quantity of phytosterols in pollen ranges widely among plants. Many are
661 dominated by a small number of $\Delta 5$ sterols such as 24-methylenecholesterol, isofucoesterol and
662 sitosterol, and contain much lower values of other sterols (Vanderplanck *et al.*, 2014; Villette *et al.*,
663 2015; Moerman *et al.*, 2017; Vanderplanck, Zerck, *et al.*, 2020a; Zu *et al.*, 2021; Furse, Martel, *et*
664 *al.*, 2023). Total sterol content can range widely, from 1.17 ± 0.59 (mg/g) in *Muscari botryoides* to
665 26.85 ± 19.85 (mg/g) in *Pyrus communis* (mean \pm SD) (Vanderplanck, Zerck, *et al.*, 2020a) and has
666 not been shown to associate with insect pollinator clades (Zu *et al.*, 2021; Furse, Martel, *et al.*,
667 2023). In the largest survey to date, sterol profiles of 122 plant species were reported showing each
668 species contained on average 9.98 ± 4.46 (mean \pm SD) different sterols. However, even in species
669 with over 20 sterols, such as *Hedera helix*, composition was still dominated by one or two common
670 sterols. Overall, sterol composition was phylogenetically conserved and higher total sterol content
671 was associated with warmer climates (Zu *et al.*, 2021).

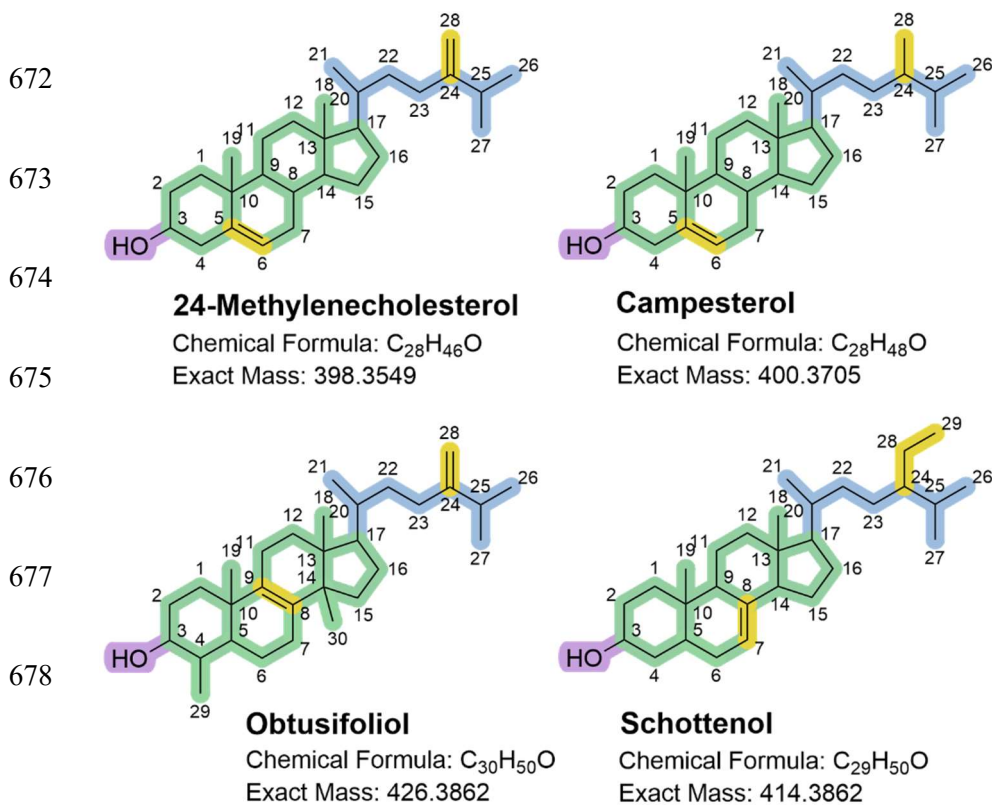


Figure 2.1. Chemical structure of four phytosterols occurring in pollen highlighting variation in the B-ring double bond and chain length at C-24 (yellow). Sterols are defined by the presence of a hydroxyl group (purple), tetracyclic ring (green) and hydrocarbon chain (blue) at C-17. Figure was produced using ChemDraw from Revvity Signals Software.

679 Bees are unable to biosynthesise their own cholesterol or modify dietary sterols like many other
680 insects. As a result, they must consume phytosterols for use in their membranes, hormone synthesis
681 and other physiological processes. This places strong selective pressure on their pollen foraging
682 choice. Experimental rearing has shown that a deficit of dietary sterols or an absence of key sterols
683 can reduce successful colony development (Herbert *et al.*, 1980; Svoboda *et al.*, 1980). For honey
684 bees in particular, the sterols 24-methylenecholesterol, sitosterol, isofucosterol and campesterol
685 have been reported in body tissues. However, only 24-methylenecholesterol diets enable the
686 reproductive success equal to that of cholesterol (Herbert *et al.*, 1980). These experiments still
687 represent the most comprehensive data on bee sterol physiology and development. Almost no work
688 has been done to answer similar questions for bumble bees or solitary bees in the intervening years.

689 A recent study by Zu *et al.*, (2021) carried out the largest effort to date cataloguing pollen sterols.
690 However, while covering a high number of plant species, selection was largely random, and all
691 samples were sourced from a single site. To make ecologically meaningful inferences about the
692 distribution and availability of sterols in different habitats and seasons, as well as how this relates
693 to the dietary needs of different bee species, a comprehensive but focussed set of species must be
694 analysed. This study sought to catalogue the interspecies variation in sterol profiles within
695 specifically UK wildflower pollens by analysing sterol content for a wide taxonomic and
696 geographic range of insect pollinated plants, addressing the following hypotheses: 1) Sterol profile
697 shows a strong phylogenetic signal, 2) Pollen from conspecific plants shows consistency in sterol
698 profiles between different collection sites, 3) Pollen varies in the quantity of sterols with
699 physiological importance for honey bees and bumble bees e.g. 24-methylenecholesterol and 4)
700 Plant species which produce pollen collected by honey bees and bumble bees display a
701 significantly different sterol profile to those which do not.

702 **Methods**

703 **Species selection**

704 Sample collection was initially led by the species list compiled by Baude *et al.*, (2016) which
705 targeted 220 plants from the Countryside Survey in Great Britain that were rewarding to pollinators
706 and common enough to contribute to landscape level floral resources. The authors included 50
707 additional species based on their potential as locally important floral resources. Of this initial list of
708 270 species, 172 were collected for this study. The remainder were not sampled due to difficulty
709 accessing live plants or extracting enough pollen material for analysis. In addition to this, 17 more
710 species were targeted due to their importance as forage plants for wild bee species in the UK (such
711 as *Bryonia cretica* for *Andrena florea*, *Reseda* spp. for *Hylaeus signatus* and *Anthyllis vulneraria*
712 for bumble bees). Many Asteraceae flowers produce copious and easily extractable quantities of
713 pollen and previous work has suggested the possibility of nutritional deficiencies within Asteraceae
714 pollen. Therefore, Asteraceae flowers were opportunistically targeted resulting in 18 more species
715 being collected. During collection visits for target species, plants which produced easily extractable
716 pollen or would contribute to wider taxonomic coverage in the dataset were also opportunistically
717 targeted. This led to the inclusion of 88 more species, 16 of which could not be identified to
718 species level. In total, 700 samples covering 295 taxonomic units (including those identified to
719 species, genus or subspecies level), were collected, 125 of which were represented by a single
720 sample. A full summary of samples collected, collection months and counties can be seen in
721 Supplementary Table S2.1.Pollen collection

722 Samples were collected from a range of sites in England including nature reserves, botanic
723 gardens, public land and private gardens from spring to early autumn from 2021-2022. Collection
724 months and counties for all samples are shown in Table S2.1 along with anonymised collector
725 codes for all 13 collectors. Flowering heads, or branch tips in the case of woody species, were cut

726 from the plant and placed in sealed sandwich bags. These were then taken back to the laboratory
727 and placed in a beaker of room temperature tap water to dehisce. The duration needed for anthers
728 to dehisce ranged from a few hours to a few days, but most were left for 24 hours. Once pollen had
729 been produced it was removed from the flower using a clean paintbrush and forceps under a
730 magnifying glass. Pollen was collected onto an ethanol-wiped glass microscope slide and visually
731 inspected for debris or insects which were subsequently removed. The pollen sample was then
732 scraped into a clean 1.5ml Eppendorf tube or glass vial using an ethanol-wiped razor blade. The
733 exact method of pollen extraction varied depending on species and floral morphology. For daisy-
734 type Asteraceae (Asteroideae), pollen was brushed from the tops of disk florets in clumps. For
735 thistle-type Asteraceae (Carduoideae), pollen was brushed from anther tube tips and tubes
736 squeezed gently to remove pollen further. For legume-type Fabaceae flowers, wing petals were
737 removed and the tip of the keel lightly squeezed, as for *Lotus corniculatus*, or all petals removed
738 entirely, as for *Trifolium pratense*. Pollen samples were then frozen at -20°C before being moved
739 to -80°C in batches. Freezing pollen has been shown to prevent nutritional degradation (Hagedorn
740 and Burger, 1968; Pernal and Currie, 2000).

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

745 A minimum of 10 mg fresh weight pollen per sample was required due to the lower end of pollen
746 total sterol content being 0.1%, i.e., 10 µg of sterol in a 10 mg sample, a level within the detectable
747 range of our analysis. At the end of the collection season all samples were transferred to 1.5 ml
748 glass vials and partitioned or combined by species, depending on whether the sample was over or
749 under 10 mg. The final weight of each sample was between 9.5-16.5 mg. Two samples were
750 included which weighed 4.9 mg and 5.4 mg (*Lysimachia vulgaris* and *L. punctata*) to provide a

751 replicate for these species which are of foraging relevance to the bee *Macropis europaea*. Both
752 species also had one >10 mg sample to which the lower weight samples were similar in
753 quantitative and qualitative sterol content.

754 **Sample preparation**

755 Sample analysis was done in collaboration with staff at RBG Kew. The samples were prepared for
756 running by EB, EM and SF. Alongside the hand collected pollen samples, three other sample types
757 were used in the sample run to monitor performance:

758 Quality Assurance (QA) samples contained a solution of reference materials compiled to assign
759 names to signals in our samples. This allowed us to assess the consistency of measurements by the
760 instrument and calculate the coefficient of variance. Reference materials verified by multi-nuclear
761 NMR were as follows: 24-methylenecholesterol, 24-methylenecycloartenol, anhelsterol (a sterol
762 found in *C. anthelmintica*, ST(28:3), that is active under 330 nm UV but whose structure has not
763 been determined formally using NMR), avenasterol, β -sitosterol, brassicasterol, campesterol,
764 cholesterol, cycloartenol, cycloeucalenol, cyclolaudenol, desmosterol, episterol, ergosterol,
765 isofucosterol, schottenol, sitostanol, spinasterol and stigmasterol. The m/z and retention time for
766 each sterol is shown in Table S2.2. Aliquots of 40 μ l were used in the run. A Quality Control (QC)
767 stock solution was made by homogenizing a set of commercially available honey bee collected
768 pollens: 95 g of *Helianthus annuus* (sunflower) pollen, 5 g of *Nymphaea* sp. pollen, 20 g of
769 *Fagopyrum esculentum* (buckwheat) pollen in 120 ml water. For this project three QC
770 concentrations were used: 100%, 50%, 25%, corresponding to 40 μ l, 20 μ l and 10 μ l aliquots of
771 the QC stock solution. These were analysed in the same way as the samples to calculate the
772 correlation between analyte concentration and signal size. Variables whose ratio was <0.75 were
773 discarded. All runs began with a blank containing LCMS quality methanol only. This was designed
774 to enable identification and removal of background noise.

775 **Sterol extraction**

776 To each vial of hand collected pollen or QC sample a 20 µl aliquot of 0.1 mg/ml (5.084
777 µMoles/sample) *d*₇-cholesterol as the internal standard (IS) was added with 300 µl methanoic KOH
778 (10 g:90 ml) and the vial vortexed for 20 seconds. Vials were then placed in drilled aluminium
779 heating blocks at 75°C in a water bath for two hours. This saponification was designed to release
780 sterols from conjugates (Evyugin *et al.*, 2023), such as sterol esters which are reported to be
781 common in pollen (reviewed in Ferrer *et al.*, 2017). The saponified material was then transferred to
782 a corresponding deep 96-well plate (96-well plate, Esslab Plate+™, 2.4 ml/well, glass-coated).
783 Pollen samples were randomised within plates and interspersed randomly with QA, QC and blank
784 (300 µl methanol and 20 µl IS) samples.

785 Extraction of the sterol fraction was carried out on the plate by SF using a 96-channel pipette
786 (liquid-handling robot, Integra Viaflow 96/384 channel pipette). To each well 500 µl DMT (250x2)
787 was added. DMT was dichloromethane (DCM) (3 parts), methanol (1 part) and triethylammonium
788 chloride (0.0005 parts, i.e. 500 mg/l), mixed and stored at room temperature out of direct sunlight.
789 A 500 µl aliquot of deionised water was then added and the mixture was agitated using the 96-
790 channel pipette. The plate was centrifuged for 2 min to separate layers (methanol + water, solid,
791 DMT + sterol). A 50 µl sample of extract (DMT & sterol) was transferred to the 384-well plate and
792 left for 30 min for the DMT to evaporate. After repeating this process four times, 150 µl of LCMS
793 grade methanol was added and the plate sealed with foil. A total of three 384-well plates were run
794 immediately in sequence, analysing 700 hand collected pollen samples and 281 analytical samples.
795 One pollen sample was later excluded because it had experienced prolonged exposure to room
796 temperature conditions during collection.

797 **Sample analysis and data processing**

798 SF designed and supervised the sample run, following (Furse, Martel, *et al.*, 2023). Instrument
799 output was also processed by SF as established in Furse, Martel, *et al.* (2023).

800 Instrument output reported mass to charge ratio (m/z), signal size and retention time. Signal size
801 output was first converted by dividing the signal area for each metabolite by the signal of internal
802 standard (d_7 -cholesterol). These relative abundance values were used for the majority of the data
803 analysis. To generate a value of total sterol production in sterol/fresh weight of pollen in mg/kg the
804 following formula was used:

805
$$\frac{\text{Sterol (mg)}}{\text{Pollen (kg)}} = \frac{\text{molecular mass of sterol} \left(\frac{\text{g}}{\text{mol}}\right) \times (\text{signal} \times 5.084e^{-12} \text{ mols}) \times 1,000,000}{\text{Starting weight (mg)}/1,000}$$

806 **Data analysis**

807 All data analysis was done in R version 4.2.3 (2023-03-15 ucrt) (R Core Team, 2023) and R studio
808 (2023.6.1.524) (Posit team, 2023). A total of 78 sterols and related compounds were identified in
809 the sample set, 24 of which were named using our reference materials, three newly named
810 (Agnosterol, Anthelsterol, Salisterol) and 51 which were identified to carbon count (CC) and
811 double bond (DB) saturation, displayed in brackets ST(CC:DB). A total of 295 taxonomic units
812 were analysed including those identified to genus, species and subspecies level. Summary statistics
813 were calculated using means of these 295 units with some then removed for species level and
814 phylogenetic analyses as detailed below.

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

819 NMDS were plotted using metaMDS(trymax=200, k=2, autotransform=FALSE). The lowest
820 number of axes was selected where stress was ≤ 0.2 (established cut off, (Bakker, 2024)). Stress
821 values reflect how well the ordination aligns with the original data. Proportion data (0-1) was used
822 for analysis, where zero denotes a sterol is absent from a sample, and one denotes it is completely
823 dominant. Proportions were arcsine square root transformed and converted into a Bray-Curtis
824 dissimilarity matrix using `vegdist(method="bray")`. Where NMDS stress value was < 0.2 a 2D
825 biplot as made using GGLOT2 (Wickham, 2016) and GGREPEL (Slowikowski, 2023) to show
826 significant ($p < 0.010$) and strong (> 0.7 or > 0.6 absolute value) associations. PERMANOVA was
827 calculated using `adonis2()` to test for significant differences between groups. `Betadisper()` and
828 `anova()` were used to confirm non-significant difference in dispersion. `Adonis2()` was also used to
829 test for pairwise comparisons between groups following Bakker (2024), using a Benjamini-
830 Hochberg correction which corrects for multiple testing to reduce the chances of false positives.
831 All functions were from VEGAN (Oksanen *et al.*, 2022) except `anova()` from the STATS package
832 (R Core Team, 2023). The STATS package from R (R Core Team, 2023) was used to calculate
833 summary statistics. Indicator Species Analysis (ISA) was carried out on arcsine square root
834 transformed data to accompany NMDS plots using `multipatt()` from the INDICESPECIES package
835 (De Cáceres and Legendre, 2009) to determine significant associations between groups and
836 individual sterols. ISA was done at the level of individual groups only and not for combinations of
837 groups.

838 **Phylogeny construction and phylogenetic signal**

839 A phylogenetic tree of the study species was constructed based on the date calibrated and rooted
840 tree from Zanne *et al.* (2014). The tree was pruned for our study species using `keep.tip()` from the
841 R package APE (Paradis and Schliep, 2019). Any synonyms present in the tree were manually
842 renamed to match the RBG Kew POWO taxonomy used in the project (19 species). In the case of
843 species which did not have a corresponding match on the Zanne phylogeny the following approach

844 was taken: Where a subspecies level match was not available, a species level branch was renamed
845 instead (2 species). Where a hybrid species was not available, a matching parent not already in the
846 dataset was renamed (5 species). Where a species match was not available, another species from
847 the same genus was renamed instead (7 species), but only where there was not already a species
848 correctly matched within that genus. Out of 295 taxonomic units, 34 could not be suitably placed
849 on the tree and were removed from phylogenetic analysis.

850 Families were checked for monophyly using `AssessMonophyly()` from the R package `MONOPHY`
851 (Schwery and O'Meara, 2016). A genus level tree was constructed from the species tree by
852 keeping only one species from each genus using `drop.tip()` from the R package `APE` (Paradis and
853 Schliep, 2019). The genus tree was plotted by creating a phy-data object using `treedata()` from
854 `GEIGER` (Pennell *et al.*, 2014). Family clades were identified using `getMRCA()` from `APE`
855 (Paradis and Schliep, 2019). Phylogeny was plotted with `ggtree()` from `GGTREE` (Yu *et al.*, 2017,
856 2018; Yu, 2020). Heatmap and bar graph associated with phylogeny were plotted using `ggplot()`
857 from `TIDYVERSE` (Wickham *et al.*, 2019). The composite graph was then plotted using `APLOT`
858 (Yu, 2023).

859 Of the 78 sterol compounds detected, the 69 that had a maximum proportion >1% were analysed
860 for phylogenetic signal (Zu *et al.*, 2021). To determine if there was a phylogenetic signal in sterol
861 production across the data, whereby closer related species would share more similar profiles than
862 expected by chance, Pagel's λ and Blomberg's K were calculated. Both metrics use a Brownian
863 model of trait evolution and in both cases a value of 0 indicates the trait has evolved independently
864 of phylogeny, therefore closely related species are not more similar to each other than distantly
865 related ones. Low phylogenetic signal can mean high rates of evolution are causing big differences
866 between closely related species or perhaps convergent evolution is causing distant relatives to be
867 more similar than close ones. High phylogenetic signal (K , $\lambda=1$) can be caused by low rates of
868 evolution or stabilising selection. Interpretation of high and low phylogenetic signal varies between

869 studies (Kamilar and Cooper, 2013). Phylo4d objects were generated from species tree and
870 proportion data using phylo4d() from PHYLOBASE (R Hackathon et al., 2020) and phyloSignal()
871 from the R package PHYLOSIGNAL (Keck *et al.*, 2016) used to calculate Pagel's λ and
872 Blomberg's K. The number of iterations was set to 999. Quantitative values (mg/kg) were used for
873 total sterol content and proportions (0-1) for all other sterol traits. To identify trends in different
874 types of sterols, proportion data was also grouped according to carbon count and position of the B-
875 ring double bond. Ergosterol was classed as NA for double bond position as it contains double
876 bonds at positions 5 and 7 and sterols with a cyclopropane ring were classed as double bond
877 absent/ Δ 0, full details are available in Table S2.3.

878 **Inter-species sterol diversity**

879 Prior to analysis of inter-species differences, 17 samples identified to only genus level where there
880 was already a species level representative in the genus were removed. For families which
881 contained at least three genera, species data were used to create a box plot showing range in total
882 sterol production (mg/kg) across families.

883 Species within the family Asteraceae were grouped according to subfamily: Asteroideae (n=21,
884 daisy-type), Cichorioideae (n=17, dandelion-type), Carduoideae (n=14, thistle-type). Subfamily
885 classifications were taken from the Open Tree of Life version 14.7 (OpenTreeOfLife *et al.*, 2019)
886 and (Mandel *et al.*, 2019). Mean sterol proportions were calculated for each species. Sterols were
887 then grouped by B-ring double bond position (absent/ Δ 0, Δ 5, Δ 7, Δ 8 or NA) and their proportions
888 summed (sterols with a cyclopropane ring were classed as absent/ Δ 0, double bond position data is
889 available in Table S2.3). Ergosterol was classed as NA for double bond position as it contains
890 double bonds at positions 5 and 7. A subtree of the species tree generated above was plotted to
891 show phylogenetic trends in the data across Asteraceae species. Proportion data (0-1) was arcsine
892 square root transformed and a Kruskal-Wallis rank sum test carried out using `kruskal.test()`, for

893 each double bond group, followed by a post-hoc Dunn test using `dunnTest()` from the FSA package
894 (Ogle *et al.*, 2023) to test for differences between Asteraceae subfamilies and the rest of the
895 dataset. Both analyses used a Benjamini-Hochberg correction for multiple testing.

896 Species within the family Rosaceae were grouped according to subfamily (Amygdaloideae (n=9),
897 Rosoideae (n=13)). Subfamily classifications were taken from the Open Tree of Life version 14.7
898 (OpenTreeOfLife *et al.*, 2019). Mean sterol proportions were calculated for each species. Sterols
899 were then grouped by carbon chain length (27, 28, 29, 30, 31) and their proportions summed
900 (carbon chain length data in Table S2.3, un-named sterols not shown). A subtree of the species tree
901 generated above was plotted to show phylogenetic trends in the data across Rosaceae.

902 Genera which contained at least five species were used to analyse intra-genus variation in sterol
903 profile. Mean values were calculated by species before being converted to proportions (0-1). Data
904 was used to calculate an NMDS and PERMANOVA to test for significant differences in sterol
905 between genera (stress value=0.099) (Figure 2.7).

906 **Intra-species sterol variation**

907 A total of 48 species were collected from at least three counties in England. As 48 groups would be
908 too many to separate on an NMDS, the species with the highest number of samples were chosen
909 for statistical comparison. Species which were sampled from four different counties and had a least
910 five samples overall were used. An NMDS and PERMANOVA were used to test for significant
911 differences between the most well sampled species (stress value=0.096) (Figure 2.10).

912 To compare the effect of collection county on total sterol production, data was subset as follows:
913 Total sterol (mg/kg) was calculated for all samples, data was filtered to only include species which
914 had been collected from at least three counties. Counties were then ranked by the number of these
915 species they included and those with ≥ 10 kept (Oxfordshire (33), Greater London (25), County

916 Durham (21), North Yorkshire (20), Sussex (10)). This led to a total of 35 species across five
917 counties being included in the analysis. A linear mixed-effects model was created with total sterol
918 production (mg/kg) as the response variable and collection county as a fixed effect. Species
919 identity was included as a random effect. Where a species had been collected more than once from
920 a county the mean value was used for that county. Total sterol data was \log_{10} transformed in order
921 to reduce non-normality of the residuals. Model was run using `lme()` from the NLME package
922 (Pinheiro, Bates and R Core Team, 2023). The residuals of the fixed effects showed normal
923 distribution (Shapiro-Wilk test: $w=0.986$, $p>0.100$) and homogeneity of variance (Levene test:
924 $F_{4,104}=0.6845$, $p>0.500$). Random effects also showed normal distribution (Shapiro-Wilk test:
925 $w=0.962$, $p>0.100$).

926 **Ecological associations of sterol profiles**

927 All available visitation data from The Database of Pollinator Interactions (DoPI) (Balfour et al.,
928 2022) were downloaded on 22-03-23 in four blocks covering all available year options. This was
929 then filtered for any plant genera present in our list of species and a total of 221 species from our
930 dataset were found to be present in the database. The insect names in the database were then
931 searched for their GBIF ID number which was used to extract higher classification data from GBIF
932 using the `get_gbifid()` and `classification()` functions in the package TAXIZE (Scott Chamberlain
933 and Eduard Szocs, 2013; Chamberlain et al., 2020).

934 Plant species with available visitation data were then grouped based on whether there were any
935 records of pollen collection visits by honey bees (*Apis mellifera*) (n=30), bumble bees (*Bombus*
936 spp.) (n=40), both (n=35) or neither (n=116). Proportion data for the six sterols, 24-
937 methylenecholesterol, β -sitosterol, campesterol, cholesterol, desmosterol and isofucosterol, was
938 arcsine square root transformed. A Kruskal-Wallis test was then carried out for each sterol group
939 with a Benjamini Hochberg correction using `kruskal.test()` from the STATS package (R Core

940 Team, 2023), followed by a post-hoc Dunn test using `dunnTest()` from the FSA package (Ogle *et*
941 *al.*, 2023). This was repeated for the same data grouped by B-ring double bond position. Finally,
942 the sterol profile of pollens belonging to each of the groups was compared by NMDS and
943 PERMANOVA to test for differences in overall sterol profile.

944 **Results**

945 **Sterol diversity**

946 All 295 taxonomic units were used for the creation of summary statistics, including those identified
947 to genus or subspecies level. Only 21 sterols occurred at >40% in at least one taxonomic unit
948 (Table 2.1). Of these, only seven had a median occurrence of >1% showing the data are strongly
949 skewed to low values even when a sterol can occur in high concentration e.g., desmosterol (median
950 0.02%, max 83.50%) and cholesterol (median 0.01%, max 61.67%) (Table 2.1). Of the 57 sterols
951 with a maximum <40% only one had a median >1% (campesterol 1.78%), despite some occurring
952 at >35% in an individual taxonomic unit (data not shown). Nine sterols did not display at
953 maximum proportion above 1% in any sample. These sterols were removed from phylogenetic
954 analysis as they are potentially intermediates in sterol metabolic pathways. A further 29 did not
955 exceed a maximum of 5% in any species (data not shown).

956 Schottenol, 24-methylenecholesterol, desmosterol, cycloartenol and isofucosterol all occurred at
957 >75% in at least one taxonomic unit. However, β -sitosterol displayed the highest median value
958 (4.68%) despite a lower maximum value. The only sterols with a median of >2.5% were β -
959 sitosterol, isofucosterol and avenasterol.

960 Most pollens contained >60 sterols, including un-named ones, with very few containing 45 or less
961 (Table 2.2). Each taxonomic unit contained 61.75 ± 7.87 (mean \pm SD) sterols in comparison with
962 9.98 ± 4.46 (mean \pm SD) reported by Zu *et al.* (2021). *Potentilla reptans* contained the fewest sterols,
963 35, contrasted with *Alnus glutinosa* which contained 76. No individual taxonomic unit was found
964 to contain all 78 sterols.

965 Pollen sterol profiles were often dominated by 1-3 major sterol components (Figure 2.2). Half of
966 the 78 detected sterols occurred as one of these top three major sterol components in at least one

967 species (Table 2.3). Of these, β -sitosterol and isofucosterol were the most common major sterol
968 components, defined as ranking in top three by proportion, in over 100 taxonomic units. In total,
969 160 taxonomic units had a single sterol component which accounted for $\geq 40\%$ of their total sterol
970 content (Figure 2.2, Table 2.4). Again, β -sitosterol was the most common dominant component in
971 the dataset. Isofucosterol, avenasterol and 24-methylenecholesterol, were also frequently dominant
972 ($>40\%$). These sterols are highlighted in Tables 2.3 and 2.4.

973 Most sterols detected were present in $>75\%$ of taxonomic units (Table 2.5). Only four sterols were
974 detected in all: campesterol, cycloartenol, ST(30:2)B and ST(30:2)C (Table 2.6). They showed a
975 wide range in maximum percentage, from 30.23% in campesterol up to 77.92% in cycloartenol,
976 both of which had a median of $>1\%$. Cyclolaudenol, ST(30:2)A, ST(30:2)E and ST(31:2)A were in
977 all but one taxonomic unit. Avenasterol, 24-methylenecholesterol and schottenol were in all but
978 two.

979 Ergosterol, a sterol specific to yeasts and not produced by higher plants and so likely a fungal
980 contaminant of pollen, was detected in 253 taxonomic units however it never accounted for
981 $>1.33\%$ in a species (*Lunaria annua*), the maximum in an individual sample (*Ranunculus acris*)
982 was 1.40% and had a median proportion across the dataset of 0.01%. Of the 12 samples where
983 ergosterol occurred $>0.75\%$ nine belonged to *Ranunculus* spp.

984 **Table 2.1.** Maximum, minimum, median and Inter Quartile Range (IQR) percentage of sterols
 985 which occurred at >40% in at least one species. Statistics were calculated from species/subspecies
 986 means, (genus where species was not available). Sterols which have a median of >2.5% are
 987 highlighted in grey. For un-named sterols, carbon count and saturation are shown in brackets.

Sterol	Max (%)	Min (%)	Median (%)	IQR (%)
24MC	91.03	0.00	1.51	11.47
Schottenol	88.24	0.00	0.25	5.04
Desmosterol	83.50	0.00	0.02	0.05
Cycloartenol	77.92	0.02	2.32	4.25
Isofucosterol	76.41	0.00	3.29	14.64
ST(29:1)C	70.94	0.00	0.13	0.33
Avenasterol	69.79	0.00	2.58	12.95
ST(30:2)B	65.65	0.02	0.22	0.50
β -Sitosterol	64.90	0.00	4.68	19.98
Cycloartanol	62.49	0.00	0.03	0.11
Episterol	61.76	0.00	0.02	0.14
Cholesterol	61.67	0.00	0.01	0.11
Cycloeucaleanol	60.85	0.00	1.67	4.59
ST(27:2)D	60.06	0.00	0.00	0.02
ST(27:1)B	59.69	0.00	0.00	0.00
Obtusifoliol	56.75	0.00	1.18	3.04
Lathosterol	55.95	0.00	0.06	0.29
ST(30:1)	49.62	0.00	0.02	0.15
24MD	45.24	0.00	0.07	0.31
ST(30:2)A	44.07	0.00	0.19	0.24
ST(30:2)E	40.37	0.00	0.49	1.24

988

989

990 **Table 2.2.** The number of sterols detected in each taxonomic unit in the dataset.

Number of sterols detected	Number of species
76-70	49
69-60	133
59-50	92
<50	21

991

992 **Table 2.3.** Sterols most commonly occurring within the top three sterols by proportion and ordered
 993 by number of taxonomic units for which this was the case. For un-named sterols, carbon count and
 994 saturation are shown in brackets.

Sterol	No. of species ranked in top 3 by proportion	Sterol	No. of species ranked in top 3 by proportion
β-Sitosterol	127	ST(28:3)B	9
Isofucosterol	120	24MCA	8
Avenasterol	98	ST(28:1)A	8
24MC	89	Cholesterol	7
Schottenol	61	ST(30:2)C	7
Cycloeucalenol	55	ST(27:1)B	3
Campesterol	46	ST(27:2)E	3
Cycloartenol	45	ST(29:1)C	3
Obtusifoliol	38	Brassicasterol	2
ST(30:2)E	23	ST(27:1)D	2
Desmosterol	20	ST(30:1)	2
Cyclolaudenol	13	ST(31:2)A	2
ST(30:2)B	13	ST(31:2)B	2
Episterol	12	ST(28:3)A	1
24MD	11	ST(29:0)C	1
ST(30:2)F	11	ST(30:2)A	1
24MP	10	ST(30:2)D	1
Cycloartanol	10	Salisterol	1
Lathosterol	10	Stigmasterol	1
ST(27:2)D	9		

995

996 **Table 2.4.** Sterols occurring as major components (>40%) and ordered by number of taxonomic
 997 units they occur in at this proportion. For un-named sterols carbon count and saturation is shown
 998 in brackets.

Sterol	Number of species with $\geq 40\%$	Sterol	Number of species with $\geq 40\%$
β -Sitosterol	34	Cycloartanol	3
24MC	25	Cholesterol	2
Avenasterol	19	Lathosterol	2
Isofucosterol	19	ST(30:2)B	2
Desmosterol	13	Episterol	1
Schottenol	8	ST(27:1)B	1
Cycloeucalenol	7	ST(29:1)C	1
Obtusifoliol	7	ST(30:1)	1
Cycloartenol	6	ST(30:2)A	1
ST(27:2)D	5	ST(30:2)E	1
24MD	3		

999 **Table 2.5.** Summary of sterol occurrence across dataset. Almost half of the 78 sterols detected
 1000 occurred in $\geq 90\%$ of sampled taxonomic units.

1001

Species present in	Number of sterols
90-100%	35
75-90%	15
50-75%	18
<50%	10

1002

1003 **Table 2.6.** Maximum, minimum, median and Inter Quartile Range (IQR) of sterols which were
 1004 present in all taxonomic units analysed (minimum species value in dataset >0). Statistics were
 1005 calculated from species means. For un-named sterols, carbon count and saturation are shown in
 1006 brackets.

1007

Sterol	Max (%)	Min (%)	Median (%)	IQR (%)
Cycloartenol	77.92	0.02	2.32	4.25
ST(30:2)B	65.65	0.02	0.22	0.50
ST(30:2)C	36.51	0.04	0.74	1.15
Campesterol	30.23	0.01	1.78	3.90

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Distribution of total sterol contents between sterol components

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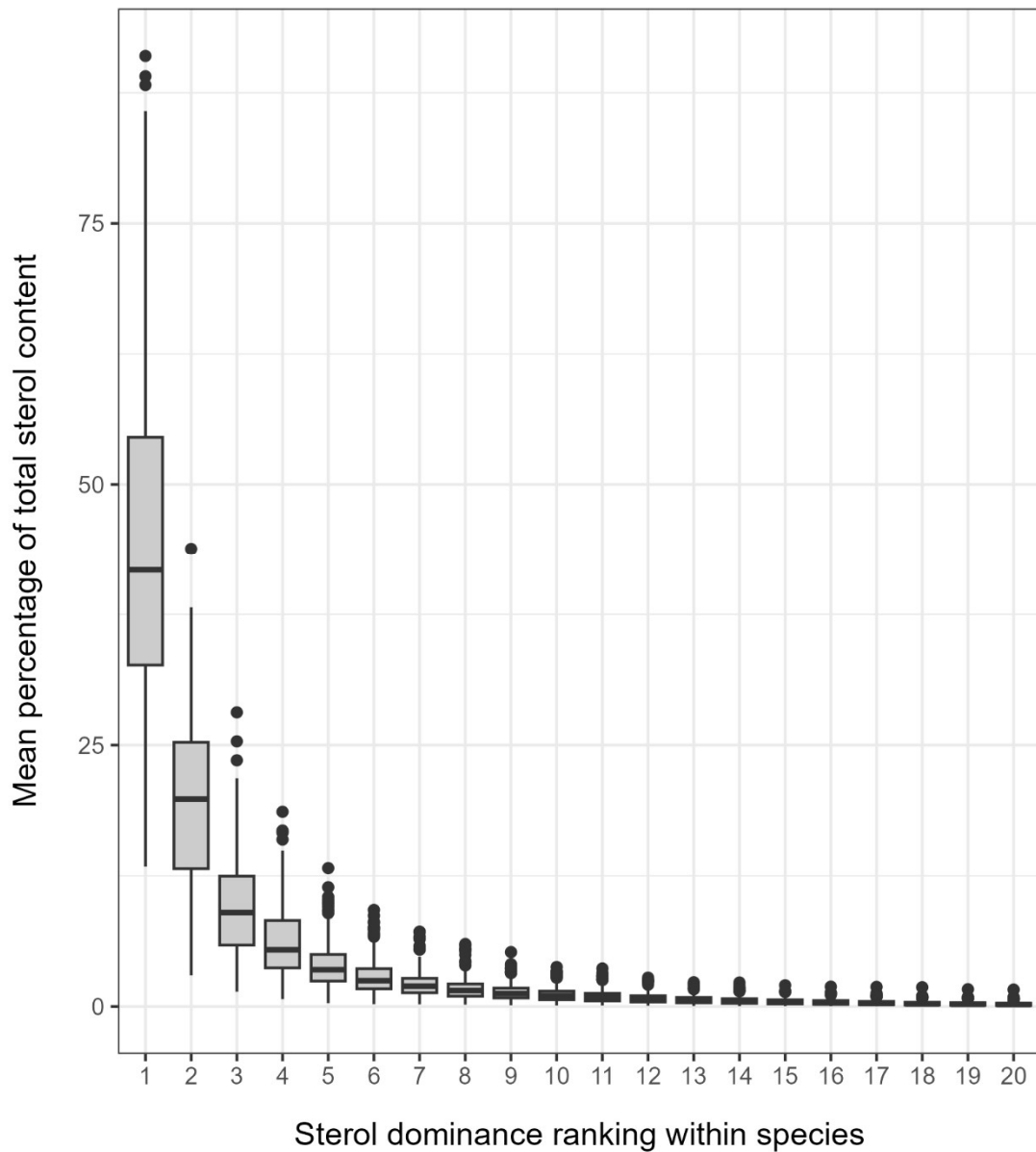
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Figure 2.2. Median percentage and Inter Quartile Range (IQR) of the most dominant 20 sterol components within each taxonomic unit, ranked by proportion in the sample. For all taxonomic units, despite most containing >60 sterols, the most dominant 20 sterols accounted for at least 90% of total sterol content. The identity of these 20 sterols varied among taxonomic units.

1032 **Inter-species sterol diversity**

1033 *Total sterol production and the proportions of individual sterols have a phylogenetic signal*

1034 Out of the 295 taxonomic units sampled, 261 could be placed on the Zanne *et al.* (2014) calibrated
1035 phylogeny using true matches, synonyms and in the cases of monotypically sampled genera, a non-
1036 matching species renamed and used as a branch tip. This covered 183 genera in 54 families.

1037 Of the 69 sterols analysed for phylogenetic signal, 31 showed significant signal for both metrics
1038 tested (K and λ), shown in Table 2.7. Ergosterol was omitted from this table despite its significant
1039 phylogenetic signal as it is not part of the sterol synthesis pathway in plants. Twenty-six sterols
1040 were significant for a single metric (all Pagel's λ except salisterol). Total sterol content, all carbon
1041 number groups except 31 and double bond groups 0, 5 and 7 also showed significant phylogenetic
1042 signal for both metrics tested (K and λ) (Table 2.7). These signals can be seen in Figure 2.3 and 2.4
1043 where trends for high total sterol content can be seen in Brassicaceae and Asteraceae.

1044 These two structural traits, carbon count and B-ring double bond position, are loosely associated
1045 with the importance of sterols for honey bee physiology as bees have evolved from relying solely
1046 on cholesterol, a C₂₇ sterol with Δ^5 double bond. Carbon chain length can affect membrane
1047 fluidity (reviewed in Furse *et al.*, 2023) and multiple bee species have been shown to contain high
1048 proportions of Δ^5 sterols in their tissues (Vanderplanck, Zerck, *et al.*, 2020a).

1049 *There are large differences in total sterol production between families*

1050 Pollen showed a hundred-fold range in total sterol content between species; *Taraxacum* sect.
1051 *Taraxacum* contained 75,423.52 mg/kg sterol/fresh weight pollen while *Malva sylvestris* contained
1052 729.98 mg/kg. Asteraceae accounted for some of the highest and lowest sterol contents (Table 2.8,
1053 Figure 2.4). Specifically, Cichorioideae subfamily flowers showed a consistently higher total sterol
1054 production than the other Asteraceae flowers (Figure 2.3).

1055 The four most frequently sampled families were Asteraceae (33 genera, 52 spp.), Rosaceae (15
1056 genera, 22 spp.), Fabaceae (13 genera, 24 spp.) and Lamiaceae (11 genera, 14 spp.). Brassicaceae
1057 showed the highest sterol content on average with all species sampled producing a higher mg/kg
1058 content of sterol than all families except Asteraceae. Asteraceae showed the highest range in sterol
1059 quantity (Table 2.8) but was still dominated by low total sterol producing species.

1060 *Asteraceae flowers produce lower proportions of $\Delta 5$ sterols than other flowers*

1061 Asteraceae species had similar levels of $\Delta 5$ and $\Delta 7$ sterols, or a dominance of $\Delta 7$ sterols in the case
1062 of Carduoideae. In contrast, the general trend of all other species (n=226) was that their sterolome
1063 was dominated by $\Delta 5$ sterols (Figure 2.5). There were significant differences between groups in all
1064 double bond classes ($\Delta 0$: $H(3)=49.772, p<0.001$, $\Delta 5$: $H(3)=55.718, p<0.001$, $\Delta 7$: $H(3)=16.974,$
1065 $p<0.001$, $\Delta 8$: $H(3)=14.047, p<0.005$, NA: $H(3)=41.833, p<0.001$). The results of further post-hoc
1066 Dunn tests showed that all Asteraceae subfamilies showed significantly higher proportions of $\Delta 0$
1067 and ΔNA sterols and lower proportions of $\Delta 5$ sterols than all other families ($\Delta 0$: $p<0.005$, NA:
1068 $p<0.001$, $\Delta 5$: $p<0.005$). Carduoideae had significantly higher $\Delta 7$ sterols than all other families
1069 ($p<0.001$) and significantly lower $\Delta 8$ than all other groups (other families: $p<0.010$, Asteroideae:
1070 $p<0.005$, Cichorioideae: $p<0.005$) though this was the lowest proportion double bond class in all
1071 groups.

1072 *Rosaceae pollens show distinct patterns in sterol carbon chain length between sub-families*

1073 As well as grouping by B-ring saturation, sterols can also be grouped according to hydrocarbon
1074 chain length. Rosaceae was the second most well sampled family after Astereaceae and when
1075 proportion data was grouped by carbon chain length, there were clear differences between the
1076 Rosaceae subfamilies Amygdaloideae and Rosoideae. Amygdaloideae species were characterised
1077 by a dominance of C28 sterols. All species sampled in this subfamily are trees and are closely
1078 related. Conversely, the Rosoideae species show a wider variety of habits but were majority

1079 dominated by C27 sterols (Figure 2.6). *Geum urbanum* and *Filipendula ulmaria* both showed
1080 profiles distinct from these two main groups, characterised by higher C30 sterols in *F. ulmaria* and
1081 higher C31 in *G. urbanum*.

1082 ***Species within a genus display similar sterol profiles despite being adapted to different habitats***
1083 ***due to the strong phylogenetic signal in sterol composition***

1084 Six genera in the dataset contained at least five different species and so were used for an NMDS to
1085 determine if sterol profiles were more similar among species of the same genus, as was expected
1086 from the strong phylogenetic signal in the data, despite growing in different habitats. Figure 2.7
1087 shows the sterol profile of all species and the resulting NMDS biplot (stress value 0.099). Species
1088 were from four different families: *Trifolium* from Fabaceae, *Plantago* and *Veronica* from
1089 Plantaginaceae, *Cirsium* from Asteraceae and *Salix* from Salicaceae. A PERMANOVA run on the
1090 NMDS data showed species grouped significantly by genus ($F_{4,20} = 14.400, p=0.001$) and a high
1091 proportion of the variance in the dataset was explained by genus ($R^2=0.742$). Pairwise comparisons
1092 showed all genus groups differed significantly from each other (all comparisons $p<0.050$).
1093 However, the extent of variation in sterols differed significantly between genera ($F_{4,20} = 3.889,$
1094 $p<0.050$). In particular, *Veronica* and *Plantago* pollen sterols varied more than the other four
1095 genera, this appeared to be driven by one to two species within each genus as seen in Figure 2.7A
1096 (*P. lanceolata* and *V. filiformis* and *V. chamaedrys*). Figure 2.7 also shows the similarities between
1097 distantly related genera, for instance *Trifolium*, *Veronica* and *Plantago* were characterised by
1098 higher levels of 24-methylenecholesterol. *Cirsium* pollen is strongly associated with stigmasterol
1099 and cycloeucalenol. ISA showed *Cirsium* was significantly associated with the most sterols (28),
1100 including lophenol, schottenol, cycloartanol, sitostanol, cholesterol and stigmasterol. It also
1101 showed the strongest individual associations. *Salix* was strongly associated with isofucosterol, β -
1102 sitosterol and 24MCA. *Trifolium* was significantly associated with avenasterol, 24-

1103 methylenecholesterol and campesterol. *Plantago* and *Veronica* were associated with the fewest
 1104 sterols, three each (Table 2.9).

1105 **Table 2.7.** Phylogenetic signal analysis on proportions of all detected sterols with a maximum
 1106 proportion >1%, total sterols (mg/kg), proportion of different carbon length sterols (light grey)
 1107 and proportion of different B-ring double bond sterols (dark grey). Only variables which were
 1108 significant for both Blomberg's *K* and Pagel's λ are shown.

Group	K	λ	p K	p λ	Group	K	λ	p K	p λ
24MC	0.202	0.951	0.001	0.001	ST(28:2)B	0.363	0.818	0.003	0.001
24MD	0.091	0.943	0.016	0.001	ST(28:2)C	0.210	0.764	0.003	0.001
24MP	0.050	0.879	0.032	0.001	ST(28:3)A	0.059	0.668	0.026	0.002
Agnosterol.	0.071	0.625	0.016	0.001	ST(28:3)B	0.979	0.817	0.001	0.001
Anthelsterol.	0.083	0.667	0.006	0.001	ST(29:0)A	0.306	1.000	0.018	0.001
Avenasterol	0.047	0.579	0.009	0.001	ST(29:0)C	0.061	0.951	0.002	0.001
β -Sitosterol	0.101	0.965	0.001	0.001	ST(29:1)A	0.259	0.998	0.012	0.001
Brassicasterol	0.227	1.000	0.004	0.001	ST(29:2)B	0.112	1.000	0.010	0.001
Campesterol	0.226	0.977	0.001	0.001	ST(29:2)D	0.131	0.961	0.007	0.001
Cycloartenol	0.128	0.986	0.013	0.001	ST(30:2)E	0.080	0.952	0.015	0.001
Desmosterol	0.091	0.950	0.015	0.001	ST(31:2)B	0.190	0.997	0.029	0.001
Isofucosterol	0.078	0.836	0.006	0.001	Total Sterol	0.070	0.814	0.010	0.001
Schottenol	0.047	0.898	0.034	0.001	$\Delta 0$	0.060	0.920	0.009	0.001
Sitostanol	0.060	0.848	0.045	0.001	$\Delta 5$	0.069	0.880	0.001	0.001
Spinasterol	0.157	0.991	0.005	0.001	$\Delta 7$	0.042	0.762	0.011	0.001
Stigmasterol	0.080	0.985	0.012	0.001	27	0.094	0.962	0.003	0.001
ST(27:1)A	0.386	0.997	0.007	0.001	28	0.252	0.980	0.001	0.001
ST(27:2)B	0.135	0.963	0.013	0.001	29	0.100	0.949	0.001	0.001
ST(28:1)A	0.104	0.797	0.023	0.001	30	0.054	0.859	0.001	0.001

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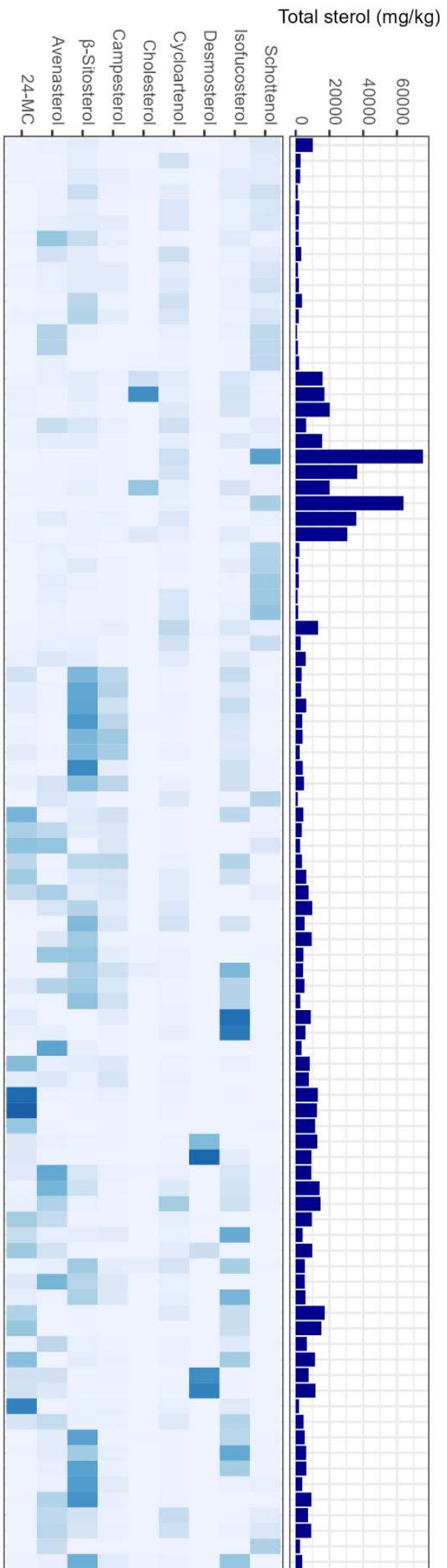
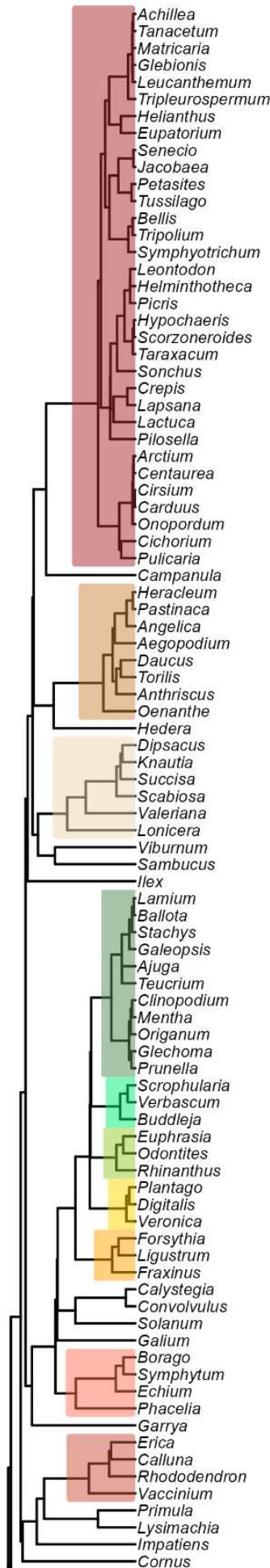
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Family

- Asteraceae
- Apiaceae
- Caprifoliaceae
- Lamiaceae
- Scrophulariaceae
- Orobanchaceae
- Plantaginaceae
- Oleaceae
- Boraginaceae
- Ericaceae
- Caryophyllaceae
- Rosaceae
- Betulaceae
- Fagaceae
- Fabaceae
- Brassicaceae
- Malvaceae
- Ranunculaceae
- Papaveraceae
- Asparagaceae
- Amaryllidaceae

Percentage



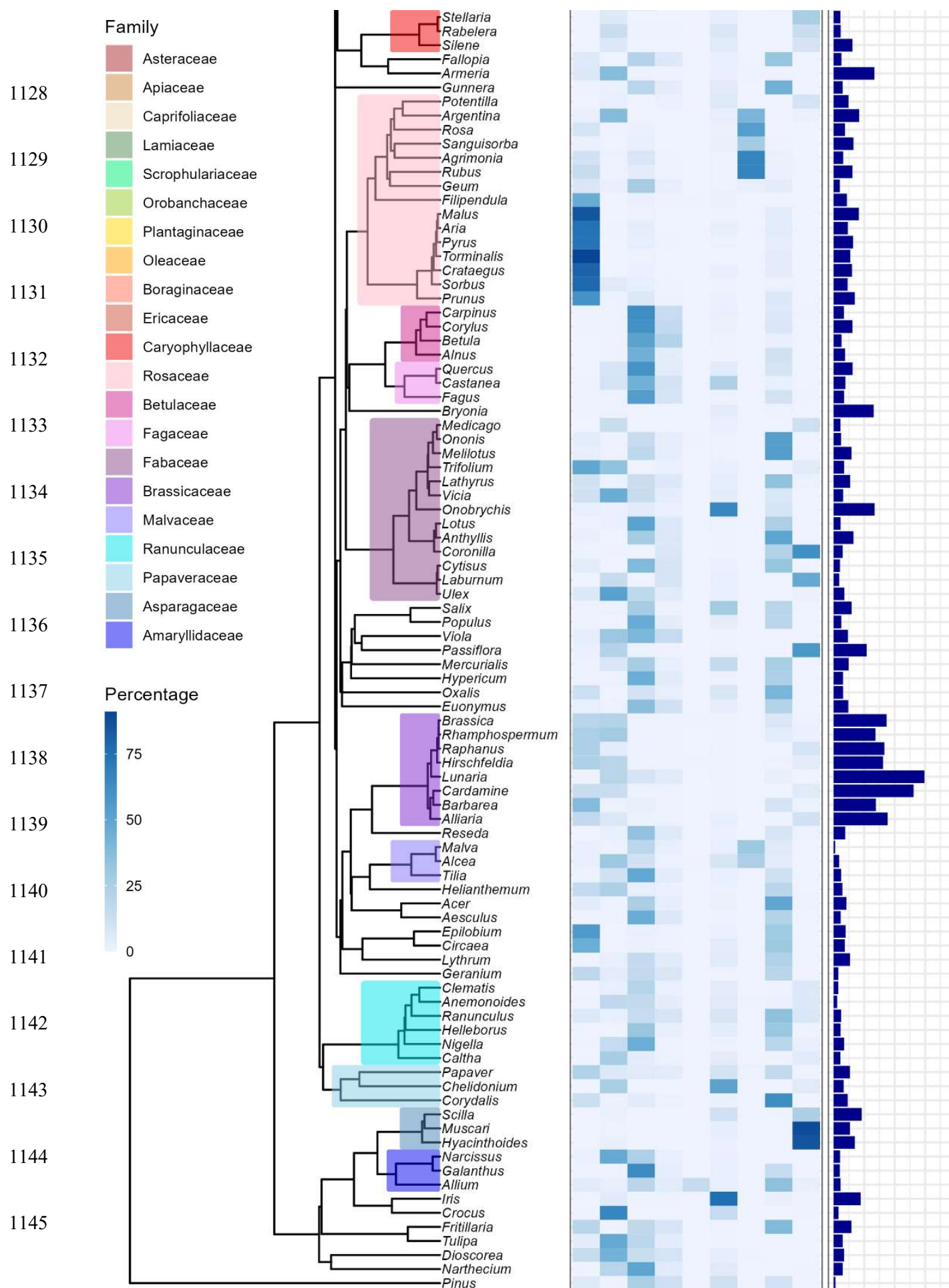


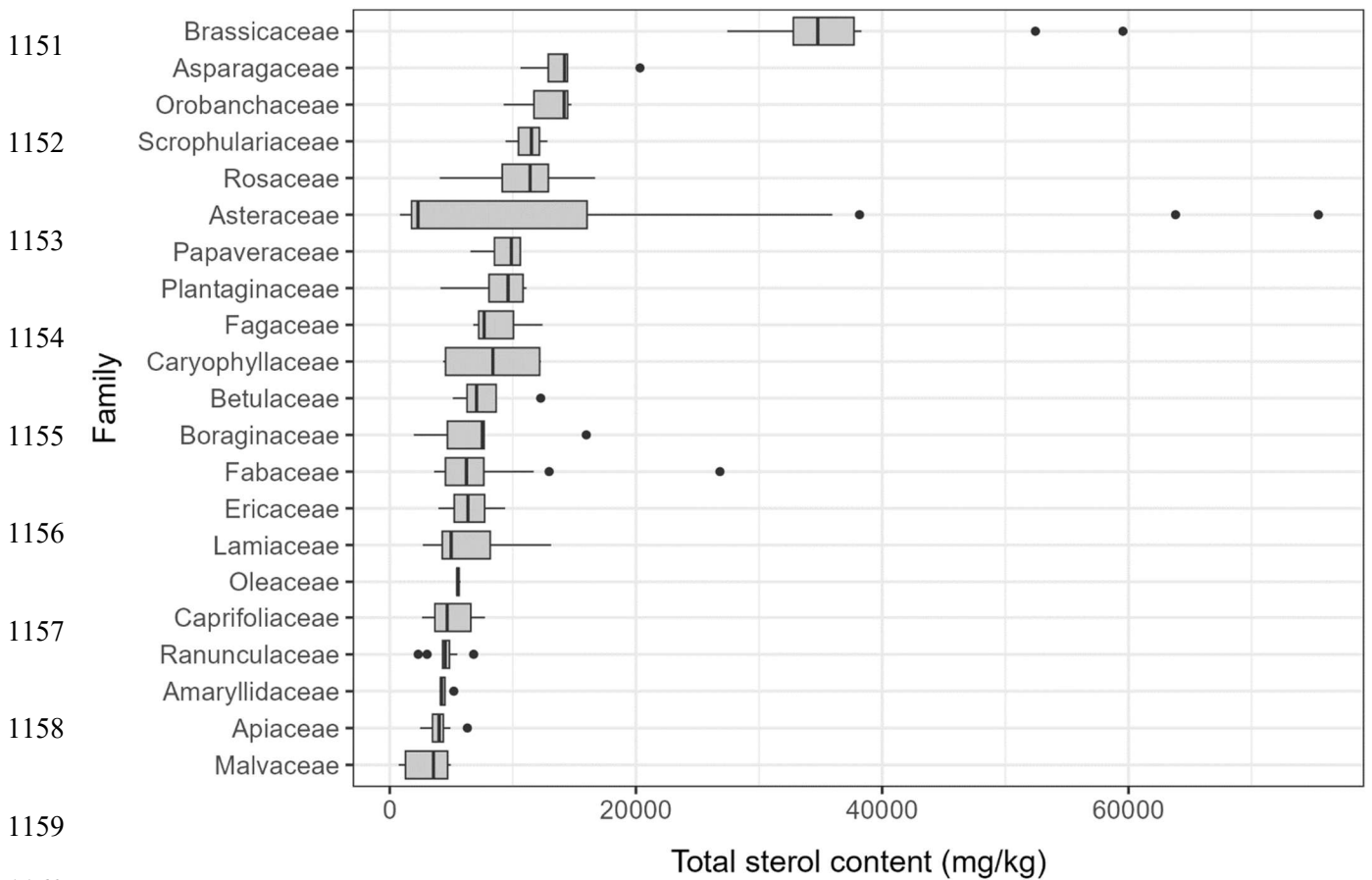
Figure 2.3. Genus phylogeny highlighting family clades which contain ≥ 3 genera. Heatmap displays proportions for sterols which have significant phylogenetic signal and are commonly major components, plus cholesterol. Bar chart shows total sterol production. 24-MC = 24-methylenecholesterol.

1146 **Table 2.8.** Species with the highest and lowest total sterol content (mg/kg). Values were calculated
 1147 as species means. Highlighted rows belong to the family Asteraceae and dominate both the highest
 1148 and lowest values in the dataset.

Species	Total Sterol (mg/kg)	Species	Total Sterol (mg/kg)
<i>Taraxacum</i> sect. <i>Taraxacum</i>	75423.521	<i>Malva sylvestris</i>	729.979
<i>Lapsana communis</i>	63824.545	<i>Centaurea cyanus</i>	827.781
<i>Lunaria annua</i>	59551.783	<i>Bellis perennis</i>	831.199
<i>Cardamine pratensis</i>	52441.401	<i>Carduus nutans</i>	1072.098
<i>Brassica rapa</i>	38312.124	<i>Pinus coulteri</i>	1136.277

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1161 **Figure 2.4.** Brassicaceae pollen showed the highest median total sterol content, but Asteraceae
 1162 pollen showed the widest range in values. Asteraceae is also the most sampled family. Only
 1163 families containing at least three genera are shown. Boxes show first and third quartile, median
 1164 and 1.5 x inter quartile range. Data averaged by species, grouped by family, ranked by mean.

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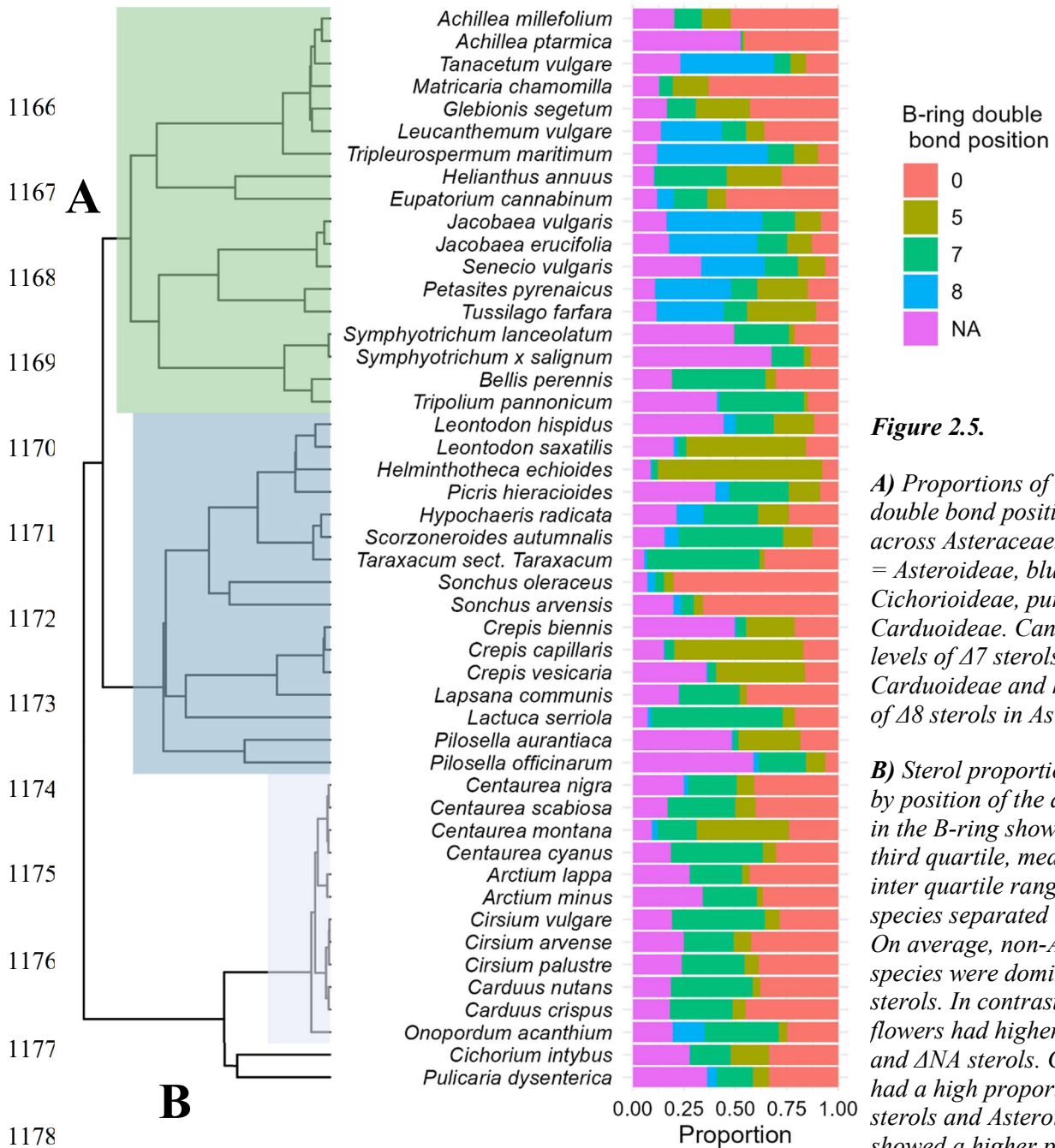
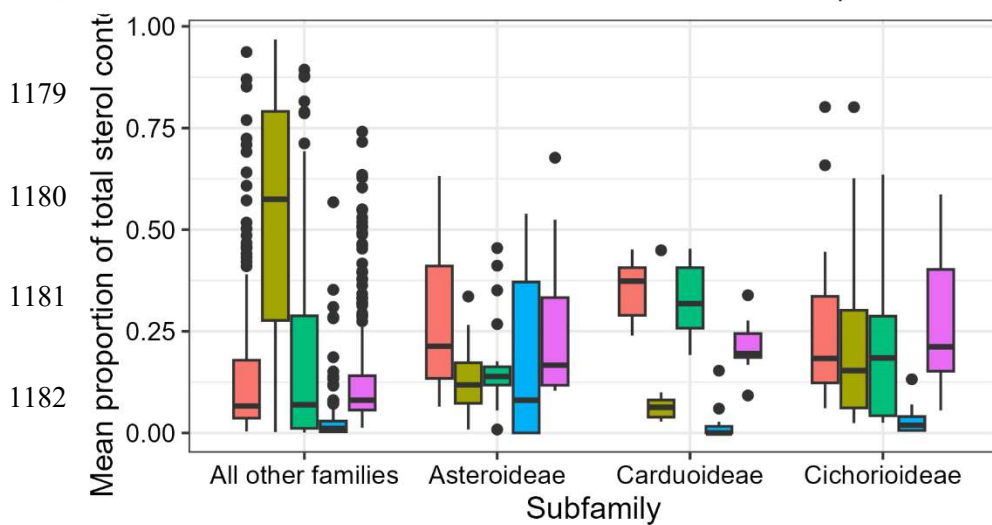
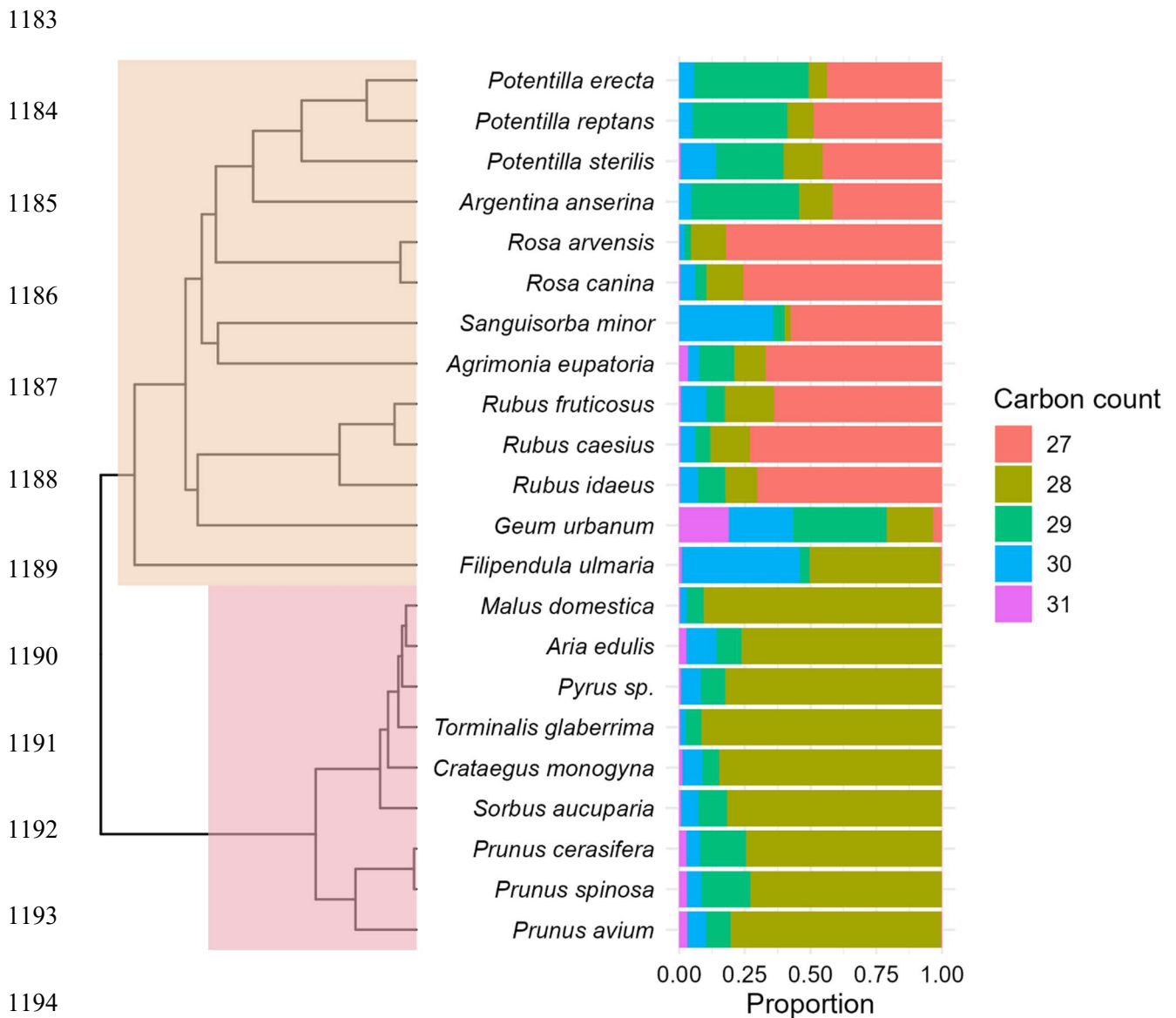


Figure 2.5.

A) Proportions of different double bond position sterols across Asteraceae. Green clade = Asteroideae, blue clade = Cichorioideae, purple clade = Carduoideae. Can see higher levels of $\Delta 7$ sterols in Carduoideae and higher levels of $\Delta 8$ sterols in Asteroideae.

B) Sterol proportions grouped by position of the double bond in the B-ring showing first and third quartile, median and 1.5 x inter quartile range. Asteraceae species separated by subfamily. On average, non-Asteraceae species were dominated by $\Delta 5$ sterols. In contrast Asteraceae flowers had higher levels of $\Delta 0$ and ΔNA sterols. Carduoideae had a high proportion of $\Delta 7$ sterols and Asteroideae also showed a higher proportion of $\Delta 8$ sterols. Replicates for each group: Asteroideae (21 spp), Cichorioideae (17 spp), Carduoideae (14 spp), all other species (226 spp).





1195 **Figure 2.6.** Proportions of sterols with different hydrocarbon chain length across Rosaceae. Peach
 1196 clade = Rosoideae and pink clade = Amygdaloideae. Rosoideae species show higher proportions
 1197 of C27 sterols and Amygdaloideae species show higher proportions of C28 sterols.

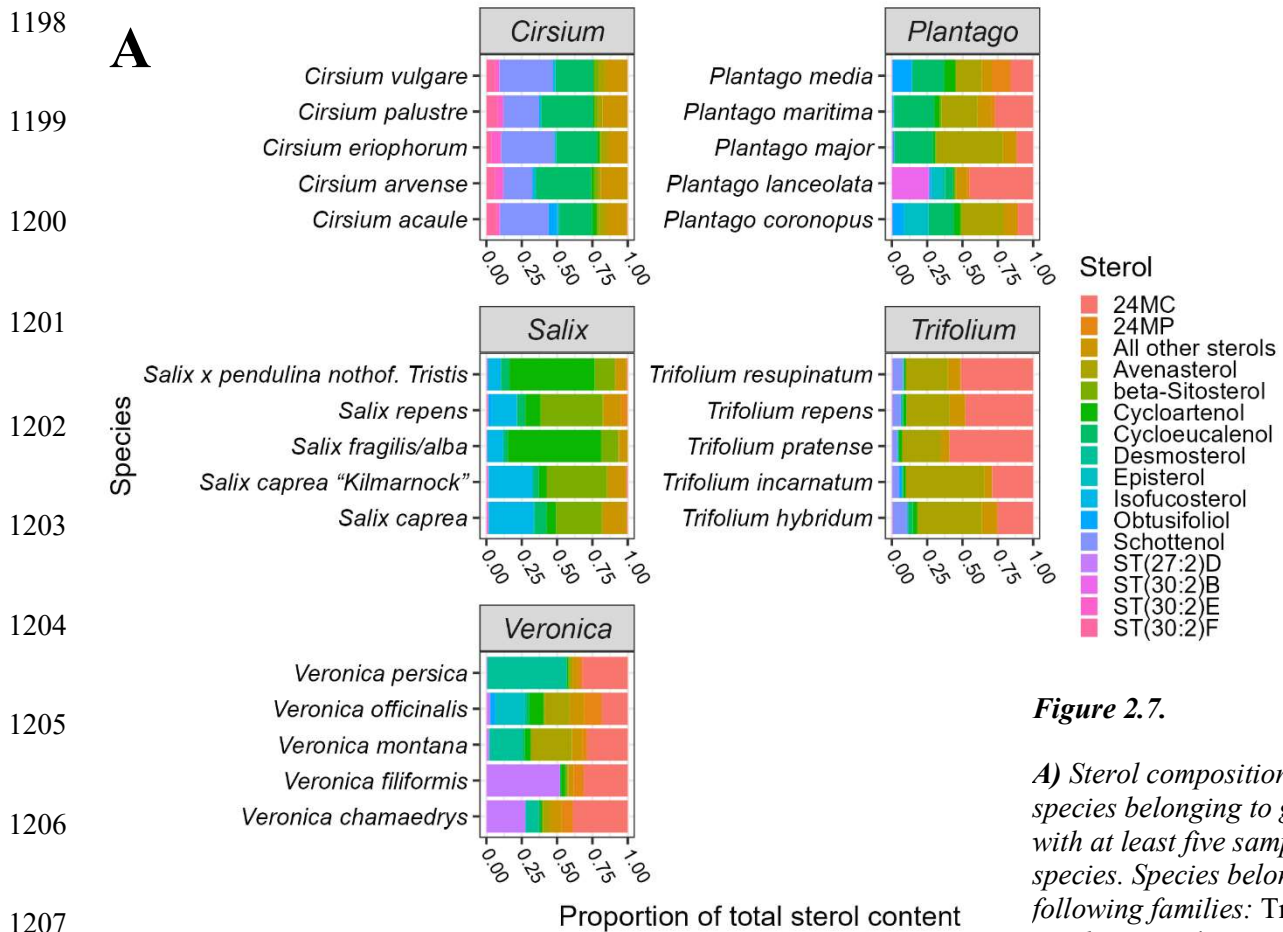
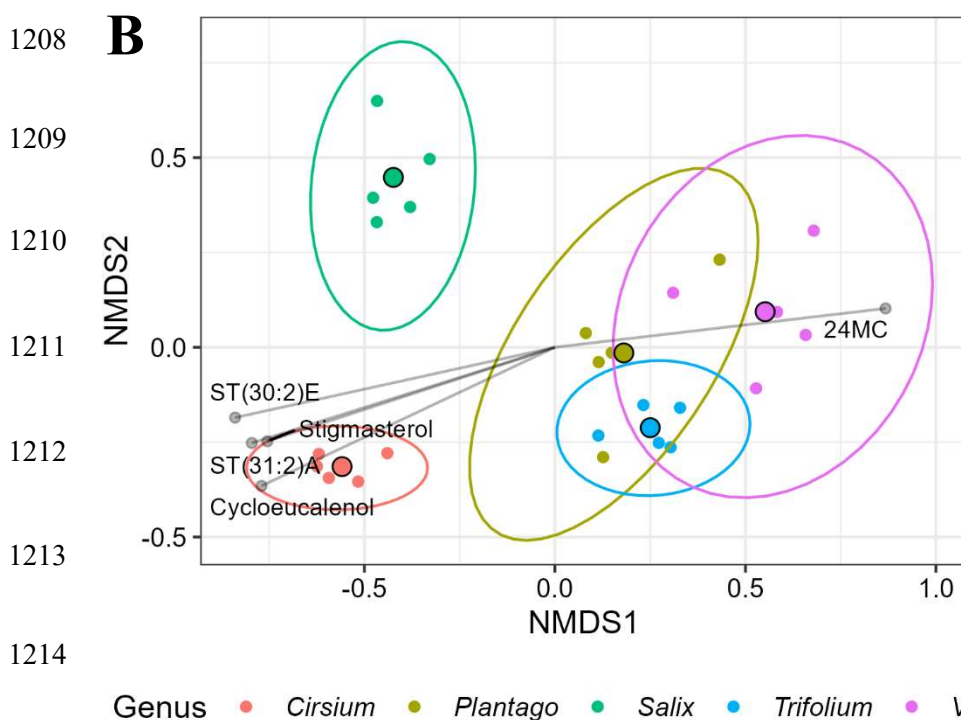


Figure 2.7.

A) Sterol composition of species belonging to genera with at least five sampled species. Species belong to the following families: Trifolium - Fabaceae, Plantago and Veronica - Plantaginaceae, Cirsium - Asteraceae and Salix - Salicaceae. All non-major sterols have been collapsed into 'All other sterols'.

B) NMDS plot of proportion data from species shown in 2.7A arcsine square root transformed. Significant ($p < 0.010$) and strong (> 0.7 absolute value) sterol correlations are plotted in grey. Stress value of plot was 0.099. PERMANOVA on NMDS data was significant ($p = 0.001$). However, variance differed significantly between groups ($p < 0.050$).



1215 **Table 2.9.** Summary of Indicator Species Analysis (ISA) results on pollen sterol data comparing
 1216 genera containing five sampled species. Out of 78 sterols tested 47 were significantly associated
 1217 with a genus group. Indicator values (IV) and p-values are shown for all 47. Proportions were
 1218 averaged by species.

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Sterol	IV	P-value	Sterol	IV	P-value
<i>Cirsium</i>			<i>Plantago</i>		
ST(28:0)D	0.985	0.005	ST(28:3)B	0.747	0.005
Lophenol	0.976	0.005	Obtusifoliol	0.678	0.050
ST(30:2)F	0.937	0.005	24MD	0.628	0.045
ST(27:1)A	0.893	0.005	<i>Salix</i>		
ST(27:1)B	0.877	0.005	Isofucosterol	0.842	0.005
ST(28:0)C	0.875	0.010	β-Sitosterol	0.794	0.005
ST(30:1)	0.861	0.005	24MCA	0.729	0.010
ST(28:2)D	0.849	0.005	Cycloartenol	0.674	0.005
ST(27:1)E	0.830	0.005	ST(29:1)B	0.597	0.005
ST(27:2)F	0.821	0.005	ST(28:1)A	0.586	0.025
Schottenol	0.810	0.005	Cyclolaudenol	0.543	0.010
Cycloartanol	0.787	0.005	<i>Trifolium</i>		
ST(28:0)E	0.787	0.005	ST(29:0)B	0.695	0.005
Sitostanol	0.776	0.005	Avenasterol	0.620	0.015
Cholesterol	0.771	0.005	Agnosterol	0.620	0.015
Stigmasterol	0.763	0.005	24MC	0.617	0.015
ST(30:2)E	0.721	0.005	Campesterol	0.549	0.030
ST(28:2)B	0.710	0.005	Anthelsterol	0.533	0.015
ST(29:3)A	0.666	0.015	<i>Veronica</i>		
ST(30:3)	0.645	0.015	ST(27:2)D	0.942	0.005
ST(29:0)C	0.644	0.005	ST(29:2)A	0.798	0.010
ST(27:2)A	0.642	0.005	24MP	0.664	0.010
ST(29:1)C	0.638	0.030			
Cycloeucalenol	0.630	0.005			
ST(28:2)C	0.618	0.015			
Lathosterol	0.575	0.015			
ST(30:2)A	0.567	0.005			
ST(31:2)A	0.543	0.040			

1220 **Intra-species sterol variation**

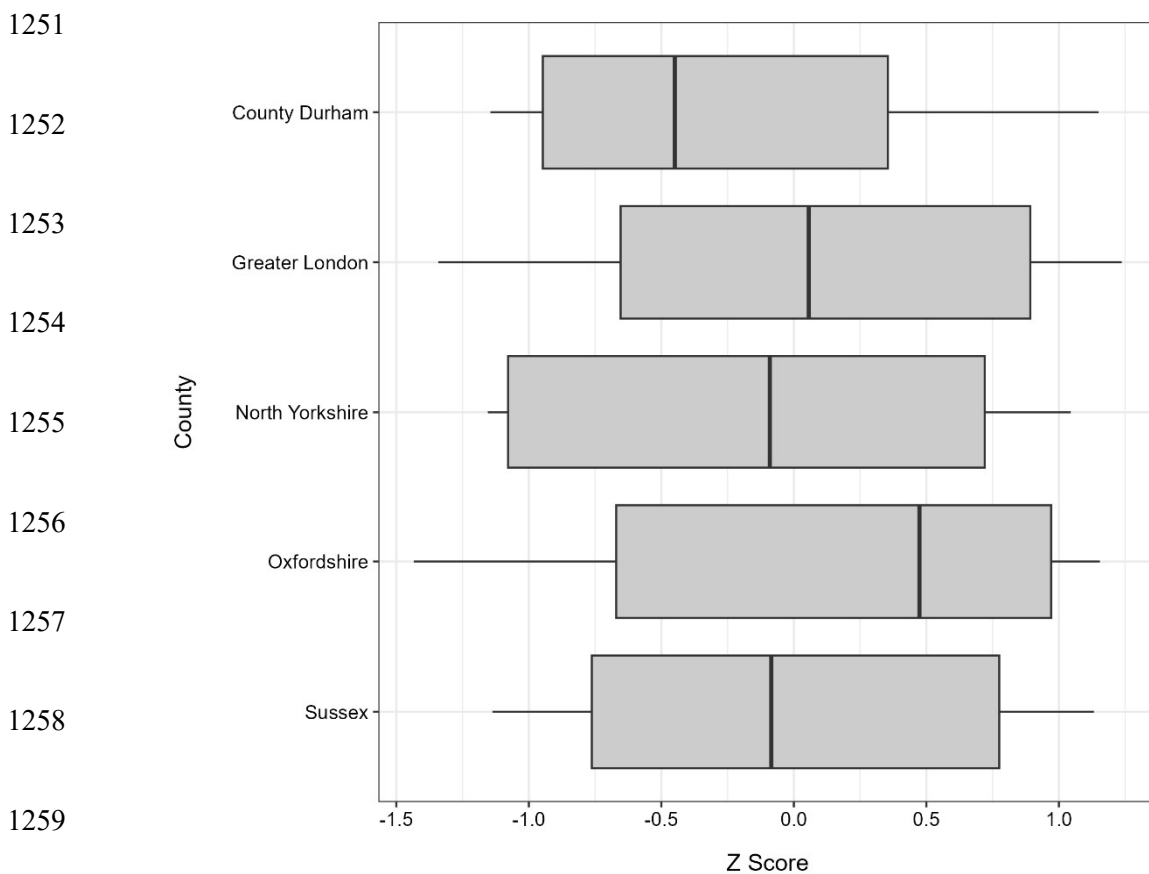
1221 *Intra-species variation in sterol composition is low, therefore species collected from multiple*
1222 *sites will be more similar to each other than other species collected from those sites*

1223 The results of the mixed effect model analysing the effect of collection county on total sterol
1224 production (mg/kg) showed no significant effect ($F_{4,70} = 1.254, p > 0.100$). To visualise this effect,
1225 total sterol content for all data used in the linear model was z-scored by species (irrelevant of
1226 county) and plotted in Figure 2.8. This scaling allows visualisation of inter-county differences
1227 while accounting for natural inter-species differences in total sterol content.

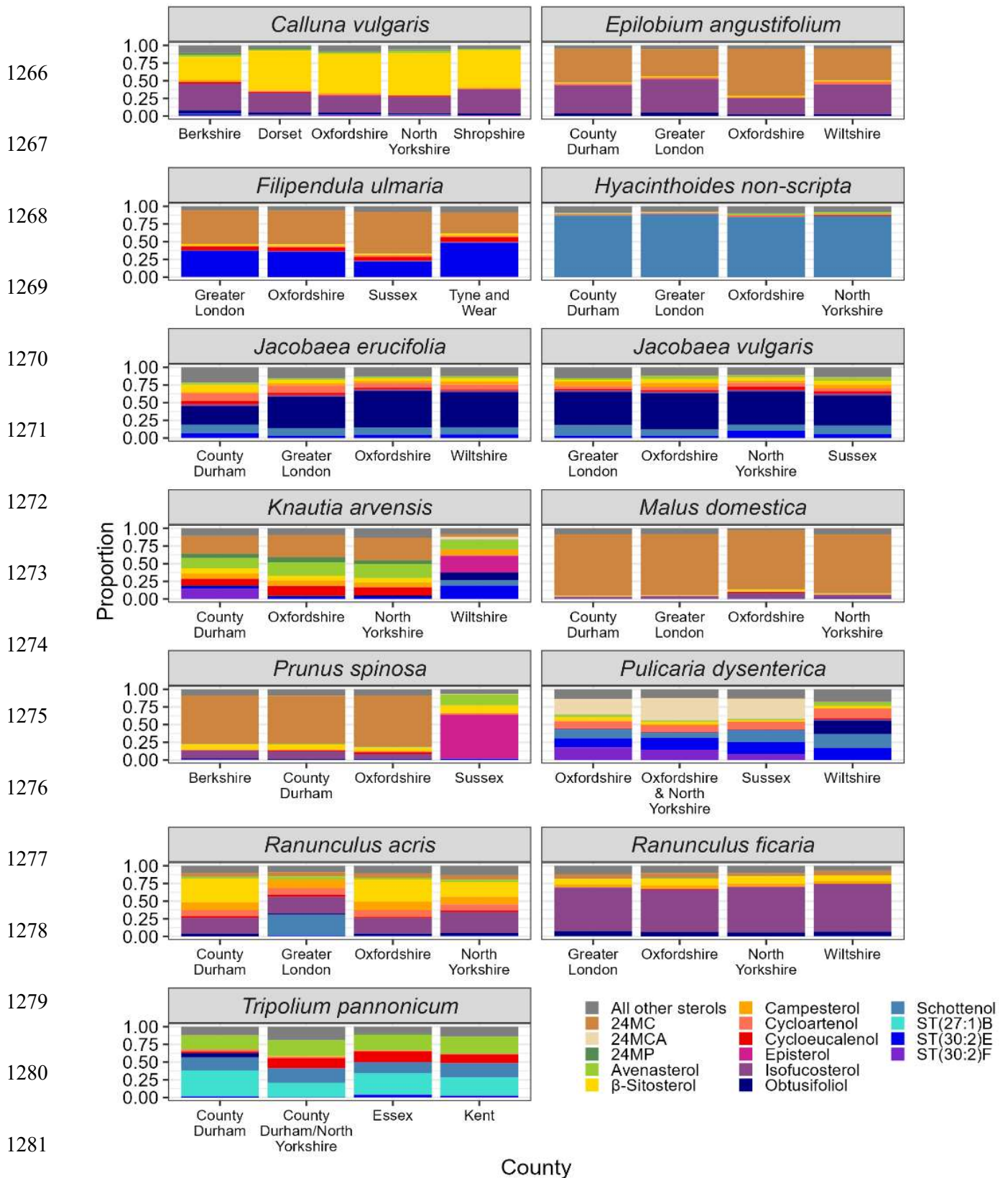
1228 Figure 2.9 shows the sterol composition of the 13 species collected from at least four counties. The
1229 graphs show a consistency in sterol profile among counties. There was variation in quantitative
1230 values, which may be caused by abiotic factors, such as soil or temperature, affecting total fat
1231 production, or a result of handling and storage procedures working at mg scale. Mean and standard
1232 deviation in total sterols for all species replicated at the county level are reported in Table S2.4.

1233 Figure 2.10 shows the NMDS plotted from the arcsine square root transformed data of species in
1234 Figure 2.9 which had at least five samples in total. There was a significant difference between the
1235 species ($F_{6,39} = 33.757, p = 0.001$), with species identity explaining a large proportion of the
1236 variance in the data ($R^2 = 0.839$). All pairwise comparisons between individual species were
1237 significantly different ($p < 0.050$) and the variance was not significantly different among species
1238 groups ($F_{6,39} = 0.600, p > 0.500$). Intraspecies variation between sites was therefore not greater than
1239 the interspecies variation in the dataset and samples still clustered by species, irrespective of
1240 collection location. Figure 2.10 shows associated correlations with individual sterols plotted as
1241 grey lines. Only sterols with significant ($p < 0.010$) and strong (> 0.6) associations are shown.
1242 *Hyacinthoides non-scripta* was associated with higher levels of schottenol and cycloartanol.
1243 *Ranunculus ficaria* was associated with higher levels of obtusifoliol, *Tripolium pannonicum* with

1244 avenasterol and *Prunus spinosa* with 24-methylenecholesterol and cyclolaudenol. ISA showed
 1245 *Tripolium pannonicum* was significantly associated with the most individual sterols (14), including
 1246 ST(27:1)B which did not occur in the other six species. *Ranunculus acris* was very strongly
 1247 associated with ergosterol, matching earlier reporting on ergosterol across the dataset.
 1248 *Hyacinthoides non-scripta* was strongly associated with schottenol, *Ranunculus ficaria* with
 1249 isofucoesterol, *Prunus spinosa* with 24-methylenecholesterol and *Calluna vulgaris* with sitostanol,
 1250 β -sitosterol and stigmasterol. A full list of all 45 associations found can be seen in Table 2.10.

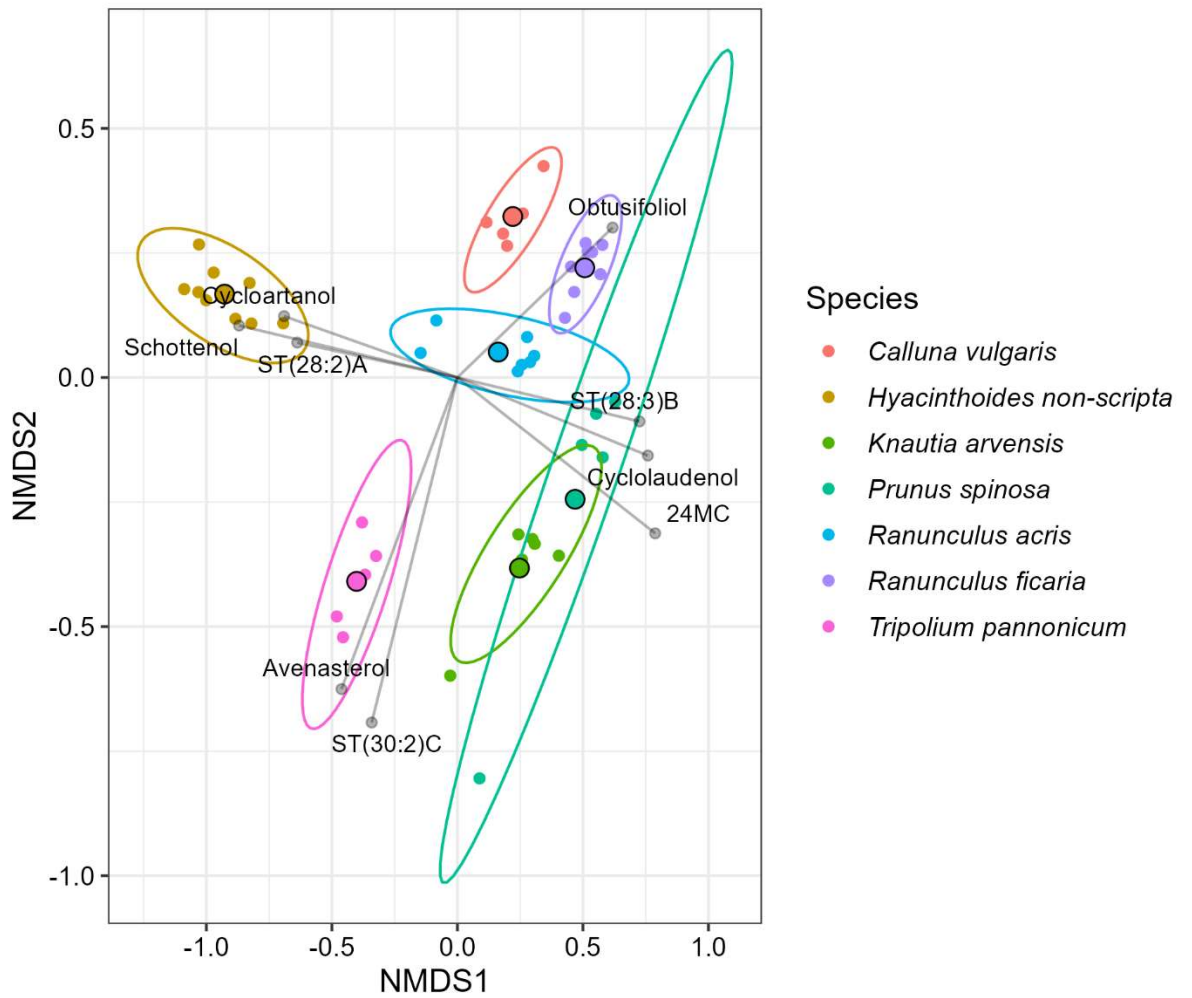


1260 **Figure 2.8.** Boxplot of data included in the mixed effect model analysing the effect of county on
 1261 total sterol production (35 plant species across five counties). Total sterol data is z-scored by
 1262 species to remove the effect of species identity. Collection county did not have a significant effect
 1263 on total sterol production. Counties are ordered alphabetically and contain the following sample
 1264 sizes: Oxfordshire (33), Greater London (25), County Durham (21), North Yorkshire (20), Sussex
 1265 (10). Boxes show first and third quartile, median and 1.5x inter quartile range.



1282 **Figure 2.9.** Sterol profiles of all species collected from at least four separate counties. Graphs are
 1283 ordered alphabetically. Species collected multiple times from the same county are represented by
 1284 mean values.

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1297 **Figure 2.10.** Lines show associations with different sterol profiles, only sterols with significant
1298 ($p < 0.0100$ and strong > 0.6) associations were used. Difference among groups was significant
1299 ($p = 0.001$) and variance was not significantly different ($p = 0.729$). Stress value of NMDS was
1300 0.096.

1301 **Table 2.10.** Summary of Indicator Species Analysis (ISA) results on pollen sterol data for the inter-
 1302 species comparison shown in Figure 2.10. Out of 78 sterols tested 45 were significantly associated
 1303 with a species group. Indicator values (IV) and p-values are shown for all 45.

Sterol	IV	P-value	Sterol	IV	P-value
<i>Calluna vulgaris</i>			<i>Ranunculus ficaria</i>		
Sitostanol	0.805	0.005	ST(29:2)B	0.734	0.005
ST(30:2)F	0.638	0.040	Isofucosterol	0.627	0.005
β -Sitosterol	0.625	0.005	ST(31:2)B	0.580	0.045
Stigmasterol	0.592	0.005	ST(30:2)A	0.530	0.005
ST(29:2)A	0.555	0.005	Obtusifoliol	0.522	0.005
ST(29:1)B	0.545	0.005	<i>Tripolium pannonicum</i>		
<i>Hyacinthoides non-scripta</i>			ST(27:1)B	0.991	0.005
ST(29:3)A	0.785	0.005	ST(28:0)C	0.758	0.010
Schottenol	0.773	0.005	ST(27:1)E	0.736	0.005
ST(28:2)A	0.703	0.005	ST(30:1)	0.687	0.005
ST(28:1)A	0.694	0.005	ST(30:2)D	0.668	0.010
ST(29:2)D	0.657	0.030	ST(29:0)A	0.643	0.005
Cycloartanol	0.607	0.005	Spinasterol	0.622	0.005
<i>Knautia arvensis</i>			ST(27:2)B	0.612	0.005
24MP	0.644	0.005	Avenasterol	0.556	0.005
ST(30:2)E	0.599	0.010	Cycloeucalenol	0.555	0.010
ST(27:1)C	0.585	0.025	ST(28:2)B	0.552	0.005
<i>Prunus spinosa</i>			ST(27:2)A	0.549	0.005
ST(28:3)B	0.586	0.020	ST(28:0)E	0.534	0.005
24MC	0.578	0.020	ST(30:2)C	0.474	0.005
Cyclolaudenol	0.498	0.005			
<i>Ranunculus acris</i>					
Ergosterol	0.826	0.005			
ST(28:3)A	0.658	0.010			
ST(27:2)E	0.627	0.005			
Cycloartenol	0.576	0.005			
Campesterol	0.572	0.005			
Lathosterol	0.572	0.010			
24MD	0.518	0.010			
ST(29:1)A	0.503	0.005			

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1305 **Ecological associations of sterol profiles**

1306 ***Pollen can contain high proportions of the sterols detected from honey bee and bumble bee***
1307 ***tissues***

1308 Figure 2.11A shows the species that produced the highest proportion of sterols reported to be
1309 important for honey bee and bumble bee development. Campesterol and cholesterol were generally
1310 detected in lower proportions than the other sterols. For many sterols, the highest producing
1311 species were dominated by one family: 24-methylenecholesterol - 6/10 Rosaceae, campesterol -
1312 6/10 Apiaceae, cholesterol - 6/10 Asteraceae subfamily Cichorioideae. The highest content of
1313 desmosterol was from flowers popular with honey bees and bumble bees such as *Rubus* spp., *Rosa*
1314 spp., *Borago officinalis* and *Symphytum* sp. β -sitosterol was not recorded more frequently by any
1315 family and three of the species in which this sterol was most abundant were wind pollinated trees.

1316 Figure 2.11B shows the ten species which produce the highest sum content when combining these
1317 six sterols. Five out of the ten species belong to Rosaceae. The tree species (*Malus domestica* and
1318 *Torminalis glaberrima*) were dominated by 24-methylenecholesterol while other Rosaceae, *Rubus*
1319 spp. and *Rosa arvensis* were dominated by desmosterol. The three *Epilobium* species displayed the
1320 highest amounts of isofucosterol but still high quantities of 24-methylenecholesterol. All ten
1321 species were dominated (>75%) by these six sterols and several are notably important to many bee
1322 species (Svoboda *et al.*, 1980; Vanderplanck, Zerck, *et al.*, 2020a).

1323 ***Pollens collected by honey bees and bumble bees do not have higher proportions of the sterols***
1324 ***detected from honey bee and bumble bee tissues***

1325 The 221 species with visitation data available from the DoPI were grouped according to whether
1326 they were visited by honey or bumble bees for pollen. The proportion of key sterols found in honey
1327 bee and bumble bee tissues was then plotted for all groups (Figure 2.12). There were no significant

1328 differences in composition between any groups analysed (24MC: $H(3)=6.694, p>0.100,$
1329 β -sitosterol: $H(3) = 3.807, p>0.100,$ campesterol: $H(3) = 5.119, p>0.100,$ cholesterol: $H(3) = 8.809,$
1330 $p>0.100,$ desmosterol: $H(3) = 4.892, p>0.100,$ isofucosterol: $H(3) = 1.969, p>0.500$). β -sitosterol
1331 and 24-methylenecholesterol displayed the highest mean proportions across the dataset while
1332 cholesterol and campesterol display consistently low proportions (Figure 2.12). The same data
1333 were then grouped according to the position of the B-ring double bond (Figure 2.13). There were
1334 no significant differences in the proportion of different B-ring double bond sterols between groups
1335 ($\Delta 0$: $H(3) = 9.025, p>0.050,$ $\Delta 5$: $H(3) = 8.819, p>0.050,$ $\Delta 7$: $H(3) = 5.207, p>0.100,$ $\Delta 8$: $H(3) =$
1336 $2.899, p>0.500,$ ΔNA : $H(3) = 2.359, p>0.500$).

1337 ***Pollens collected by honey bees and bumble bees do not have a different sterol profile to pollens***
1338 ***which are not collected by them***

1339 For plant species with pollinator visitation data available from DoPI (221 plant species), an NMDS
1340 was carried out on the arcsine square root transformed sterol proportions data. When species were
1341 grouped by pollen collection (honey bee, bumble bee, both, neither), the stress value of the NMDS
1342 was too high to draw reliable conclusions from plotting (stress value = 0.228) and pollen sterol
1343 composition did not significantly differ between groups ($F_{3,217}=1.330, p>0.050$). Groups had
1344 comparable variances ($F_{3,217}=2.483, p>0.050$).

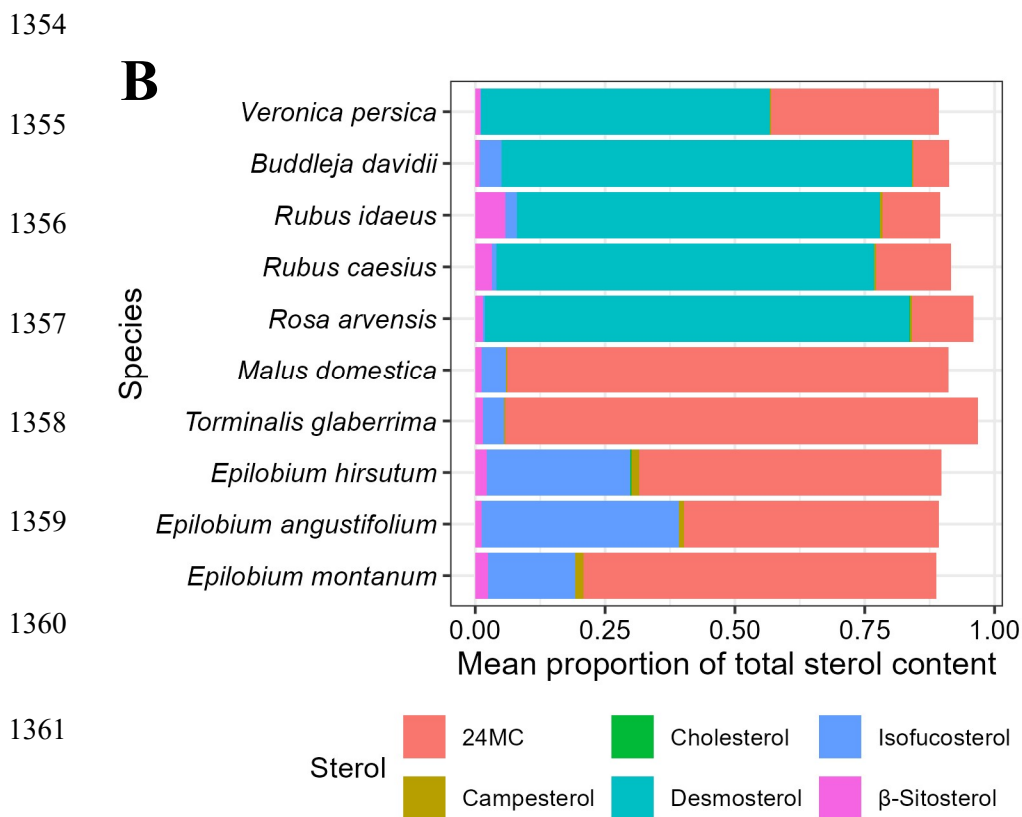
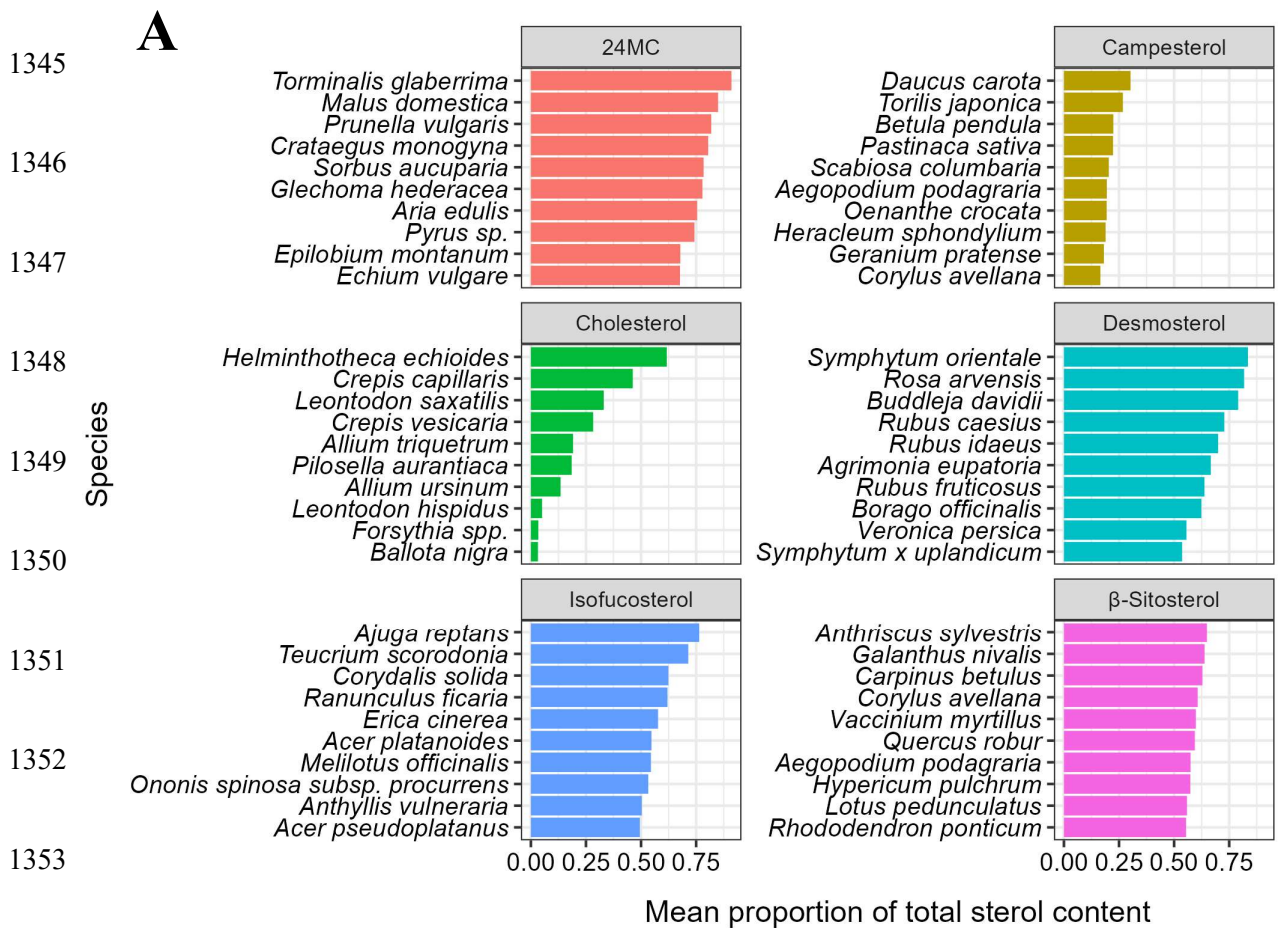


Figure 2.11.

A) The top 10 species with highest proportions for six sterols detected in honey bee and bumble bee tissues. Campesterol and cholesterol are generally present in low proportion compared to the others which are the dominant sterol in multiple species.

B) The 10 species which contained the highest total proportion of all six sterols detected in honey bee and bumble bee tissues. Desmosterol and 24-methylenecholesterol dominate composition across these species.

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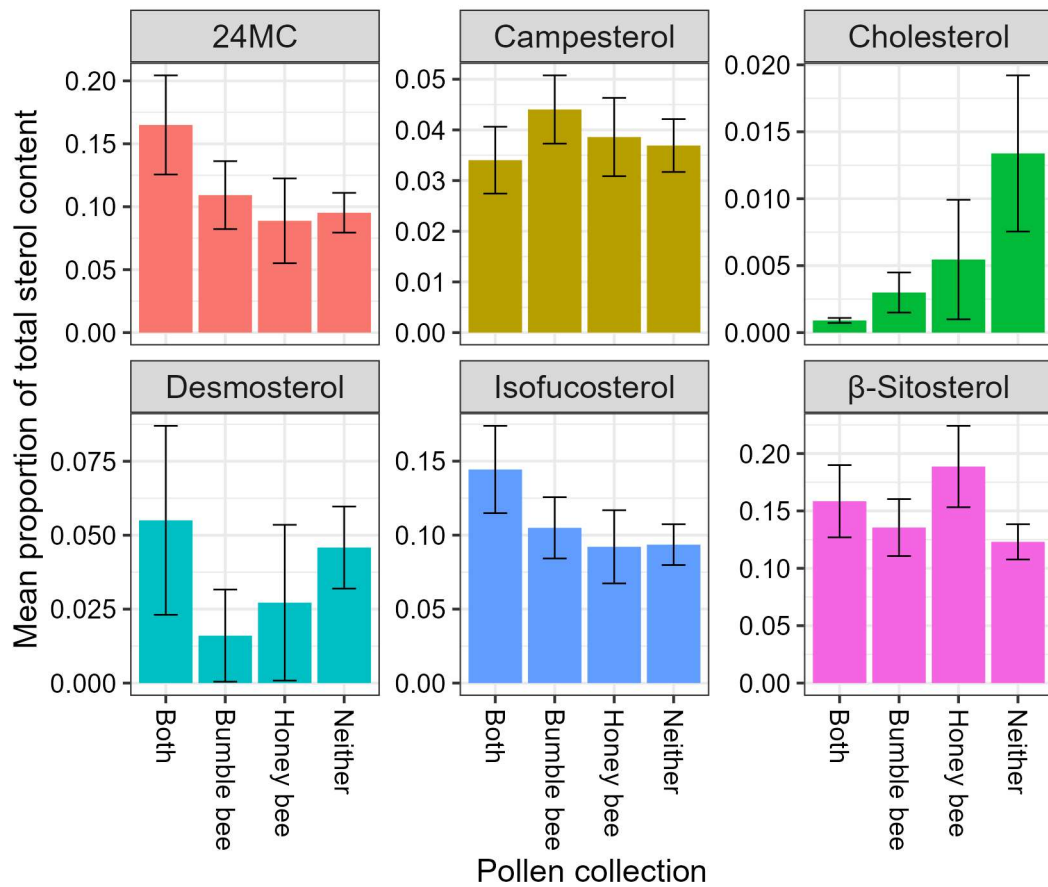
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Figure 2.12. Plant species for which pollen visitation data were available from Database of Pollinator Interactions grouped by whether there is reported pollen collection by bumble bees and honey bees, both or neither. The mean proportion of sterols is plotted with \pm SEM.

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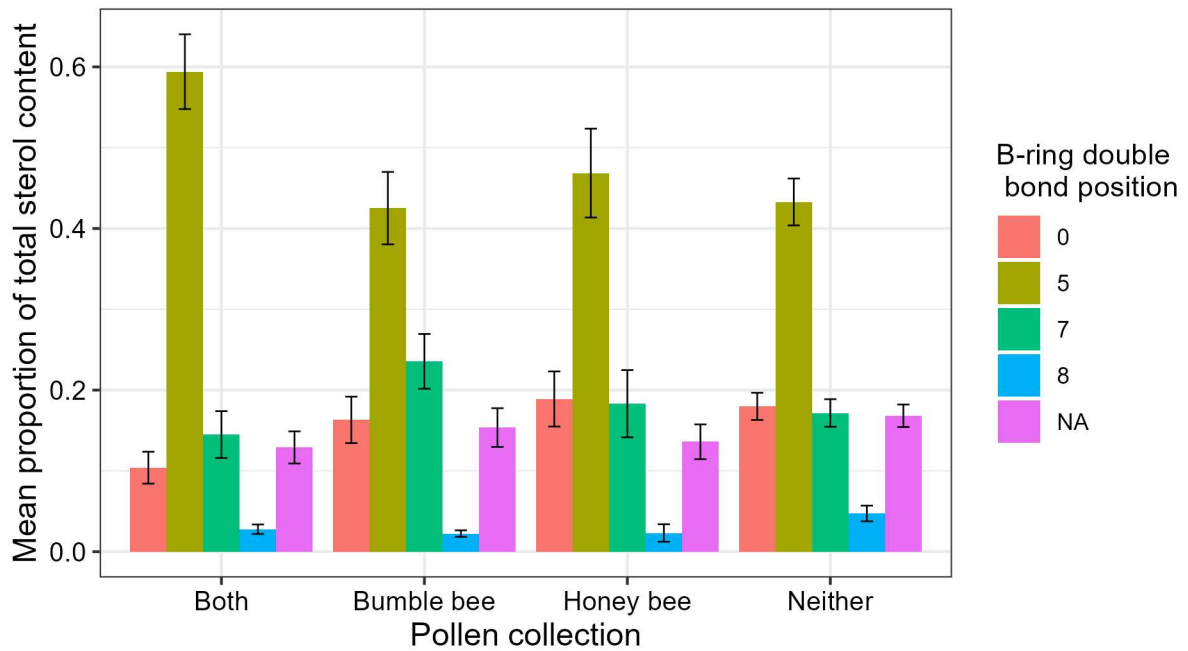
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Figure 2.13. Plant species for which pollen visitation data were available from the Database of Pollinator Interactions grouped by whether there has been pollen collection reported either by bumble bees or honey bees, both or neither. The total proportion of sterols with double bond at C-5, 7 or 8 in the B-ring or none is then plotted. Sterols for which double bond position could not be determined were classed as NA. There was a general dominance of $\Delta 5$ sterols in the dataset.

1394 **Discussion**

1395 Pollen has previously been shown to display a sterol profile distinct from other vegetative plant
1396 tissues (Villette *et al.*, 2015; Furse, Martel, *et al.*, 2023). It has been proposed that the sterols
1397 contained in pollen are the result of a truncated pathway of sterol synthesis from other tissues,
1398 leading to a higher diversity of biosynthetic intermediaries. The 78 sterol compounds reported here
1399 represent both named sterols from reference materials and unknown sterols which are likely
1400 intermediate steps in the sterol synthesis pathways. In total 38 of these sterols never occurred in
1401 >5% in any species and represent potential intermediate synthesis steps.

1402 Our analysis showed a high proportion of Δ^5 sterols in pollen across species including β -sitosterol,
1403 24-methylenecholesterol and isofucosterol, all of which had a mean percentage of >10% of total
1404 sterol across the dataset which concurs with Villette *et al.*, (2015). These sterols have been
1405 suggested as membrane components affecting fluidity and are abundant in different bee species
1406 (Furse, Koch, *et al.*, 2023). Campesterol was one of four sterols detected in all species. In bees,
1407 which are unable to synthesise cholesterol, campesterol can be used in the synthesis of the
1408 moulting hormone makisterone A (24-methyl-20-hydroxyecdysone), a C28 ecdysteroid (Svoboda
1409 and Feldlauffer, 1991; Furse, Koch, *et al.*, 2023). Bees may therefore have adapted their hormone
1410 biosynthesis pathways to the reliable availability of campesterol in the landscape.

1411 Ergosterol is not biosynthesised by animals or plants and so the frequent detection of ergosterol in
1412 samples (254/295 species) is caused by fungi within the pollen material. Its consistent presence in a
1413 low proportion (max 1.30%, mean 0.05%.) between the different collectors and storage facilities in
1414 this study suggests it is a natural part of the pollen microbiome and not the result of collection
1415 methods. As a result it would be frequently consumed by pollinivores (Steffan *et al.*, 2019),
1416 potentially as an essential dietary component as demonstrated in *Scaptotrigona depilis* which is
1417 reliant upon a brood yeast species for steroid synthesis precursors (Paludo *et al.*, 2018).

1418 The function of pollen as the male gametophyte requires the rapid biosynthesis of membrane
1419 components to support pollen tube elongation after germination. While almost two thirds of species
1420 contained >60 sterols in their pollen, most were dominated by 1-3 major sterol components. In
1421 total, 54% of taxonomic units had at least one sterol which accounted for $\geq 40\%$ of their total sterol
1422 content. This suggests plants rely on a few dominant sterols to fulfil their needs during germination
1423 and pollen tube growth rather than diversity. The sterols which most frequently occurred as major
1424 components were β -sitosterol, isofucosterol, avenasterol and 24-methylenecholesterol suggesting
1425 these are useful to pollen biochemistry across a wide range of plants. β -sitosterol, isofucosterol and
1426 avenasterol are all components of the same biosynthesis pathway, though the final product of this
1427 pathway, stigmasterol, never reached >40% in any species. Previous work has shown
1428 cycloeucaenol is the major neosynthesised sterol in germinating *Nicotiana tabacum* pollen grains,
1429 however, the pollen tubes also showed high levels of 24-methylenedesmosterol and 24-
1430 methylenecholesterol which originated in the pollen (Villette *et al.*, 2015).

1431 The significant phylogenetic signal across 31/69 tested sterols confirms the finding reported in Zu
1432 *et al.* (2021) that sterol profiles are similar between related taxa. When grouped by carbon number
1433 and double bond position, sterol groupings retain phylogenetic signal.

1434 While there are trends within certain families, such as Asparagaceae which displays a high
1435 proportion of schottenol compared to the rest of the dataset, there is also divergence within more
1436 highly sampled families. Many genera within Rosaceae which are trees (*Prunus*, *Malus*, *Pyrus*,
1437 *Crataegus*, *Aria* and *Torminalis*), displayed elevated 24-methylenecholesterol, however, other
1438 Rosaceae display elevated levels of desmosterol. These sterols are not part of the same synthesis
1439 pathway and suggest different dominant sterol synthesis pathways in the two subfamilies. We can
1440 also see patterns that spread across closely related families, such as elevated β -sitosterol in
1441 Betulaceae and Fagaceae. These families share ecological similarities as catkin producing trees and
1442 appear to have retained a common ancestral sterol synthesis pathway. Other well sampled families

1443 such as Fabaceae however show little consistency in sterol profile among genera, suggesting that
1444 the strong phylogenetic constraint to sterol biosynthesis is not universal.

1445 Total sterol content showed a hundred-fold range across the dataset with some of the highest and
1446 lowest total sterol production in Asteraceae. Specifically, Cichorioideae showed a consistently
1447 higher total sterol content than the other Asteraceae subfamilies Carduoideae and Asteroideae.
1448 Brassiceae showed consistently high sterol production across all species. The evolutionary cause of
1449 this variation remains unknown, but drivers may include stigma-ovule distance, which could
1450 strongly influence sterol requirements during pollen tube growth, as suggested for protein content
1451 (Roulston, Cane and Buchmann, 2000), or a compensatory mechanism for attracting pollinators to
1452 potentially poor quality pollen. However, while Cichorioideae species produced comparatively low
1453 proportions of $\Delta 5$ sterols, Carduoideae and Asteroideae produced similar levels without a high
1454 total sterol production. The compensation may instead have been that Cichorioideae produced
1455 some of the highest cholesterol proportions in the dataset (*Helminthotheca echioides*: 60.05%,
1456 *Crepis capillaris*: 44.21%, *Leontodon saxatilis*: 31.33%, *Crepis vesicaria*: 27.77%) (Figure
1457 2.11A). This has been proposed as facilitating the transition from carnivory to pollinivory in
1458 Apidae evolution (Santerre, 2023). Bees evolved from sphecid wasps which obtained their required
1459 cholesterol from carnivory. As bees evolved from carnivorous to pollinivorous diets, cholesterol
1460 rich pollens may have provided an intermediary step prior to evolving to use longer chain
1461 phytosterols in their tissues, as suggested by Dötterl and Vereecken (2010). *Dasygaster* species,
1462 which occupy a basal position in bee evolution, are also ancestrally oligolectic on Asteraceae
1463 flowers (Michez *et al.*, 2008).

1464 It is also possible that total sterol content was influenced by abiotic factors, as previous work has
1465 linked increased total content of sterol derivatives to higher temperature exposure (Narayanan *et*
1466 *al.*, 2016) and adaptation to higher temperatures (Zu *et al.*, 2021). Temperature was not measured
1467 at individual sites during this study but all samples were collected within England therefore

1468 seasonal temperature fluctuations are likely greater than geographic ones. Species which were
1469 present in both our dataset (A) and that of Vanderplanck *et al.* (2019) (B) showed a higher total
1470 sterol content in our data (mean \pm SD(*Lamium album* (A=4814 \pm 672 mg/kg,
1471 B=3060 \pm 1400 mg/kg), *Prunus avium* (A= 14348 \pm 5079 mg/kg, B=11940 \pm 470 kg/g), *Salix caprea*
1472 (A= 6896 \pm 782 mg/kg, B=6520 \pm 440 mg/kg), *Sorbus aucuparia* (A= 9085 \pm 2083 mg/kg,
1473 B=8610 \pm 1830 mg/kg), suggesting there is an impact of abiotic factors or analysis method on total
1474 sterol content.

1475 In the UK, many Asteraceae pollens are not frequently collected by honey bees or bumble bees
1476 despite their availability and instead are targeted by specialist solitary bees. This ‘Asteraceae
1477 paradox’, referring to the more frequent collection of Asteraceae pollen by specialists compared to
1478 generalists, is thought to be the result of chemical defences and nutritional deficiencies (Müller and
1479 Kuhlmann, 2008; Vanderplanck, Gilles, *et al.*, 2020). Our results showed pollen contained
1480 proportions of Δ 5: Δ 7 sterols clearly distinct from the trend shown by the rest of the dataset, with
1481 Carduoideae flowers producing especially low proportions of Δ 5 sterols, such as cholesterol,
1482 desmosterol, campesterol, β -sitosterol, isofucosterol and stigmasterol. This supports the hypothesis
1483 that some Asteraceae pollen is nutritionally less suitable for species of bees that typically forage
1484 for pollen which contains high levels of Δ 5 sterols such as honey bees and bumble bees. However,
1485 the family is widespread, with long flowering times and abundant floral rewards, therefore there
1486 are benefits to specialist bees which have adapted to using Asteraceae pollen (Müller and
1487 Kuhlmann, 2008).

1488 Genera analysed included species from a wide range of habitats. For instance, *Plantago maritima*
1489 is strictly coastal, however, *P. lanceolata* occurs in a wide range of habitats and *P. media* prefers
1490 meadows. These species therefore grow under distinct abiotic conditions, potentially influencing
1491 the availability of soil nutrients and moisture, but nevertheless showed a conserved sterol profile.
1492 This indicates that sterol biosynthesis is strongly genetically controlled. All three species also

1493 shared a similar floral anatomy lending credence to sterol production being governed by stigma-
1494 ovule distance. However, the size of individual florets and flowering heads in *Cirsium* show more
1495 range in size, from the small *C. palustre* to medium *C. vulgare* and large *C. eriophorum*, but still
1496 recorded a similar sterol profile across the species. The consistently high proportions of 24-
1497 methylenecholesterol and avenasterol in *Trifolium* raises an interesting question about their use in
1498 planting schemes. For example, does planting multiple clover species, as is common in many seed
1499 mixes, introduce any additional nutritional benefits beyond redundancy and a continuous supply of
1500 its sterols? The design of agricultural seed mixes is often hindered by the absence of nutritional
1501 data for many species, it is possible that *Trifolium* spp. do not provide the diversity of the sterols
1502 required to support many solitary bees. In addition, only long tongued species can access some
1503 *Trifolium* spp., such as *T. pratense*, preventing visitation by many bee species. Cataloguing the
1504 diversity of sterols available and interpreting the nutritional value of species through flowering
1505 time and the physiological requirements of bees will help to inform more effective seed mixes for a
1506 wider range of species.

1507 As pollen collection was time intensive and challenging, 125 out of 295 taxonomic units in the
1508 dataset are represented by a single sample (though these samples are composites from multiple
1509 flowers, and often plants). The data shows there is consistency in the sterol profile of multiple-
1510 sampled species, therefore single samples are sufficiently representative for inclusion in the
1511 dataset. This data further supports the findings from our intra-genus comparisons that growing
1512 conditions do not significantly alter the sterol profile of pollen. The variation in total sterol
1513 production between replicated species could be a result of collection method as samples were not
1514 freeze dried and humidity may have skewed signal conversion, which relied on fresh weight
1515 measurements.

1516 Previous work has shown no association between pollinator identity and pollen sterol profile (Zu *et*
1517 *al.*, 2021). However it is becoming clear that bee species have differing sterol requirements and

1518 therefore plants pollinated by ‘bees’ may be expected to share little in common nutritionally
1519 (Vanderplanck *et al.*, 2018). This work compared plants which had reported pollen collection by
1520 honey bees and bumble bees but also found no trends in pollen sterol profile. Again, this adds
1521 support to a plant physiology rather than pollinator identity driven mechanism of sterol synthesis in
1522 pollen. However, the physiological requirements of bees mean pollen sterol profile is still likely to
1523 contribute in some regard to foraging choice alongside morphology, flowering time and other
1524 nutritional information.

1525 This dataset provides a major contribution to our understanding of pollinator nutrition in the UK
1526 landscape and raises questions about the evolution of pollen sterol pathways across angiosperms,
1527 such as whether the variation shown between species is determined by inherited genes controlling
1528 sterol synthesis and metabolism. It indicates that abiotic factors do not greatly influence sterol
1529 profiles and that instead they are the result of physiological and evolutionary constraints. However,
1530 to determine how sterol biosynthesis may be affected by predicted global changes in temperature
1531 and rainfall, experimentally rearing plants under controlled conditions would provide stronger
1532 evidence for this. Our dataset also focussed on cataloguing insect-pollinated UK flora, however,
1533 future studies should target tropical species which contain a wider range of floral morphologies and
1534 pollination syndromes. This may contribute to understanding the relationship between different
1535 pollinator groups and pollen sterols. The strong phylogenetic signal and within-genus consistency
1536 in sterol profile invites further investigation into the mechanisms regulating sterol production in
1537 pollen from an evolutionary perspective. Further, an assessment of stigma-ovule distance in the
1538 study species may prove enlightening for the trends in total sterol production. Finally, the low
1539 proportions of $\Delta 5$ sterols in Asteraceae flowers suggests the sterol requirements of Asteraceae-
1540 specialist solitary bees may be different to that of common eusocial species (*Apis mellifera* and
1541 *Bombus* spp.) and provides more support to the nutritional deficit argument of the Asteraceae
1542 paradox.

1543 **Acknowledgements**

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1551 assisted by Flynn Bizzell, Harry Savage, Nell Miles, Poppy Emson, Sarah Bannister and Léa
1552 Beaupère.

1553 **Supplementary**

1554 **Table S2.1.** Collection details for all pollen samples. Multi-site samples are those where smaller
 1555 samples were combined to reach 10 mg. In these cases, collection month and year are given for
 1556 each county. In total 13 people collected pollen samples, each has been given a numeric code
 1557 between 1-13 as a collector ID.

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Acer campestre</i>	Oxfordshire	Apr-22	2	No	1
<i>Acer platanoides</i>	Oxfordshire	Apr-21	2	No	1
<i>Acer platanoides</i>	Sussex	Mar-22	5	No	1
<i>Acer pseudoplatanus</i>	County Durham	Apr-22	8	No	1
<i>Acer pseudoplatanus</i>	County Durham	May-22	3	No	1
<i>Acer pseudoplatanus</i>	County Durham	May-22	8	No	1
<i>Acer pseudoplatanus</i>	Greater London	Apr-22	5	No	2
<i>Acer pseudoplatanus</i>	Oxfordshire	May-21	2	No	2
<i>Acer sp.</i>	Oxfordshire	Apr-21	2	No	1
<i>Achillea millefolium</i>	Oxfordshire	Aug-21	12	Yes	1
<i>Achillea millefolium</i>	Oxfordshire	Jul-22	12	Yes	1
<i>Achillea ptarmica</i>	Greater London	Jun-22	5	No	1
<i>Aegopodium podagraria</i>	Greater London	May-22	5	No	1
<i>Aegopodium podagraria</i>	Oxfordshire	Jun-22	2	No	1
<i>Aegopodium podagraria</i>	Oxfordshire	May-22	2	No	1
<i>Aesculus hippocastanum</i>	County Durham	May-22	8	No	1
<i>Aesculus hippocastanum</i>	North Yorkshire	May-22	3	No	1
<i>Aesculus hippocastanum</i>	Oxfordshire	May-21	2	No	2
<i>Agrimonia eupatoria</i>	Oxfordshire & Sussex	Jul & Aug - 21 (O), Jul - 22 (S)	12 & 5	Yes	1
<i>Ajuga reptans</i>	Oxfordshire	May-21	2	No	1
<i>Alcea rosea</i>	Oxfordshire	Aug-21	1	No	2
<i>Alliaria petiolata</i>	Oxfordshire	Apr-22	2	No	1
<i>Alliaria petiolata</i>	Oxfordshire	May-21	2	No	1
<i>Allium triquetrum</i>	Oxfordshire	May-21	2	No	1
<i>Allium ursinum</i>	North Yorkshire	May-22	3	No	1
<i>Allium ursinum</i>	Oxfordshire	Apr-21	2	No	1
<i>Allium ursinum</i>	Oxfordshire	May-21	2	No	1

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Alnus glutinosa</i>	Greater London	Feb-22	5	No	3
<i>Alnus glutinosa</i>	Oxfordshire	Feb-22	2	No	1
<i>Alnus glutinosa</i>	Oxfordshire	Mar-21	2	No	3
<i>Alnus glutinosa</i>	Oxfordshire	Mar-21	4	No	1
<i>Alnus glutinosa</i>	Oxfordshire	Mar-21	6	No	1
<i>Alnus glutinosa</i>	Oxfordshire	Mar-22	2	No	1
<i>Anemonoides nemorosa</i>	Greater London	Mar-22	5	No	1
<i>Anemonoides nemorosa</i>	Oxfordshire	Apr-22	2	No	1
<i>Anemonoides nemorosa</i>	Oxfordshire	Mar-22	2	Yes	1
<i>Anemonoides nemorosa</i>	Sussex	Mar-22	5	No	1
<i>Anemonoides</i> spp.	West Yorkshire	Apr-21	10	No	1
<i>Angelica sylvestris</i>	Oxfordshire	Aug-21	12	No	1
<i>Anthriscus sylvestris</i>	Oxfordshire	Apr-22	2	No	1
<i>Anthyllis vulneraria</i>	County Durham	Jun-22	3	No	1
<i>Anthyllis vulneraria</i>	Sussex	Jul-22	5	No	1
<i>Arctium</i> agg.	North Yorkshire	Jul-22	3	No	1
<i>Arctium lappa</i>	Oxfordshire	Aug-21	12	No	2
<i>Arctium lappa</i>	Oxfordshire	Jul-22	2	No	1
<i>Arctium minus</i>	County Durham	Jul-22	8	No	1
<i>Arctium minus</i>	Oxfordshire	Aug-21	12	No	1
<i>Arctium minus</i>	Oxfordshire	Jul-22	2	No	1
<i>Arctium minus</i>	Sussex	Jul-22	5	No	1
<i>Argentina anserina</i>	Oxfordshire	May-21	2	No	1
<i>Aria edulis</i>	Oxfordshire	May-21	2	No	1
<i>Armeria maritima</i>	Dorset	Jul-22	2	No	1
<i>Ballota nigra</i>	Oxfordshire	Jul-21	2	No	1
<i>Ballota nigra</i>	Oxfordshire	Sep-21	12	No	1
<i>Barbarea vulgaris</i>	Oxfordshire	May-21	2	No	1
<i>Bellis perennis</i>	Oxfordshire	Feb-21	2	No	1
<i>Bellis perennis</i>	Oxfordshire	Mar-21	2	No	2
<i>Betula pendula</i>	County Durham	Apr-22	8	No	2
<i>Betula pendula</i>	North Yorkshire	May-22	3	No	1
<i>Betula pendula</i>	Oxfordshire	Apr-21	2	No	2
<i>Borago officinalis</i>	Oxfordshire	Aug-21	1	No	3
<i>Borago officinalis</i>	Oxfordshire	Sep-22	2	No	2
<i>Brassica napus</i>	North Yorkshire	Apr-22	3	No	1

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Brassica napus</i>	Oxfordshire	Apr-22	2	No	1
<i>Brassica napus</i>	West Yorkshire	Jul-22	3	No	1
<i>Brassica oleracea</i>	Oxfordshire	May-21	2	No	1
<i>Brassica rapa</i>	Oxfordshire	May-22	2	No	1
<i>Brassica</i> sp.	Oxfordshire	Jun-21	2	No	1
<i>Brassica</i> sp.	Oxfordshire	May-21	2	No	1
<i>Bryonia cretica</i>	Oxfordshire	Jun-21	2	No	1
<i>Bryonia cretica</i> subsp. <i>Dioica</i>	Greater London	Jul-22	5	No	1
<i>Buddleja davidii</i>	Oxfordshire	Aug-21	12	No	1
<i>Buddleja davidii</i>	Oxfordshire	Sep-21	2	No	1
<i>Calluna vulgaris</i>	Berkshire	Aug-22	2	No	1
<i>Calluna vulgaris</i>	Dorset	Jul-22	2	No	1
<i>Calluna vulgaris</i>	North Yorkshire	Aug-22	3	No	1
<i>Calluna vulgaris</i>	North Yorkshire	Jul-22	3	No	1
<i>Calluna vulgaris</i>	Oxfordshire	Sep-21	2	No	1
<i>Calluna vulgaris</i>	Shropshire	Aug-21	2	No	1
<i>Caltha palustris</i>	Oxfordshire	Apr-21	7	No	1
<i>Calystegia sepium</i>	Greater London	Jun-22	5	No	1
<i>Calystegia sepium</i>	North Yorkshire	Jul-22	3	No	1
<i>Calystegia sepium/silvatica</i>	Oxfordshire	Aug-21	2	No	1
<i>Calystegia sepium/silvatica</i>	Oxfordshire	Sep-21	12	No	1
<i>Calystegia silvatica</i>	Oxfordshire	Sep-21	2	No	1
<i>Calystegia silvatica</i>	Oxfordshire	Sep-21	12	No	1
<i>Calystegia</i> sp.	West Yorkshire	Jul-22	3	No	1
<i>Campanula latifolia</i>	North Yorkshire	Jul-22	3	No	1
<i>Campanula persicifolia</i>	Oxfordshire	Jun-21	2	No	1
<i>Campanula rotundifolia</i>	Greater London	Jul-22	5	No	1
<i>Campanula rotundifolia</i>	North Yorkshire	Jul-22	3	No	2
<i>Campanula trachelium</i>	Oxfordshire	Aug-21	2	No	1
<i>Cardamine pratensis</i>	Greater London	Mar-22	5	No	1
<i>Cardamine pratensis</i>	Oxfordshire	Apr-21	2	No	1
<i>Carduus crispus</i>	Oxfordshire	Jul-21	12	No	1
<i>Carduus nutans</i>	Oxfordshire	Jun-21	1	No	1
<i>Carduus nutans</i>	Oxfordshire	Jun-21	2	No	1

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Carpinus betulus</i>	Greater London	Mar-22	5	No	1
<i>Carpinus betulus</i>	Oxfordshire	Apr-21	2	No	1
<i>Carpinus betulus</i>	Oxfordshire	Apr-21	7	No	3
<i>Carpinus betulus</i>	Oxfordshire	Mar-21	11	No	1
<i>Castanea sativa</i>	Greater London	Jul-22	5	No	1
<i>Centaurea cyanus</i>	Dorset	Jul-21	12	No	1
<i>Centaurea montana</i>	Oxfordshire	Jun-21	2	No	1
<i>Centaurea nigra</i>	County Durham	Jul-22	3	No	2
<i>Centaurea nigra</i>	Oxfordshire	Jul-21	1	No	2
<i>Centaurea nigra</i>	Oxfordshire	Jun-21	2	No	2
<i>Centaurea scabiosa</i>	North Yorkshire	Jul-22	3	No	1
<i>Centaurea scabiosa</i>	Oxfordshire	Jul-21	12	No	1
<i>Centaurea scabiosa</i>	Wiltshire	Jul-21	2	No	1
<i>Chelidonium majus</i>	Oxfordshire	May-21	2	No	1
<i>Cichorium intybus</i>	Oxfordshire	Jul-21	1	No	1
<i>Cichorium intybus</i>	Oxfordshire	Jul-21	2 & 12	Yes	1
<i>Circaea lutetiana</i>	Oxfordshire	Jul - 22 & Aug- 22	9	Yes	1
<i>Circaea lutetiana</i>	Oxfordshire & Greater London	Jul - 21 (O), Jun - 22 (GL)	2 & 5	Yes	1
<i>Cirsium acaule</i>	Sussex	Jul-22	5	No	2
<i>Cirsium arvense</i>	County Durham	Jul-22	3	No	1
<i>Cirsium arvense</i>	Oxfordshire	Jul-22	9	No	1
<i>Cirsium arvense</i>	Oxfordshire	Jun-21	2	No	1
<i>Cirsium eriophorum</i>	Oxfordshire	Aug-21	12	No	1
<i>Cirsium eriophorum</i>	Oxfordshire	Aug-22	2	No	1
<i>Cirsium eriophorum</i>	Wiltshire	Jul-21	2	No	1
<i>Cirsium palustre</i>	North Yorkshire	Aug-22	3	No	1
<i>Cirsium palustre</i>	North Yorkshire	Jul-22	3	No	1
<i>Cirsium palustre</i>	Oxfordshire	Jul-21	12	No	1
<i>Cirsium palustre</i>	Oxfordshire	Jul-22	2	No	1
<i>Cirsium vulgare</i>	County Durham	Jul-22	3	No	1
<i>Cirsium vulgare</i>	Greater London	May-22	5	No	1
<i>Cirsium vulgare</i>	Oxfordshire	Jul-21	12	No	1
<i>Clematis vitalba</i>	Essex	Jun-22	5	No	1
<i>Clematis vitalba</i>	Oxfordshire	Jul-21	2	No	1
<i>Clinopodium vulgare</i>	Oxfordshire	Aug-21	12	No	1
<i>Convolvulus arvensis</i>	Greater London	Jun-22	5	No	1
<i>Convolvulus arvensis</i>	North Yorkshire	Jul-22	3	No	1
<i>Cornus sanguinea</i>	Greater London	May-22	5	No	1

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Cornus sanguinea</i>	North Yorkshire	Jun-22	3	No	1
<i>Cornus sanguinea</i>	Oxfordshire	May-22	2	No	1
<i>Coronilla varia</i>	Oxfordshire	Sep-21	2 & 12	No	1
<i>Corydalis solida</i>	Oxfordshire	Mar-21	2	No	2
<i>Corylus avellana</i>	Greater London	Feb-22	5	No	1
<i>Corylus avellana</i>	Greater London	Jan-22	5	No	4
<i>Corylus avellana</i>	Oxfordshire	Feb-22	2	No	3
<i>Corylus avellana</i>	Oxfordshire	Mar-22	2	No	1
<i>Crataegus monogyna</i>	Greater London	May-22	5	No	1
<i>Crataegus monogyna</i>	Oxfordshire	May-21	2	No	2
<i>Crepis biennis</i>	Oxfordshire	May-21	2	No	1
<i>Crepis capillaris</i>	County Durham	Jun-22	3	No	1
<i>Crepis capillaris</i>	Oxfordshire	Jun-22	2	No	1
<i>Crepis capillaris</i>	Sussex	Jun-22	5	No	1
<i>Crepis vesicaria</i>	North Yorkshire	May-22	3	No	1
<i>Crepis vesicaria</i>	Oxfordshire	Jun-21	2	No	1
<i>Crocus</i> sp.	Oxfordshire	Mar-21	2	No	1
<i>Crocus vernus</i>	Oxfordshire	Feb-21	2	No	2
<i>Crocus vernus</i>	Oxfordshire	Mar-22	2	No	2
<i>Crocus x luteus</i>	Oxfordshire	Feb-21	2	No	1
<i>Cytisus scoparius</i>	County Durham	Apr-22	8	No	1
<i>Cytisus scoparius</i>	North Yorkshire	May-22	3	No	1
<i>Cytisus scoparius</i>	Tyne and Wear	Apr-22	8	No	1
<i>Daucus carota</i>	Oxfordshire & North Yorkshire	Jul - 21(O), Jul - 22 (NY)	12 & 3	Yes	1
<i>Digitalis purpurea</i>	County Durham	Jun-22	8	No	1
<i>Digitalis purpurea</i>	Oxfordshire	Jul-21	2	No	1
<i>Dioscorea communis</i>	Oxfordshire	Jun-22	2	No	1
<i>Dipsacus fullonum</i>	County Durham	Jul-22	8	No	1
<i>Dipsacus fullonum</i>	County Durham/North Yorkshire	Aug-22	8	No	1
<i>Dipsacus fullonum</i>	Oxfordshire	Sep-21	12	No	1
<i>Dipsacus laciniatus</i>	Oxfordshire	Aug-21	2	No	1
<i>Echium vulgare</i>	County Durham	Jun-22	3	No	1
<i>Echium vulgare</i>	Oxfordshire	Jul-21	2	No	1
<i>Echium vulgare</i>	Oxfordshire	Jun-21	2	No	1
<i>Epilobium angustifolium</i>	County Durham	Jul-22	3	No	1

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Epilobium angustifolium</i>	Greater London	Jul-22	5	No	1
<i>Epilobium angustifolium</i>	Oxfordshire	Aug-21	12	No	1
<i>Epilobium angustifolium</i>	Wiltshire	Jul-21	12	No	1
<i>Epilobium hirsutum</i>	Greater London	Aug-22	5	No	1
<i>Epilobium hirsutum</i>	West Yorkshire	Jul-22	3	No	1
<i>Epilobium montanum</i>	Greater London	May-22	5	No	1
<i>Epilobium parviflorum</i>	West Yorkshire & Sussex	Jun - 22 (S), Jul - 22 (WY)	3 & 5	Yes	1
<i>Erica cinerea</i>	Berkshire	Aug-22	2	No	1
<i>Erica cinerea</i>	Dorset	Jul-22	2	No	1
<i>Erica tetralix</i>	Berkshire	Aug-22	2	No	1
<i>Erica tetralix</i>	Dorset	Jul-22	2	No	1
<i>Erica tetralix</i>	North Yorkshire	Aug-22	3	No	1
<i>Euonymus europaeus</i>	Oxfordshire	Apr & May - 22	2	No	1
<i>Eupatorium cannabinum</i>	Oxfordshire	Aug-21	12	No	2
<i>Euphrasia officinalis</i>	County Durham	Aug-22	3	No	1
<i>Fagus sylvatica</i>	Oxfordshire	Apr-21	7	No	2
<i>Fallopia baldschuanica</i>	Oxfordshire	Jul-22	2	No	1
<i>Filipendula ulmaria</i>	Greater London	Jul-22	5	No	1
<i>Filipendula ulmaria</i>	Oxfordshire	Jul-21	12	No	1
<i>Filipendula ulmaria</i>	Sussex	Jun-22	5	No	1
<i>Filipendula ulmaria</i>	Tyne and Wear	Jun-22	8	No	1
<i>Forsythia</i> spp.	West Yorkshire	Apr-21	10	No	1
<i>Fraxinus excelsior</i>	Greater London	Apr-22	5	No	1
<i>Fraxinus excelsior</i>	Oxfordshire	Apr-22	2	No	1
<i>Fritillaria meleagris</i>	Oxfordshire	Apr-21	2	No	2
<i>Galanthus nivalis</i>	Oxfordshire	Feb-21	2	No	2
<i>Galanthus nivalis</i>	Oxfordshire	Feb-22	2	No	1
<i>Galanthus nivalis</i>	Oxfordshire	Mar-21	2	No	2
<i>Galanthus nivalis</i>	Oxfordshire	Mar-22	2	No	1
<i>Galeopsis tetrahit</i>	Sussex	Aug-22	5	No	1
<i>Galium verum</i>	Oxfordshire	Aug-22	9	No	1
<i>Garrya elliptica</i>	Greater London	Jan-22	5	No	1
<i>Geranium macrorrhizum</i>	Oxfordshire	May-21	2	No	1
<i>Geranium pratense</i>	Oxfordshire	Jun-21	2	No	1

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Geranium pyrenaicum</i>	Oxfordshire	May-21	2	Yes	1
<i>Geranium robertianum</i>	Greater London	Apr-22	5	No	1
<i>Geranium robertianum</i>	Oxfordshire	May-22	2	No	1
<i>Geum urbanum</i>	County Durham & Greater London	May-22	8 & 5	Yes	1
<i>Geum urbanum</i>	Oxfordshire	May-22	2	No	1
<i>Glebionis segetum</i>	Dorset	Jul-21	12	No	1
<i>Glechoma hederacea</i>	County Durham	Apr-22	3	No	1
<i>Glechoma hederacea</i>	Oxfordshire	Apr-21	2	No	1
<i>Glechoma hederacea</i>	Oxfordshire	May-21	2	No	1
<i>Gunnera tinctoria var. tinctoria</i>	Greater London	NA	5	No	1
<i>Hedera helix</i>	Wiltshire, Oxfordshire & Great London	Sep - 21 (W & O), Sep - 22 (GL)	12 & 5	Yes	1
<i>Helianthemum nummularium</i>	Oxfordshire	Jun-21	2 & 1	Yes	1
<i>Helianthemum nummularium</i>	Oxfordshire	Sep-21	2	No	1
<i>Helianthus annuus</i>	NA	Sep-21	2	No	4
<i>Helleborus argutifolius</i>	Oxfordshire	Apr-21	2	No	1
<i>Helminthotheca echioides</i>	Oxfordshire	Jul-21	12	No	1
<i>Helminthotheca echioides</i>	Oxfordshire	Sep-22	2	No	1
<i>Heracleum sphondylium</i>	Greater London	Jun-22	5	No	1
<i>Heracleum sphondylium</i>	Oxfordshire	Jun-21	2	No	1
<i>Hirschfeldia incana</i>	North Yorkshire	Jun-22	3	No	1
<i>Hyacinthoides non-scripta</i>	County Durham	Apr-22	8	No	1
<i>Hyacinthoides non-scripta</i>	Greater London	Apr-22	5	No	1
<i>Hyacinthoides non-scripta</i>	North Yorkshire	Apr-22	3	No	1
<i>Hyacinthoides non-scripta</i>	North Yorkshire	May-22	3	No	1
<i>Hyacinthoides non-scripta</i>	Oxfordshire	Apr-21	2	No	2

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Hyacinthoides non-scripta</i>	Oxfordshire	Apr-22	2	No	1
<i>Hyacinthoides non-scripta</i>	Oxfordshire	May-21	2	No	2
<i>Hyacinthoides x massartiana</i>	County Durham	Apr-22	3	No	1
<i>Hyacinthoides x massartiana</i>	North Yorkshire	May-22	3	No	1
<i>Hyacinthoides x massartiana</i>	Oxfordshire	Apr-21	2	No	1
<i>Hypericum hirsutum</i>	Oxfordshire	Jul-21	12	Yes	1
<i>Hypericum perforatum</i>	North Yorkshire	Aug & Sep - 2022	3	No	1
<i>Hypericum perforatum</i>	Oxfordshire	Jul-21	12	No	1
<i>Hypericum pulchrum</i>	Sussex	Jun-22	5	No	1
<i>Hypochaeris radicata</i>	North Yorkshire	Jun-22	3	No	1
<i>Hypochaeris radicata</i>	Oxfordshire	Jun-21	1	No	1
<i>Hypochaeris radicata</i>	Oxfordshire	Jun-22	2	No	1
<i>Hypochaeris radicata</i>	Oxfordshire & Greater London	Jun - 21 (O), Jul - 22 (GL)	2 & 5	Yes	1
<i>Ilex aquifolium</i>	Greater London	Apr-22	5	No	1
<i>Ilex aquifolium</i>	Oxfordshire	May-22	2	No	1
<i>Impatiens glandulifera</i>	County Durham	Sep-22	3	No	1
<i>Impatiens glandulifera</i>	Greater London	Aug-22	5	No	1
<i>Impatiens glandulifera</i>	Oxfordshire	Jul-21	12	No	1
<i>Iris foetidissima</i>	Oxfordshire	Jun-22	2	No	1
<i>Iris pseudacorus</i>	Greater London	May-22	5	No	2
<i>Iris pseudacorus</i>	Oxfordshire	May-22	2	No	1
<i>Jacobaea erucifolia</i>	County Durham	Aug-22	3	No	1
<i>Jacobaea erucifolia</i>	Greater London	Jul-22	5	No	1
<i>Jacobaea erucifolia</i>	Oxfordshire	Aug-22	2	No	1
<i>Jacobaea erucifolia</i>	Wiltshire	Sep-21	12	No	1
<i>Jacobaea vulgaris</i>	Greater London	Jun-22	5	No	1
<i>Jacobaea vulgaris</i>	North Yorkshire	Jul-22	3	No	1
<i>Jacobaea vulgaris</i>	Oxfordshire	Jun-22	2	No	1
<i>Jacobaea vulgaris</i>	Sussex	Jul-22	5	No	1
<i>Knautia arvensis</i>	County Durham	Jul-22	3	No	1
<i>Knautia arvensis</i>	North Yorkshire	Jul-22	3	No	1

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Knautia arvensis</i>	Oxfordshire	Jul-21	1	No	1
<i>Knautia arvensis</i>	Oxfordshire	Jul-21	2	No	1
<i>Knautia arvensis</i>	Oxfordshire	Jun-21	2	No	1
<i>Knautia arvensis</i>	Wiltshire	Jul-21	1	No	1
<i>Laburnum anagyroides</i>	Oxfordshire	May-21	2	No	1
<i>Lactuca serriola</i>	Oxfordshire	Sep-21	2	No	1
<i>Lamium album</i>	County Durham	Apr-22	3	No	1
<i>Lamium album</i>	Oxfordshire	Apr-21	2	No	1
<i>Lamium album</i>	Oxfordshire	Aug-21	2	No	1
<i>Lamium album</i>	Oxfordshire	Aug-21	12	No	1
<i>Lamium galeobdolon</i>	Oxfordshire	Apr-21	2	No	1
<i>Lamium galeobdolon</i> subsp. <i>Argentatum</i>	Oxfordshire	Apr-22	2	No	1
<i>Lamium purpureum</i>	Greater London	Apr-22	5	No	1
<i>Lapsana communis</i>	County Durham	Jun-22	8	No	1
<i>Lapsana communis</i>	Oxfordshire	Jun-21	2	Yes	1
<i>Lapsana communis</i>	Oxfordshire	Sep-21	12 & 2	Yes	1
<i>Lathyrus latifolius</i>	Oxfordshire	Jul-21	12	No	2
<i>Lathyrus pratensis</i>	Oxfordshire	Jul-21	2	Yes	1
<i>Lathyrus pratensis</i>	Sussex	Jun-22	5	No	1
<i>Leontodon hispidus</i>	Oxfordshire	Jun-22	2	No	1
<i>Leontodon hispidus</i>	Oxfordshire	Sep-21	2	No	1
<i>Leontodon hispidus</i>	Sussex	Jun-22	5	No	1
<i>Leontodon saxatilis</i>	Greater London	Jun-22	5	No	1
<i>Leontodon</i> sp.	Oxfordshire	Aug-21	12	No	1
<i>Leucanthemum vulgare</i>	North Yorkshire	May-22	3	No	1
<i>Leucanthemum vulgare</i>	Oxfordshire	Jul-21	12	No	1
<i>Leucanthemum vulgare</i>	Oxfordshire	Jun-21	1	No	1
<i>Leucanthemum vulgare</i>	Oxfordshire	Jun-21	2	No	1
<i>Leucanthemum vulgare</i>	Oxfordshire	May & Jun - 21	2 & 1	Yes	1
<i>Leucanthemum vulgare</i>	Sussex	Jun-22	5	No	1
<i>Ligustrum</i> sp.	Oxfordshire	Jul-21	12	No	1
<i>Ligustrum vulgare</i>	Oxfordshire	Jun-22	2	No	1
<i>Lonicera caprifolium</i>	Oxfordshire	Jun-21	2	No	1

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Lonicera periclymenum</i>	North Yorkshire	Aug-22	3	No	1
<i>Lonicera periclymenum</i>	Oxfordshire	Jun-21	13	No	1
<i>Lotus corniculatus</i>	North Yorkshire	Jun-22	3	No	1
<i>Lotus corniculatus</i>	Oxfordshire	Aug-21	1 & 12	No	1
<i>Lotus corniculatus</i>	Oxfordshire	Jul & Aug - 21	1 & 12	Yes	1
<i>Lotus corniculatus</i>	Oxfordshire	Jun-21	1	No	1
<i>Lotus corniculatus</i>	Oxfordshire	Jun-21	2 & 1	Yes	1
<i>Lotus corniculatus</i>	Oxfordshire	Sep-22	2	No	2
<i>Lotus corniculatus</i> subsp. <i>Corniculatus</i>	North Yorkshire	May-22	3	No	1
<i>Lotus pedunculatus</i>	North Yorkshire	Jul-22	3	No	1
<i>Lotus pedunculatus</i>	Sussex	Jun & Jul - 22	5	Yes	1
<i>Lotus pedunculatus</i>	Sussex	Jun-22	5	No	1
<i>Lunaria annua</i>	Oxfordshire	May-21	2	No	1
<i>Lysimachia arvensis</i>	County Durham	Jun-22	3	No	1
<i>Lysimachia nemorum</i>	Oxfordshire & Sussex	Jul - 22 (O), Jun - 22 (S)	9 & 5	Yes	1
<i>Lysimachia punctata</i>	North Yorkshire	Jul-22	3	No	1
<i>Lysimachia punctata</i>	Oxfordshire	Jul-21	2	No	1
<i>Lysimachia vulgaris</i>	Oxfordshire	Aug-21	2	No	1
<i>Lysimachia vulgaris</i>	Oxfordshire	Jul-21	12	No	1
<i>Lythrum salicaria</i>	Oxfordshire	Aug & Sep - 21	2	Yes	1
<i>Malus domestica</i>	County Durham	Apr-22	3	No	1
<i>Malus domestica</i>	Greater London	Apr-22	5	No	1
<i>Malus domestica</i>	North Yorkshire	Apr-22	3	No	1
<i>Malus domestica</i>	Oxfordshire	May-21	2	No	1
<i>Malus</i> sp.	Oxfordshire	Apr-22	2	No	3
<i>Malva moschata</i>	Dorset	Jul-21	12	No	1
<i>Malva moschata</i>	North Yorkshire	Jul-22	3	No	1
<i>Malva moschata</i>	Oxfordshire	Jul-21	1	No	1
<i>Malva moschata</i>	Oxfordshire	Jul-21	12	No	1
<i>Malva moschata</i>	Oxfordshire	Jun-21	2	No	1
<i>Malva sylvestris</i>	Greater London	Jun-22	5	No	1
<i>Malva sylvestris</i>	Oxfordshire	Jun-21	1	No	1
<i>Malva sylvestris</i>	Oxfordshire	Jun-21	2	No	3
<i>Matricaria chamomilla</i>	County Durham	May-22	8	No	1
<i>Matricaria chamomilla</i>	Oxfordshire	Aug-21	2	No	1

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Medicago sativa</i>	Oxfordshire	Sep-21	12	No	1
<i>Medicago sativa</i>	Oxfordshire	Sep-22	2	No	1
<i>Melilotus officinalis</i>	Oxfordshire	Aug-21	2 & 12	No	1
<i>Melilotus</i> sp.	Oxfordshire	Aug-21	1	No	1
<i>Melilotus</i> sp.	Oxfordshire	Aug-21	2	No	1
<i>Mentha aquatica</i>	County Durham	Aug-22	3	No	1
<i>Mentha aquatica</i>	Oxfordshire	Aug-21	12	No	1
<i>Mercurialis perennis</i>	Greater London	Apr-22	5	No	1
<i>Mercurialis perennis</i>	North Yorkshire	Apr-22	3	No	2
<i>Mercurialis perennis</i>	Oxfordshire	Apr-21	2	No	2
<i>Muscari armeniacum</i>	Wiltshire	Apr-21	2	No	1
<i>Narcissus pseudonarcissus</i>	Greater London	Feb-22	5	No	1
<i>Narcissus</i> sp.	Oxfordshire	Apr-21	2	No	1
<i>Narcissus</i> sp.	Oxfordshire	Feb-21	2	No	3
<i>Narcissus</i> sp.	Oxfordshire	Mar-21	2	No	1
<i>Narcissus</i> sp.	Oxfordshire	Mar-21	4	No	2
<i>Narcissus</i> sp.	Oxfordshire	Mar-21	6	No	1
<i>Narcissus</i> sp.	Oxfordshire	Mar-22	2	No	2
<i>Narthecium ossifragum</i>	Northumberland	Jul-22	8	No	1
<i>Nigella damascena</i>	Wiltshire	Sep-21	12	No	1
<i>Odontites vernus</i>	Oxfordshire	Aug-21	1	No	1
<i>Odontites vernus</i>	Wiltshire	Jul-21	2	No	1
<i>Odontites vernus</i>	Wiltshire & Oxfordshire	Aug - 21 (W & O)	1 & 2	Yes	1
<i>Oenanthe crocata</i>	Greater London	May-22	5	No	1
<i>Oenanthe crocata</i>	Oxfordshire	May-22	2	No	1
<i>Onobrychis viciifolia</i>	County Durham	Jul-22	3	No	1
<i>Onobrychis viciifolia</i>	Wiltshire	Aug-21	1	No	1
<i>Onobrychis viciifolia</i>	Wiltshire	Jul-21	2 & 12	No	1
<i>Ononis spinosa</i> subsp. <i>Procurrens</i>	North Yorkshire	Jun-22	3	No	1
<i>Ononis spinosa</i> subsp. <i>Procurrens</i>	Oxfordshire	Sep-21	2	No	1
<i>Onopordum acanthium</i>	Oxfordshire	Jul-21	2	No	1
<i>Origanum vulgare</i>	Oxfordshire	Aug-21	12	No	1
<i>Oxalis acetosella</i>	North Yorkshire	May-22	3	No	1
<i>Papaver cambricum</i>	Oxfordshire	Jun-21	2 & 1	No	1
<i>Papaver cambricum</i>	Oxfordshire	May-21	2	No	1

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Papaver rhoeas</i>	Greater London	Jun-22	5	No	1
<i>Papaver rhoeas</i>	Oxfordshire	Jun-21	2	No	1
<i>Passiflora caerulea</i>	Oxfordshire	Aug-21	12	No	1
<i>Pastinaca sativa</i>	County Durham	Jul-22	3	No	1
<i>Pastinaca sativa</i>	Oxfordshire	Jul-22	2	No	1
<i>Petasites hybridus</i>	North Yorkshire	Apr-22	3	No	2
<i>Petasites hybridus</i>	Oxfordshire	Apr-22	2	No	2
<i>Petasites pyrenaicus</i>	Oxfordshire	Feb-22	2	No	1
<i>Phacelia tanacetifolia</i>	Oxfordshire	Sep-21	2	No	1
<i>Picris hieracioides</i>	Oxfordshire	Aug-21	1	No	1
<i>Pilosella aurantiaca</i>	County Durham	Jun-22	3	No	1
<i>Pilosella officinarum</i>	Oxfordshire	Jun-22	2	No	1
<i>Pilosella officinarum</i>	Oxfordshire, County Durham & Greater London	Aug - 21 (O), Jun - 22 (CD), May - 22 (GL)	12, 3 & 5	Yes	1
<i>Pinus coulteri</i>	Greater London	2022	5	No	1
<i>Plantago coronopus</i>	Dorset	Jul-22	2	No	1
<i>Plantago coronopus</i>	Greater London	May-22	5	No	1
<i>Plantago coronopus</i>	North Yorkshire	Jun-22	8	No	1
<i>Plantago lanceolata</i>	Greater London	May-22	5	No	1
<i>Plantago lanceolata</i>	North Yorkshire	May-22	3	No	2
<i>Plantago lanceolata</i>	Oxfordshire	Aug-21	12	No	1
<i>Plantago lanceolata</i>	Oxfordshire	May-21	2	No	3
<i>Plantago major</i>	Oxfordshire	Jul-21	12	No	1
<i>Plantago maritima</i>	County Durham	Jun-22	8	No	1
<i>Plantago maritima</i>	Dorset	Jul-22	2	No	1
<i>Plantago media</i>	Oxfordshire	Jun & Jul - 21	2, 1 & 12	Yes	1
<i>Plantago media</i>	Oxfordshire	Jun-21	2	No	1
<i>Plantago media</i>	Oxfordshire	Jun - 21, Aug - 21	1 & 12	Yes	1
<i>Populus tremula</i>	Oxfordshire	Mar-22	2	Yes	1
<i>Potentilla erecta</i>	Berkshire	May-22	2	No	1
<i>Potentilla reptans</i>	Oxfordshire	Jun-22	2	No	1
<i>Potentilla sterilis</i>	Oxfordshire & North Yorkshire	Apr - 22 (O), Mar - 22 (NY)	2 & 3	Yes	1
<i>Primula veris</i>	Oxfordshire	Apr-21	2	No	2
<i>Primula vulgaris</i>	County Durham & North Yorkshire	Apr - 22 (CD & NY)	8 & 3	Yes	1
<i>Primula vulgaris</i>	Oxfordshire	Feb-21	2	No	1

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Primula vulgaris</i>	Oxfordshire	Mar-21	2	No	3
<i>Primula vulgaris</i>	Oxfordshire	Mar-21	6	No	1
<i>Primula vulgaris</i>	Oxfordshire	Mar-22	2	No	1
<i>Primula vulgaris</i>	Sussex	Mar-22	5	No	1
<i>Prunella vulgaris</i>	Oxfordshire	Jun-22	2	No	1
<i>Prunus avium</i>	Greater London	Mar-22	5	No	1
<i>Prunus avium</i>	Oxfordshire	Apr-21	2	No	2
<i>Prunus avium</i>	Oxfordshire	Mar-21	6	No	1
<i>Prunus avium</i>	Sussex	Mar-22	5	No	1
<i>Prunus cerasifera</i>	Oxfordshire	Mar-21	2	No	6
<i>Prunus cerasifera</i>	Oxfordshire	Mar-22	2	No	1
<i>Prunus spinosa</i>	Berkshire	Apr-21	2	No	1
<i>Prunus spinosa</i>	County Durham	Apr-22	3	Yes	1
<i>Prunus spinosa</i>	Oxfordshire	Apr-21	2 & 6	No	1
<i>Prunus spinosa</i>	Oxfordshire	Mar-21	2, 11 & 4	Yes	1
<i>Prunus spinosa</i>	Sussex	Mar-22	5	No	1
<i>Pulicaria dysenterica</i>	Oxfordshire	Aug-21	2	No	1
<i>Pulicaria dysenterica</i>	Oxfordshire & North Yorkshire	Sep - 21 (O), Sep - 22 (NY)	12 & 3	Yes	1
<i>Pulicaria dysenterica</i>	Sussex	Aug-22	5	No	1
<i>Pulicaria dysenterica</i>	Wiltshire	Sep-21	12	No	1
<i>Pyrus</i> sp.	Oxfordshire	Apr-21	2	No	1
<i>Quercus robur</i>	Greater London	Apr-22	5	No	1
<i>Rabelera holostea</i>	North Yorkshire	Apr-22	3	No	1
<i>Rabelera holostea</i>	North Yorkshire	May-22	3	No	1
<i>Rabelera holostea</i>	Oxfordshire	Apr-22	2	No	1
<i>Rabelera holostea</i>	Oxfordshire	May-21	2	No	3
<i>Ranunculus acris</i>	County Durham	May-22	8	No	1
<i>Ranunculus acris</i>	Greater London	May-22	5	No	2
<i>Ranunculus acris</i>	North Yorkshire	Jun-22	3	No	1
<i>Ranunculus acris</i>	North Yorkshire	May-22	3	No	1
<i>Ranunculus acris</i>	Oxfordshire	Jun-21	1	No	1
<i>Ranunculus acris</i>	Oxfordshire	May-21	2	No	1
<i>Ranunculus bulbosus</i>	Greater London	Apr-22	5	No	1
<i>Ranunculus bulbosus</i>	Greater London	NA	5	No	1
<i>Ranunculus bulbosus</i>	North Yorkshire	May-22	3	No	1
<i>Ranunculus bulbosus</i>	Oxfordshire	Apr-21	2	No	2
<i>Ranunculus bulbosus</i>	Oxfordshire	May-21	2	No	1
<i>Ranunculus ficaria</i>	Greater London	Mar-22	5	No	1

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Ranunculus ficaria</i>	North Yorkshire	Apr-22	3	No	1
<i>Ranunculus ficaria</i>	North Yorkshire	Mar-22	3	No	1
<i>Ranunculus ficaria</i>	Oxfordshire	Mar-21	2	No	4
<i>Ranunculus ficaria</i>	Wiltshire	Mar-22	2	No	1
<i>Ranunculus repens</i>	Greater London	May-22	5	No	1
<i>Ranunculus repens</i>	North Yorkshire	Jun-22	3	No	1
<i>Ranunculus repens</i>	Oxfordshire	Jul-21	12	No	1
<i>Ranunculus repens</i>	Oxfordshire	May-21	2	No	1
<i>Ranunculus</i> sp.	Oxfordshire	Mar-21	2	No	1
<i>Raphanus raphanistrum</i>	Greater London	Jun-22	5	No	1
<i>Raphanus raphanistrum</i> subsp. <i>Sativus</i>	Greater London	Sep-22	5	No	1
<i>Reseda lutea</i>	County Durham	Jul-22	3	No	1
<i>Reseda lutea</i>	Greater London	May-22	5	No	1
<i>Reseda lutea</i>	North Yorkshire	Jun-22	3	No	1
<i>Reseda luteola</i>	Greater London	May-22	5	No	1
<i>Reseda luteola</i>	Tyne and Wear	May-22	8	No	1
<i>Rhamphospermum arvense</i>	North Yorkshire	May-22	3	No	1
<i>Rhamphospermum arvense</i>	Oxfordshire	Apr-22	2	No	1
<i>Rhamphospermum arvense</i>	Oxfordshire	Jun-22	2	No	1
<i>Rhinanthus minor</i>	North Yorkshire	Jun-22	3	No	1
<i>Rhinanthus minor</i>	Oxfordshire	Jul-21	2	No	1
<i>Rhinanthus minor</i>	Oxfordshire	Jun-21	2	No	2
<i>Rhododendron ponticum</i>	Greater London	May-22	5	No	1
<i>Rhododendron ponticum</i>	Oxfordshire	Jun-21	2	No	1
<i>Rosa arvensis</i>	Sussex	Jun-22	5	No	2
<i>Rosa canina</i>	Greater London	May-22	5	No	1
<i>Rosa canina</i>	North Yorkshire	Jun-22	3	No	1
<i>Rosa canina</i>	Oxfordshire	Jun-21	1 & 13	Yes	1
<i>Rosa canina</i>	Oxfordshire	Jun-22	2	No	1
<i>Rubus caesius</i>	Wiltshire	Sep-21	12	No	1
<i>Rubus fruticosus</i>	Oxfordshire	Aug-21	12	No	1
<i>Rubus fruticosus</i>	Oxfordshire	Jun-21	13	No	1
<i>Rubus fruticosus</i>	Wiltshire	Jul-21	1	No	1

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Rubus idaeus</i>	Oxfordshire	Jun-22	2	No	1
<i>Rubus idaeus</i>	Sussex	Jun-22	5	No	1
<i>Salix caprea</i>	Oxfordshire	Mar-21	4	No	1
<i>Salix caprea</i>	Oxfordshire	Mar-22	2	No	1
<i>Salix caprea</i> "Kilmarnock"	Greater London	Mar-22	5	No	1
<i>Salix caprea/cinerea</i>	Berkshire	Apr-21	2	No	1
<i>Salix caprea/cinerea</i>	County Durham	Apr-22	3	No	3
<i>Salix caprea/cinerea</i>	Oxfordshire	Mar-21	2	No	2
<i>Salix caprea/cinerea</i>	Oxfordshire	Mar-21	4	No	2
<i>Salix caprea/cinerea</i>	Sussex	Mar-22	5	No	1
<i>Salix fragilis/alba</i>	Greater London	Apr-22	5	No	1
<i>Salix repens</i>	Greater London	Apr-22	5	No	1
<i>Salix</i> sp.	County Durham	Apr-22	8	No	1
<i>Salix</i> sp.	Oxfordshire	Apr-22	2	No	2
<i>Salix</i> sp.	Oxfordshire	Mar-21	2	No	2
<i>Salix x pendulina</i> <i>nothof. Tristis</i>	Oxfordshire	Apr-21	2	No	2
<i>Sambucus nigra</i>	Oxfordshire	Jun-21	1	No	2
<i>Sambucus nigra</i>	Oxfordshire	Jun-21	2	No	1
<i>Sanguisorba minor</i>	County Durham	Jun-22	8	No	1
<i>Sanguisorba minor</i>	Greater London	Jun-22	5	No	2
<i>Sanguisorba minor</i>	Oxfordshire	Apr-22	2	No	1
<i>Scabiosa columbaria</i>	Wiltshire	Jul-21	1	No	1
<i>Scilla forbesii</i>	Oxfordshire	Mar-21	2	No	2
<i>Scilla siberica</i>	Oxfordshire	Mar-21	2	No	1
<i>Scorzoneroides autumnalis</i>	County Durham	Sep-22	3	No	1
<i>Scorzoneroides autumnalis</i>	Greater London	Jul-22	5	No	1
<i>Scorzoneroides autumnalis</i>	Oxfordshire	Aug-21	12	Yes	1
<i>Scrophularia vernalis</i>	Oxfordshire	May-21	2	No	1
<i>Senecio vulgaris</i>	Greater London & North Yorkshire	Jun - 22 (NY), Aug - 22 (GL)	8 & 5	Yes	1
<i>Senecio/Jacobea</i> sp.	Oxfordshire	Jul-21	12	No	2
<i>Silene dioica</i>	County Durham	May-22	3	No	1
<i>Silene dioica</i>	County Durham	May-22	8	No	1
<i>Silene dioica</i>	North Yorkshire	May-22	3	No	1
<i>Silene dioica</i>	Oxfordshire	Jun-21	2	No	1

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Silene dioica</i>	Oxfordshire	Jun, Jul & Aug - 21	2, 12 & 1	Yes	1
<i>Silene dioica</i>	Oxfordshire	May-21	2	No	1
<i>Silene latifolia</i>	Oxfordshire	Aug-21	12	No	1
<i>Silene latifolia</i>	Oxfordshire	Jun-21	2	No	1
<i>Solanum dulcamara</i>	Greater London	May-22	5	No	1
<i>Solanum dulcamara</i>	Oxfordshire	Jun-22	2	No	1
<i>Solanum lycopersicum</i>	NA	Jul-21	2	No	1
<i>Solanum nigrum</i>	Greater London	Jun-22	5	No	1
<i>Solanum tuberosum</i>	North Yorkshire	Jul-22	3	No	1
<i>Sonchus arvensis</i>	County Durham	Aug-22	3	No	1
<i>Sonchus arvensis</i>	North Yorkshire	Jun-22	8	No	1
<i>Sonchus arvensis</i>	Sussex	Jul-22	5	No	1
<i>Sonchus oleraceus</i>	Greater London	May-22	5	No	1
<i>Sonchus oleraceus</i>	Oxfordshire, County Durham & North Yorkshire	Jun - 22 (NY), Jul - 22 (CD), Jul - 22 (O)	3 & 8	Yes	1
<i>Sorbus aucuparia</i>	County Durham	May-22	8	No	1
<i>Sorbus aucuparia</i>	Oxfordshire	May-21	2	No	3
<i>Stachys sylvatica</i>	Oxfordshire	Jun-22	2	No	1
<i>Stachys sylvatica</i>	Sussex	Jun-22	5	No	1
<i>Stellaria graminea</i>	Oxfordshire	Jun-22	2	No	1
<i>Succisa pratensis</i>	County Durham	Aug-22	8	No	1
<i>Succisa pratensis</i>	Greater London	Sep-22	5	No	1
<i>Succisa pratensis</i>	Oxfordshire	Aug-21	12	No	1
<i>Succisa pratensis</i>	Oxfordshire	Sep-21	12	No	1
<i>Symphyotrichum lanceolatum</i>	Oxfordshire	Sep-21	2	No	1
<i>Symphyotrichum lanceolatum</i>	Oxfordshire	Sep-21	12	No	1
<i>Symphyotrichum x salignum</i>	County Durham	Sep-22	3	No	1
<i>Symphytum orientale</i>	Oxfordshire	Apr-21	2	No	1
<i>Symphytum</i> sp.	Oxfordshire	Jun-21	1	No	1
<i>Symphytum x uplandicum</i>	Greater London	May-22	5	No	1
<i>Tanacetum vulgare</i>	County Durham	Jul-22	8	No	1
<i>Tanacetum vulgare</i>	County Durham	Sep-22	3	No	1
<i>Tanacetum vulgare</i>	North Yorkshire	Jul-22	3	No	1
<i>Taraxacum</i> sect. <i>Taraxacum</i>	Greater London	Apr-22	5	No	1

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Taraxacum</i> sect. <i>Taraxacum</i>	Oxfordshire	Apr-21	2	No	1
<i>Teucrium scorodonia</i>	Sussex	Jun-22	5	No	1
<i>Tilia platyphyllos</i>	Greater London	Jun-22	5	No	3
<i>Tilia x europaea</i>	Oxfordshire	Jun-21	2	No	1
<i>Torilis japonica</i>	North Yorkshire	Jul-22	3	No	1
<i>Torminalis glaberrima</i>	Greater London	May-22	5	No	1
<i>Trifolium hybridum</i>	Oxfordshire	Sep-22	2	No	1
<i>Trifolium incarnatum</i>	Oxfordshire	Sep-22	2	No	3
<i>Trifolium pratense</i>	Oxfordshire	Jul-21	1 & 12	No	2
<i>Trifolium pratense</i>	Oxfordshire	Jul-21	2	No	1
<i>Trifolium pratense</i>	Oxfordshire	Jul-21	12	No	1
<i>Trifolium pratense</i>	Oxfordshire	Jul-21	12 & 1	Yes	1
<i>Trifolium pratense</i>	Oxfordshire	Sep-21	2	No	1
<i>Trifolium pratense</i>	Oxfordshire	Sep-22	2	No	3
<i>Trifolium repens</i>	Oxfordshire	Aug-21	2	No	1
<i>Trifolium repens</i>	Oxfordshire	Aug-21	2 & 12	No	1
<i>Trifolium repens</i>	Oxfordshire	Aug-21	12	No	1
<i>Trifolium repens</i>	Oxfordshire	Sep-22	2	No	1
<i>Trifolium resupinatum</i>	Oxfordshire	Sep-22	2	No	2
<i>Tripleurospermum inodorum</i>	Oxfordshire	Jul-21	2	No	1
<i>Tripleurospermum inodorum</i>	Oxfordshire	Sep-22	2	No	1
<i>Tripleurospermum maritimum</i>	Dorset	Jul-22	2	No	1
<i>Tripolium pannonicum</i>	County Durham	Aug-22	3	No	1
<i>Tripolium pannonicum</i>	County Durham/North Yorkshire	Aug-22	8	No	1
<i>Tripolium pannonicum</i>	Essex	Sep-22	5	No	2
<i>Tripolium pannonicum</i>	Kent	Sep-22	5	No	1
<i>Tulipa sylvestris</i>	Oxfordshire	Apr-21	2	No	1
<i>Tulipa sylvestris</i>	Oxfordshire	Apr-21	7	No	2
<i>Tussilago farfara</i>	County Durham	Mar-22	3	No	1
<i>Ulex europaeus</i>	Greater London	Mar-22	5	No	1
<i>Ulex europaeus</i>	Oxfordshire	Mar-22	2	No	1
<i>Ulex europaeus</i>	Oxfordshire	May-21	2	No	2

Species	County collected	Month collected	Collector ID	Multi-site sample	Number of samples
<i>Ulex europaeus</i>	Sussex	Mar-22	5	No	1
<i>Ulex gallii</i>	Dorset	Sep-22	2	No	1
<i>Ulex minor</i>	Berkshire & Dorset	Aug - 22 (B), Sep - 22 (D)	2	Yes	1
<i>Ulex minor</i>	Hampshire	Aug-22	5	No	1
<i>Vaccinium myrtillus</i>	Berkshire	May-22	2	No	1
<i>Vaccinium myrtillus</i>	North Yorkshire	May-22	3	No	2
<i>Valeriana rubra</i>	Oxfordshire	Jun-21	2	No	2
<i>Verbascum thapsus</i>	Oxfordshire	Jul - 21, Aug - 21	12	Yes	1
<i>Veronica chamaedrys</i>	Greater London	May-22	5	No	1
<i>Veronica chamaedrys</i>	North Yorkshire	May-22	3	No	1
<i>Veronica chamaedrys</i>	Oxfordshire	May-21	2	No	1
<i>Veronica filiformis</i>	Oxfordshire	Apr-22	2	No	1
<i>Veronica montana</i>	Oxfordshire	May-22	2	No	1
<i>Veronica officinalis</i>	Oxfordshire	Jun-22	2	No	1
<i>Veronica officinalis</i>	Sussex	Jun-22	5	No	1
<i>Veronica persica</i>	Oxfordshire	Mar-21	2	No	1
<i>Viburnum lantana</i>	Oxfordshire	Apr-21	2	No	1
<i>Viburnum lantana</i>	Oxfordshire	May-21	2	No	1
<i>Vicia cracca</i>	Oxfordshire	Jul - 21, Aug - 21, Jul - 22, Aug - 22	12 & 2	Yes	1
<i>Vicia sativa</i>	Oxfordshire	May-22	2	No	1
<i>Vicia sativa</i>	Oxfordshire, County Durham & North Yorkshire	May - 21 (O), Apr - 22 (O), Jun - 22 (NY), May - 22 (CD)	2 & 3	Yes	1
<i>Vicia sepium</i>	North Yorkshire	Apr-22	3	No	1
<i>Vicia sepium</i>	Oxfordshire	May-22	2	No	2
<i>Viola odorata</i>	Oxfordshire	Mar-21	2	No	1
<i>Viola odorata</i>	Oxfordshire	Mar - 21, Mar - 22	2	Yes	1
<i>Viola riviniana</i>	North Yorkshire	Apr-22	3	No	1
<i>Viola riviniana</i>	Sussex	Apr-22	5	No	1

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1559 **Table S2.2.** Retention time, mass to charge ratio (*m/z*) and structure description of sterols (carbon
 1560 count (*CC*): double bond equivalents (*DB*)) used in Quality Assurance (*QA*) mixture.

Sterol	Structure	<i>m/z</i>	Retention time (min)
24-methylenecholesterol	(28:2)	398.3549	5.9
24-methylenecycloartenol	(31:2)	440.4018	7.5
Anthelsterol	(29:3)	410.3549	5.3
Avenasterol	(29:2)	412.3705	6.9
β-sitosterol	(29:1)	414.3862	9.7
Brassicasterol	(28:2)	398.3549	6.6
Campesterol	(28:1)	400.3705	8.2
Cholesterol	(27:1)	386.3549	7.0
Cycloartenol	(30:2)	426.3862	8.3
Cycloeucalenol	(30:2)	426.3862	7.7
Cyclolaudenol	(31:2)	440.4018	9.5
Desmosterol	(27:2)	384.3392	5.1
Episterol	(28:2)	398.3549	5.7
Ergosterol	(28:3)	396.3392	5.5
Isofucosterol	(29:2)	412.3705	7.5
Schottenol	(29:1)	414.3862	9.0
Sitostanol	(29:0)	416.4018	11.1
Spinasterol	(29:2)	412.3705	8.0
Stigmasterol	(29:2)	412.3705	8.5

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1562 **Table S2.3.** *Summary of double bond positions of identifiable sterols.*

Sterol	Carbons	Double bond
Cholesterol	27	5
Desmosterol	27	5
Lathosterol	27	7
24MC	28	5
24MD	28	5
Brassicasterol	28	5
Campesterol	28	5
Episterol	28	7
Lophenol	28	7
24MP	29	0
Sitostanol	29	0
Isofucosterol	29	5
Stigmasterol	29	5
β -Sitosterol	29	5
Avenasterol	29	7
Schottenol	29	7
Spinasterol	29	7
Cycloartanol	30	0
Cycloartenol	30	0
Cycloeucalenol	30	0
Obtusifoliol	30	8
24MCA	31	0
Cyclolaudenol	31	0

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Table S2.4. Mean, standard deviation, maximum and minimum total sterols (mg/kg) of species collected from at least three counties. Data ordered alphabetically by species.

Species	Total samples	Counties collected from	Mean	SD	Max	Min
<i>Acer pseudoplatanus</i>	7	3	8610.555	1345.595	10928.204	6953.336
<i>Aesculus hippocastanum</i>	4	3	4604.565	547.779	5235.141	4002.777
<i>Anemonoides nemorosa</i>	4	3	2307.654	170.936	2524.541	2127.581
<i>Arctium minus</i>	4	3	2219.324	704.018	3265.202	1739.239
<i>Betula pendula</i>	5	3	5116.401	2029.067	6597.529	1589.767
<i>Brassica napus</i>	3	3	33451.561	3396.653	37013.049	30248.107
<i>Calluna vulgaris</i>	6	5	6348.888	1154.772	7264.602	4134.187
<i>Centaurea scabiosa</i>	3	3	1199.632	216.622	1449.554	1065.771
<i>Cirsium vulgare</i>	3	3	1709.678	211.229	1953.153	1575.384
<i>Cornus sanguinea</i>	3	3	3930.186	1241.603	5233.460	2761.165
<i>Crepis capillaris</i>	3	3	22428.861	3475.129	25239.075	18543.120
<i>Cytisus scoparius</i>	3	3	4110.612	972.251	5233.257	3544.539
<i>Dipsacus fullonum</i>	3	3	4412.749	449.283	4812.914	3926.736
<i>Epilobium angustifolium</i>	4	4	8474.809	1572.140	10182.083	6475.260
<i>Erica tetralix</i>	3	3	5238.576	1340.619	6386.278	3765.090
<i>Filipendula ulmaria</i>	4	4	8687.125	2123.704	11335.502	6174.779
<i>Hyacinthoides non-scripta</i>	9	4	14194.948	2261.074	16081.941	10057.008
<i>Hyacinthoides x massartiana</i>	3	3	12877.678	202.285	13110.376	12743.781
<i>Hypochaeris radicata</i>	4	3	6191.681	595.632	6762.113	5397.867
<i>Impatiens glandulifera</i>	3	3	2591.728	303.325	2939.297	2380.482
<i>Jacobaea erucifolia</i>	4	4	1870.751	460.026	2323.470	1283.104
<i>Jacobaea vulgaris</i>	4	4	1895.421	325.238	2235.333	1568.926
<i>Knautia arvensis</i>	6	4	3593.105	298.439	4050.026	3207.376
<i>Leucanthemum vulgare</i>	6	3	2171.353	207.509	2517.856	1977.685
<i>Malus domestica</i>	4	4	16512.809	3748.606	19858.413	11137.434
<i>Malva moschata</i>	5	3	1273.185	293.156	1523.881	783.989
<i>Mercurialis perennis</i>	5	3	9731.803	1206.430	11267.924	7884.401
<i>Odontites vernus</i>	3	3	14163.904	2713.539	16473.212	11175.233
<i>Plantago coronopus</i>	3	3	8646.771	1684.472	10471.891	7151.846
<i>Plantago lanceolata</i>	7	3	9205.729	1645.184	11387.386	6920.123
<i>Primula vulgaris</i>	8	3	7611.531	1654.301	10863.400	5842.855
<i>Prunus avium</i>	5	3	14347.664	5079.107	20662.503	9089.118
<i>Prunus spinosa</i>	5	4	12668.479	2747.687	14883.978	8061.504
<i>Pulicaria dysenterica</i>	4	4	2985.718	1141.451	4642.911	2208.354
<i>Ranunculus acris</i>	7	4	4619.536	659.699	5517.671	3768.286

Species	Total samples	Counties collected from	Mean	SD	Max	Min
<i>Ranunculus bulbosus</i>	6	3	4846.928	910.858	6033.132	4058.682
<i>Ranunculus ficaria</i>	8	4	5482.621	628.629	6584.106	4669.943
<i>Ranunculus repens</i>	4	3	4308.205	661.074	4985.550	3410.640
<i>Reseda lutea</i>	3	3	9977.461	2052.981	11566.781	7659.555
<i>Rosa canina</i>	4	3	6078.973	1957.445	8818.577	4485.807
<i>Sanguisorba minor</i>	4	3	12942.587	1427.023	14137.584	11224.995
<i>Scorzoneroides autumnalis</i>	3	3	15686.667	6467.893	23147.111	11656.491
<i>Silene dioica</i>	6	3	12283.416	804.685	13402.136	11328.085
<i>Sonchus arvensis</i>	3	3	38158.330	4914.281	41611.095	32532.080
<i>Succisa pratensis</i>	4	3	2616.959	208.286	2848.998	2434.762
<i>Tripolium pannonicum</i>	5	4	1308.602	618.596	1716.578	255.504
<i>Ulex europaeus</i>	5	3	7592.451	1999.135	9858.110	4941.572
<i>Veronica chamaedrys</i>	3	3	10931.137	1507.200	12666.230	9946.364

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Chapter 3: Sterol profiles of UK bumble bee species, their tagma and collected pollen

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Abstract

Bumble bees (*Bombus* spp.) are an economically and ecologically important group of pollinators. Currently, different bumble bee species are experiencing expansions and severe declines in range. Previous work has demonstrated the importance of dietary sterols for bumble bee colony development. However, their sterol requirements appear to be flexible in terms of their ratio, allowing potential intra-species variation between habitat types. This study focused on quantifying the intra- and inter-species variation in sterol requirements within UK *Bombus* and its relationship with existing phylogenetic and pollen data. The results showed little meaningful variation among species and the maintenance of a consistent sterol profile, dominated by 24-methylenecholesterol, β -sitosterol and isofucosterol, across a range of foraging environments. This study provides much needed data on the sterol nutritional requirements of UK bumble bees, which can be used in the context of pollen sterol data to provide recommendations for planting and interpreting foraging choice data. The consistency of requirements between species suggests similar plants can be used to support a wide range of *Bombus* spp. and species absence from certain habitats is not caused by un-met sterol requirements.

1590 **Introduction**

1591 Sterols are an essential group of lipids serving structural and metabolic functions, namely as
1592 components of the cell membrane phospholipid bilayer, as precursors to steroid hormones and as
1593 signalling molecules. Characterised by a 3B-hydroxyl group, a tetracyclic ring and a hydrocarbon
1594 tail with 8-10 carbons, sterols are apolar molecules which slot into the phospholipid bilayer and
1595 stabilise membranes by increasing rigidity. More recently, sterols have also been shown to regulate
1596 morphology by modifying Hedgehog family proteins, which regulate cell fates during growth
1597 (Gallet *et al.*, 2003) as shown in *Drosophila*.

1598 More than half of all insects eat plants and herbivory is present in 8/30 insect orders, notably
1599 Orthoptera, Hemiptera, Coleoptera, Diptera, Lepidoptera and Hymenoptera. Arthropods, 90% of
1600 which are insects, are unable to synthesise sterols and instead obtain sterols, vital to their
1601 successful development (Clark and Bloch, 1959; Cooke and Sang, 1970), from their diet and from
1602 maternal loading during oogenesis (Carvalho *et al.*, 2010; Jing and Behmer, 2020). Cholesterol
1603 (C27) is the dominant tissue sterol in animals and is synthesised by vertebrates *de novo*. However,
1604 cholesterol is not produced in high quantities in plants, which instead contain predominantly longer
1605 chain phytosterols (C28-29). Consequently, herbivorous insects cannot obtain enough cholesterol
1606 through direct dietary means alone. Despite this, most herbivorous insects, particularly
1607 lepidopterans, have cholesterol dominated tissues (Behmer and Nes, 2003; Jing and Behmer,
1608 2020). They achieve this by metabolising longer chain dietary phytosterols through dealkylation
1609 into cholesterol to meet their physiological requirements. These dietary sterols are primarily used
1610 as bulk membrane components. Cholesterol is also used to synthesise C27 ecdysteroid moulting
1611 hormones such as ecdysone (20-hydroxyecdysone (20E)) (Figure 3.1).

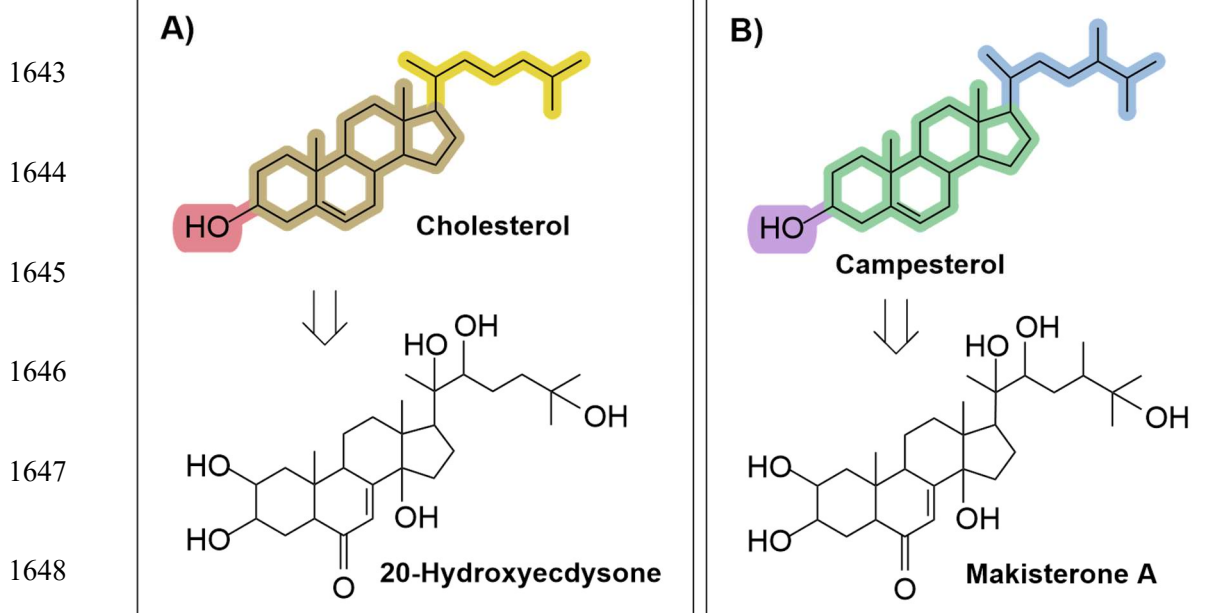
1612 Honey bees are unable to alter dietary sterols through dealkylation and instead must utilise sterols
1613 from the available diet pool (Herbert *et al.*, 1980; Svoboda, Herbert and Thompson, 1983). They,

1614 therefore, acquire a variety of different pollen phytosterols for their physiological requirements and
1615 instead of converting cholesterol into ecdysone, they convert campesterol into makisterone A or β -
1616 sitosterol into makisterone C (Svoboda and Feldlaufer, 1991; Jing and Behmer, 2020) (Figure 3.1).
1617 Honey bees have adapted their physiology to a pollenivorous diet and survive equally well on 24-
1618 methylenecholesterol as cholesterol (Herbert *et al.*, 1980). The sterol profile of honey bee prepupae
1619 contains a dominance of 24-methylenecholesterol (~50%) as well as β -sitosterol (~20%),
1620 isofucoesterol (~15%), campesterol (~8%) and <1% desmosterol (Svoboda *et al.*, 1980). This
1621 profile shows some plasticity under artificial feeding conditions. For instance, when fed only
1622 cholesterol, prepupae showed increased cholesterol levels from 0.6% to 17.2% (Svoboda *et al.*,
1623 1980). However, there appear to be limits to this plasticity, as 24-methylenecholesterol was always
1624 the dominant sterol in prepupal tissues, even when it was absent from the diet. Similarly, feeding
1625 with any mono-sterol diet led to compensation by nurse bees to maintain larvae sterol ratios using
1626 sterols from their endogenous pools rather than directly using dietary sterols (Svoboda *et al.*,
1627 1980). When honey bees are allowed to forage from wild pollens their sterol profile is still
1628 dominated by 24-methylenecholesterol, isofucoesterol and β -sitosterol even when these sterols are
1629 not abundant in their main pollen source (Svoboda *et al.*, 1983). Maintaining a balance of
1630 phytosterols therefore appears to be of physiological importance in bees, though further testing in
1631 more bee species is required to investigate this general claim.

1632 The sterol composition of different body compartments, tagmata, has not yet been investigated.
1633 The head, thorax and abdomen contain different organ systems and are therefore dominated by
1634 different tissue types, for instance muscle in the thorax. In *Drosophila*, it was found that sterols
1635 were retained in the central nervous system and gut apical membrane during periods of sterol
1636 scarcity (Carvalho *et al.*, 2010). Even if bees show consistent sterol profiles, the tagmata may show
1637 distinct sterol profiles within this. Similarly, differences in sterol usage between reproductive
1638 organs may lead to distinct sterol profiles between male and female bees of the same species.

1639 However, it has been shown that honey bee ovaries contain high levels of 24-methylenecholesterol,
1640 isofucoesterol and sitosterol, matching previous prepupae findings (Feldlaufer, Svoboda and E.W.
1641 Herbert, 1986).

1642



1649 **Figure 3.1.**

1650 **A)** Moulting hormone synthesis from sterol precursors in most insects.

1651 **B)** Moulting hormone synthesis from sterol precursors in honey bees.

1652 Figure was produced using ChemDraw from Revvity Signals Software.

1653 *Bombus* is a genus of bees containing 15 subgenera and 265 species (Arbetman *et al.*, 2017).

1654 Bumble bees live in eusocial colonies which go through one or multiple worker-reproductive
1655 producing cycles and can persist for several months. Their long flight period and wide habitat
1656 coverage necessitates a flexible foraging strategy to fulfil dietary needs across the flowering
1657 season.

1658 UK *Bombus* contains six cuckoo bumble bees (subgenus *Psithyrus*) which are social parasites of

1659 other bumble bee species. They kill or suppress established queens and utilise the workers to raise

1660 their own young. Cuckoos do not produce workers or have corbiculate legs for pollen collection
1661 and instead are fed *via* the foraging of their host species. As a result, they are predicted to have the
1662 same nutritional needs as their host to ensure healthy development of their offspring. As they do
1663 not forage for their offspring, their pollen choices may also be under less strong selection.

1664 Habitat preference, emergence time, colony cycle duration and tongue length all impact floral
1665 resource availability for bumble bees. The selection pressure on bumble bees to obtain nutritionally
1666 complete diets for their offspring is high as they do not store food overwinter and, unlike
1667 honeybees, cannot alter the composition of food post-forage. All UK bumble bees are highly
1668 generalist foragers however some species can exhibit strong preferences, such as *Bombus*
1669 *monticola* for *Vaccinium myrtillus*, *B. jonellus* for heathers and *B. distinguendus* for Fabaceae,
1670 whilst others such as *B. terrestris* do not. When foraging, bumble bees display lower floral
1671 constancy than honey bees and do not add salivary enzymes or microorganisms to pollen for
1672 storage as honey bees do. Current evidence suggests bumble bees are unable to distinguish food
1673 sources on the basis of sterol content (Vanderplanck *et al.*, 2014).

1674 The conservation status of UK bumble bees is not uniform; some species such as *Bombus*
1675 *hypnorum* are continuing to expand their range since entering the UK, whereas *B. distinguendus*
1676 has declined hugely and is now one of the rarest bumble bees in the UK (Edwards and Jenner,
1677 2018). Causes behind these differences have been attributed to association with declined flower-
1678 rich habitat, late emergence times and, in some cases, colony size (Goulson *et al.*, 2005; National
1679 Biodiversity Data Centre, 2022). Bumble bees are often one of the target taxa of seed mixes
1680 designed to increase floral resources in amenity and agricultural settings (Edwards and Jenner,
1681 2018). However, species selection for these mixes is often based on “educated guesses” rather than
1682 an understanding of the nutritional resources on offer (Williams and Lonsdorf, 2018).

1683 This study examines how sterol profile differs between UK bumble bees species (*Bombus* spp.),
1684 addressing the following hypotheses: 1) There are significant inter-species differences in the sterol
1685 profiles of UK bumble bee species, 2) Common bumble bees species maintain a consistent sterol
1686 profile across different foraging environments, 3) Bumble bee body tagmata (head, thorax,
1687 abdomen) display distinct sterol profiles, 4) The sterol profiles of pollen collected from bumble bee
1688 corbiculae align with the collecting bee's profile, 5) The sterol profiles of cuckoo bumble bees
1689 match that of their host(s) and 6) Plant species with pollens frequently collected by bumble bees
1690 show a sterol profile containing a dominance of sterols important to bumble bees.

1691 **Methods**

1692 **Sample collection**

1693 Female workers of all UK bumble bee species were targeted for collection from 2020 to 2023. A
1694 minimum collection target of 5 individuals per species was set and specimens collected with
1695 landowner permission. Only four species were represented by <5 individuals (male or female)
1696 (*Bombus barbutellus*, *B. campestris*, *B. ruderarius* and *B. sylvarum*). Rare species (*Bombus*
1697 *distinguendus* and *B. sylvarum*) were collected only with prior arrangement with the Bumblebee
1698 Conservation Trust. Queens from abundant and common species ('The big 7': *Bombus hortorum*,
1699 *B. hypnorum*, *B. lapidarius*, *B. lucorum*, *B. pascuorum*, *B. pratorum* and *B. terrestris*) were
1700 collected to assess tagma composition. These samples were only used in analysis of tagma
1701 composition and not compared to samples which had been analysed as whole bodies. Female
1702 workers of *Bombus lapidarius*, *B. terrestris* and *B. pascuorum* were collected from three different
1703 sites to facilitate intra-species analysis. These sites were an urban garden environment (Royal
1704 Botanic Gardens, Kew), a semi-natural, dry, calcareous grassland site (sites near Rolleston Camp
1705 and Bulford Camp, Salisbury Plain Training Area, Wiltshire)(Natural England, 2015), and a semi-
1706 natural grass moor and heather moorland site (Harthope Valley, Cheviot hills, Northumberland
1707 National Park, Northumberland)(Natural England, 2013). For cuckoo species, which do not have
1708 workers, queens were collected, with males collected when this was not possible. For *Bombus*
1709 *ruderarius* and *B. campestris*, no female workers/queens could be collected and so males were
1710 collected instead. To ensure there was not a difference between the sterols of male and females of
1711 the same species, males were opportunistically collected from well sampled common species
1712 (*Bombus hortorum*, *B. lapidarius*, *B. lucorum*, *B. ruderatus*, *B. rupestris*, *B. vestalis*) to enable
1713 statistical comparison. Individuals of *Bombus terrestris* and the *B. lucorum* complex (*B. lucorum*,
1714 *B. cryptarum*, *B. magnus*) were combined into *Bombus terrestris/lucorum* for analysis as many

1715 samples had indeterminate characters. Similarly, *Bombus hortorum* and *B. ruderatus* were
1716 combined into *B. hortorum/ruderatus*. This led to a total of 18 species, including the two species
1717 aggregates, being collected. A total of 286 individuals were collected with 223 used for whole
1718 body analysis and 63 for segment analysis. A full summary of sample collection details can be
1719 found in Table S3.1.

1720 Bees were captured using a net and euthanized in a -20°C freezer. They were then stored at -20°C
1721 and moved to -80°C storage in batches. After sample collection, bees were defrosted for dissection
1722 and the full gut, crop, sting and venom sacs removed from the abdominal cavity. This was to
1723 prevent misinterpretation of sterols detected from gut contents with those incorporated into body
1724 tissues. Any visible parasites were also removed. Ovaries, where present, were retained. A hind leg
1725 was also removed from all samples for future barcoding of specimens with indeterminate
1726 characters. For any male specimens, genitalia were retained, and guts removed. A set of queen
1727 bumble bees were also separated into body tagmata (head, thorax, abdomen). Any loose pollen in
1728 the corbiculae or on the body was removed by brushing and full pollen baskets collected into 1.5ml
1729 glass vials. Dissected specimens were then refrozen at -80°C.

1730 Species with indeterminate characters were barcoded for later identification as follows. Hind legs
1731 were stored at -80°C in a 1.5ml eppendorf. DNA was extracted from legs using a Qiagen DNeasy
1732 Blood & Tissue Kit following the supplementary protocol 'Purification of total DNA from insects
1733 using the DNeasy® Blood & Tissue Kit'. Legs were homogenised using four 2.3mm ceramic
1734 beads in an MP FastPrep-24™ 5G bead beating grinder and lysis system in five cycles of 35s at 6
1735 m/second followed by 180s rest. The genomic DNA was then sent to SureScreen Scientifics for
1736 PCR, barcoding and species identification.

1737 **Sample preparation**

1738 All whole-body and tagma samples were covered in 5ml or 3ml of deionised water respectively
1739 and re-frozen at -80°C before being freeze dried. Samples were then transferred to plastic bead-
1740 beating vials containing four 2.3mm steel beads and homogenised at 30rpm in a Qiagen tissue
1741 ruptor for 3 minutes in two 1.5 minute intervals. Homogenised tissue was then transferred back to
1742 their glass vial in a 1:50 (mg:µl) dilution of GCTU and re-frozen at -80°C. For each sample, 60µl
1743 of homogenate was transferred to a 96-well plate for sterol extraction.

1744 Paired pollen baskets weighing >20mg were separated and those weighing 10-20mg were
1745 combined for sterol analysis. Pollens were suspended in 200µl KOH and vortexed to disperse.
1746 They were then boiled at 75°C for two hours. Twelve which had dried out due to methanol
1747 evaporation during that time were re-suspended in 150 µl methanol. All saponified material was
1748 then transferred to a 96-well plate for sterol extraction.

1749 **Sample run**

1750 Samples were analysed in collaboration with staff at Royal Botanic Gardens (RBG), Kew. Sample
1751 run was prepared by EB, RTS and SF. Samples were run across two 384-well plates comprising
1752 eight individual 96-well plates. Sample order was randomised within each 96-well plate. Each
1753 plate also contained samples designed to monitor instrument functioning: Blanks (60µl GCTU)
1754 were used to detect and remove background noise. All runs began with a blank. A stock solution of
1755 reference materials verified by multi-nuclear NMR (QA) were used to calculate coefficient of
1756 variance. Reference materials were available for: 24-methylenecholesterol, 24-
1757 methylenecycloartenol, anthelsterol (a sterol found in *C. anthelmintica*, ST(28:3), that is active
1758 under 330 nm UV but whose structure has not been determined formally using NMR), avenasterol,
1759 β-sitosterol, brassicasterol, campesterol, cholesterol, cycloartenol, cycloeucalenol, cyclolaudenol,
1760 desmosterol, episterol, ergosterol, isofucosterol, schottenol, sitostanol, spinasterol and stigmasterol.

1761 The m/z and retention time for each sterol is shown in Table S3.2. Aliquots of 40 μl were used in
1762 the run. A QC solution of homogenised commercially available honey bee collected pollens
1763 (*Helianthus annuus*, *Nymphaea* sp., *Fagopyrum esculentum*) and bumble bee adults, larvae and
1764 pupae in GCTU (6M guanidine + 1.5M thiourea to make 1 L dissolved in deionised water) was run
1765 at a series of concentrations to test the range of linearity of the instrument. Three concentrations of
1766 QC were used: 100%, 50%, 25%, corresponding to 40 μl , 20 μl and 10 μl aliquots of the QC stock
1767 solution. These were analysed in the same way as the samples to calculate the correlation between
1768 analyte concentration and signal size. Variables whose ratio was <0.75 were discarded.

1769 All samples were then extracted as 96-well plates by SF (96-well plate, Esslab Plate+™,
1770 2.4 ml/well, glass-coated) using a 96-channel pipette (liquid-handling robot, Integra Viaflow
1771 96/384 channel pipette) as follows. First 150 μl of internal standard (d_7 -cholesterol) was added to
1772 all samples. For plate A, whole-body bumble bee samples, the concentration of d_7 -cholesterol was
1773 5 mg/l ($1.99098e^{-9}$ Moles/sample). For plate C, body tagmata and pollen samples, it was 1 mg/l
1774 ($3.98196e^{-10}$ Moles/sample). Then 500 μl DMT (250 $\mu\text{l} \times 2$) was added to all samples. DMT was
1775 made from dichloromethane (DCM) (3 parts), methanol (1 part) and triethylammonium chloride
1776 (0.0005 parts, i.e. 500 mg/l), mixed and stored at room temperature out of direct sunlight. Followed
1777 by 500 μl deionised water before agitating using the multi-channel pipette. Layers were separated
1778 by spinning for 2 min (methanol + water, Solid, DMT + sterol) and 50 μl of extract (DMT &
1779 sterol) transferred to a 384-well plate and left for 30 min for the DMT to evaporate. Once the 384-
1780 well plate was full, 150 μl of LCMS quality methanol was added and the plate sealed with foil.

1781 **Sample analysis and data processing**

1782 The sample run was completed under the supervision of SF following the protocol established in
1783 Furse, Martel, *et al.*, (2023). Instrument output was also processed by SF as established in Furse,
1784 Martel, *et al.* (2023).

1785 Instrument output (m/z) was converted to mg/g first by dividing the signal area for each metabolite
1786 by the signal of internal standard (d_7 -cholesterol) then using the following formula:

$$1787 \quad \frac{\text{Sterol (mg)}}{\text{Tissue (g)}} = \frac{\text{sterol molecular mass} \left(\frac{\text{g}}{\text{mol}} \right) \times (\text{signal} \times \text{mol of } d_7\text{cho.}) \times 1,000}{\text{Starting weight (mg)}/1,000}$$

1788 The amount of mol of d_7 -cholesterol was $1.99098e^{-9}$ Moles/sample for plate A and $3.98196e^{-10}$
1789 Moles/sample for plate C.

1790 Due to the impact of the COVID-19 pandemic a second analysis run was needed to analyse
1791 samples of *Bombus distinguendus* and *B. jonellus* which were collected a year later than intended.
1792 Ten samples which had also shown anomalous results in the first run were also re-analysed as part
1793 of this run. The second analysis detected a different selection of low-abundance sterols compared
1794 to the previous run. As the primary aim of this work was to enable comparison between species
1795 and sites, any sterols which were not detected in both runs were therefore removed from data
1796 analysis to enable the most accurate comparisons. This led to the removal of two sterols from the
1797 second run (stigmaterol and ST(30:3)) and ten from the first (24MCA, sitostanol, ST(27:2)C,
1798 ST(29:0)A, ST(29:0)B, ST(29:2)A, ST(29:2)B, ST(29:2)C, ST(30:2)A and ST(30:2)C).

1799 **Phylogenetic analysis**

1800 The most comprehensive phylogeny of the *Bombus* genus to date was used with permission from
1801 Hines (2008). Phylogeny was trimmed using keep.tip() from APE (Paradis and Schliep, 2019) to
1802 only include sampled species. *Bombus sylvestris* was not available in the phylogeny and so not
1803 included in these analyses. Subgenera were defined following Williams *et al.* (2008). To plot
1804 *Bombus lucorum/terrestris* and *B. hortorum/ruderatus* on the phylogeny, branch tips for *Bombus*
1805 *terrestris* and *B. hortorum* were used.

1806 Following Zu *et al.*, (2021), phylo4d objects were created from the trimmed Hines (2008)
1807 phylogenetic tree and sterol proportion data, total sterols (mg/g) and sterol proportions grouped by
1808 carbon count and B-ring double bond position using phylo4d() from PHYLOBASE (R Hackathon
1809 et al., 2020). Ergosterol was classed as NA for double bond position as it contains double bonds at
1810 positions 5 and 7. Sterols with either a saturated B-ring or cyclopropane ring were classed as $\Delta 0$.
1811 This was used to calculate Pagel's λ and Blomberg's K using phyloSignal() from the R package
1812 PHYLOSIGNAL (number of iterations: 999).

1813 The phylogenetic tree was plotted by creating a phy-data object using treedata() from GEIGER
1814 (Pennell *et al.*, 2014). Family clades were identified using getMRCA() from APE (Paradis and
1815 Schliep, 2019). Phylogeny was plotted with ggtree() from GGTREE (Yu *et al.*, 2017, 2018; Yu,
1816 2020). Heatmap and bar graph associated with phylogeny were plotted using ggplot() from
1817 TIDYVERSE (Wickham *et al.*, 2019). The composite graph was then plotted using APLOT (Yu,
1818 2023).

1819 **Data analysis**

1820 All data analysis was done in R version 4.2.3 (2023-03-15 ucrt) (R Core Team, 2023) and R studio
1821 (2023.6.1.524) (Posit team, 2023). For each data type (whole-body, individual tagma, corbiculate
1822 pollen), a Bray-Curtis dissimilarity matrix was used to identify outliers using disana() from the
1823 LABDSV package (Roberts, 2023). Any data point with a minimum dissimilarity over 0.5
1824 compared to all other points for that group was identified as an outlier, as per author guidance, and
1825 so was removed. No samples exceeded this threshold so all data was included in the analysis.
1826 Summary statistics were calculated from species means using the STATS package from R (R Core
1827 Team, 2023).

1828 To carry out statistical comparisons of sterol profile between groups, proportion data (0-1) was
1829 used, where zero denotes a sterol is absent from a sample, and one denotes it is completely

1830 dominant. Data were arcsine square root transformed and used to calculate a Bray-Curtis
1831 dissimilarity matrix using `vegdist(method="bray")`. The matrix was used to plot an NMDS using
1832 `metaMDS(autotransform=FALSE)`. The lowest number of axes was selected where stress was ≤ 0.2
1833 (established cut off, (Bakker, 2024)). The stress value reflects how well the ordination represents
1834 the original data. `Adonis2()` was used to carry out a PERMANOVA testing for significant
1835 differences between groups. Differences in variance between groups which could invalidate
1836 comparisons were tested using `anova()` and `betadisper()`. Pairwise comparisons between groups
1837 were carried out following Bakker (2024), using `adonis2()` with a Benjamini-Hochberg correction.
1838 All functions were from VEGAN (Oksanen *et al.*, 2022) except `anova()` from the STATS package
1839 (R Core Team, 2023). A 2D biplot was created from NMDS with a stress value was < 0.2 . Strong
1840 (> 0.7 or > 0.6 absolute value) and significant ($p < 0.010$) sterol associations were plotted over the
1841 datapoints using `ggplot()` and `ggrepel()` from GGLOT2 (Wickham, 2016) and GGREPEL
1842 (Slowikowski, 2023).

1843 The INDICESPECIES package (De Cáceres and Legendre, 2009) was used to carry out Indicator
1844 Species Analysis (ISA) using `multipatt()` to determine significant associations between groups and
1845 individual sterols. ISA was done at the level of individual groups only and not for combinations of
1846 groups.

1847 ***Differences in sterol profile among UK bumblebee species***

1848 The six species with the highest number of samples, including male and female specimens, were
1849 used in an NMDS (`trymax=200`, `k=3`) to compare sterol profile between species. The first two
1850 dimensions of the NMDS are plotted in Figure 3.3. All available whole-body samples were used in
1851 an ISA to test for associations between individual sterols and species.

1852 ***Bumble bee body tagmata (head, thorax, abdomen) sterol profiles***

1853 Tagmata were analysed by NMDS (trymax = 1000, maxit=1000, k=2) and PERMANOVA to test
1854 for significant differences in sterol profile between head, thorax and abdomen. The strata argument
1855 was used to control for the effect of tagma being from the same individual. Species identity was
1856 also included as a fixed effect in the model.

1857 ***Sex differences in sterol profile***

1858 Where collection of females was not possible, male bumble bees were collected instead. For each
1859 species which had at least one male and female specimen (*Bombus barbutellus*,
1860 *B. hortorum/ruderatus*, *B. lapidarius*, *B. monticola*, *B. terrestris/lucorum*, *B. rupestris*,
1861 *B. sylvestris*, *B. vestalis*) an NMDS and PERMANOVA was calculated using means for each
1862 species-sex combination to prevent the often-higher female sample sizes affecting the results
1863 (trymax=200, k=2). Sex and species identity were included as fixed effects. *Bombus vestalis*, had
1864 highest number of male specimens (11) and a comparable number of female specimens (8) and was
1865 therefore used for a separate NMDS and PERMANOVA (trymax=200, k=2).

1866 ***Intra-species sterol profile across different foraging environments***

1867 To determine whether there were significant intra-species differences driven by habitat, female
1868 workers of a set of species that are common in a range of habitats were collected from an urban
1869 garden environment (Royal Botanic Gardens, Kew), a semi-natural, dry, calcareous grassland site
1870 (sites near Rollestone Camp and Bulford Camp, Salisbury Plain Training Area, Wiltshire)(Natural
1871 England, 2015), and a semi-natural grass moor and heather moorland site (Harthope Valley,
1872 Cheviot hills, Northumberland National Park, Northumberland)(Natural England, 2013). These
1873 species were *Bombus terrestris/lucorum*, *B. pascuorum*, and *B. lapidarius*. Between site
1874 comparisons were plotted for *Bombus pascuorum*, *B. lapidarius* and *B. terrestris/lucorum*
1875 individually using NMDS (trymax = 200, k = 2) and sites were compared statistically using
1876 PERMANOVA.

1877 ***Sterol requirements of cuckoo bumble bees and their host(s)***

1878 Cuckoo bumble bees occupy a distinct phylogenetic and ecological group and so were compared to
1879 the rest of the dataset in an NMDS (trymax = 200, k=2) and PERMANOVA to determine if they
1880 displayed a distinct sterol profile. Male and female specimens were included in this analysis. ISA
1881 was also carried out on the data. *Bombus vestalis* was the best sampled cuckoo species and so was
1882 used in a separate NMDS (trymax = 200, k=3) and PERMANOVA to determine if it clustered
1883 more closely with host versus non-host bumble bee species. All other cuckoo species were
1884 removed from this analysis.

1885 ***Sterol profiles of bumble bee macro-parasites***

1886 During gut removal, several bumble bee specimens were found to contain macroparasites in their
1887 abdomen. These were identified as *Sphaerularia bombi* (n=3) and conopid fly larvae (family
1888 Conopidae) which were grouped into large (n=4) and small (n=4). Twelve individuals also had
1889 external mites present (*Parasitellus fucorum*). Parasites were removed to prevent interference with
1890 the sterol profile of the bumble bee. To make opportunistic use of these parasites, their sterol
1891 profiles were analysed to determine if they were utilising phytosterols in their tissues. Given the
1892 minimum weight required for sterol analysis by our method, parasites of the same type were
1893 pooled from multiple individuals and analysed as a single composite sample. No statistical analysis
1894 of these results was possible as there was only a single analysed sample of each parasite type.

1895 ***Comparison between bumble bee collected pollen and whole-body tissue sterol profiles***

1896 A total of 40 pollen baskets large enough to analyse for their individual sterol content were
1897 collected from females. An NMDS was calculated using this data (n=40), all whole bee species
1898 (n=18) and all 295 pollen species detailed in Chapter 2 (n=295) (trymax = 500, maxit=500, k=2).
1899 ISA was then carried out to test for associations between these groups and individual sterols. As

1900 the pollen analysis had produced a wider range of sterols, only those identified in both analyses
1901 (24-methylenecholesterol, β -sitosterol, campesterol, cholesterol, cycloartenol, cyclolaudenol,
1902 desmosterol, ergosterol and isofucosterol) were used to calculate total sterols and then re-calculate
1903 proportions for the pollen and bee data.

1904 ***Comparison between bumble bee favoured pollens and the sterol profiles of bumble bees***

1905 Visitation data was collated from all available years in The Database of Pollinator Interactions
1906 (DoPI) (Balfour *et al.*, 2022). All reports of pollen collection by *Bombus* spp. were combined and
1907 plant species ranked by a) the number of bumble bee species reported to collect from that flower
1908 and b) the total number of unique records of any bumble bee collecting from that flower. There
1909 was very high overlap in these lists, so the top 15 records from both were combined. Pollen sterol
1910 data were available from previous analyses for all but one species (*Calendula* sp.). *Melilotus*
1911 *altissimus* was replaced by a mean of available *Melilotus* species (*Melilotus* sp., *Melilotus*
1912 *officinalis*). *Symphytum officinale* was replaced by a mean of available *Symphytum* species
1913 (*Symphytum* sp., *Symphytum orientale*, *Symphytum x uplandicum*). Sterol profile data for these
1914 species were compared to data for all other available pollen species from Chapter 2 by
1915 PERMANOVA to determine if they had a significantly different sterol profile. Data were plotted
1916 using NMDS (trymax = 200, k=3).

1917 **Results**

1918 **Trends in sterol profile across UK bumble bee species**

1919 Across all species, whole-body tissues were dominated by 24-methylenecholesterol, isofucosterol
1920 and β -sitosterol (Table 3.1). Cholesterol was present in very low proportions across all species
1921 (<0.5 median) compared to campesterol (median 6.635%). Both are potential moulting hormone
1922 precursors. Ergosterol, a fungal sterol not synthesised by animals or plants, was detected in all
1923 species at a median proportion of 2.078%. Sterol profile appeared to be consistent among species
1924 (Figure 3.2) and among subgenera. No sterols, carbon count/B-ring double bond groups or total
1925 sterol (mg/g) showed a significant phylogenetic signal.

1926 **Differences in sterol profile between UK bumblebee species**

1927 UK bumble bees are all generalist foragers but display differences in forage and habitat preference.
1928 It may therefore be expected that these species show distinct sterol profiles as a result of their
1929 foraging differences. However, due to the many shared forage preferences between species, for
1930 instance *Trifolium pratense*, their close relatedness and collective eusociality, it may be expected
1931 that these differences are in sterol proportions rather than presence or absence of certain sterols.
1932 The ISA testing for significant associations between species and sterols analysed showed that only
1933 two sterols were significantly associated with individual bumble bee species. Campesterol was
1934 associated with *Bombus ruderarius* (IV=0.316, $p<0.050$) and 24-methylenecholesterol with
1935 *B. distinguendus* (IV=0.283, $p<0.050$). It is worth noting the indicator value for both these
1936 associations is low (<0.4) and *Bombus ruderarius* was represented only by a single sample.

1937 The first two dimensions of the NMDS comparing the six most sampled species are plotted in
1938 Figure 3.3A (stress=0.135). The results of PERMANOVA showed significant differences between
1939 species ($F_{5,145}=2.735$, $p=0.001$) and no difference in variance between species ($F_{5,145}=1.943$,

1940 $p>0.050$). Significant differences in variance can lead to potential misinterpretation of
1941 PERMANOVA results, specifically, whether the result is caused by differences in mean or
1942 dispersion between groups. Therefore, visual inspection of the data by NMDS is used to aid
1943 interpretation. Species identity explained a low proportion of the variation in the data ($R^2=0.086$).
1944 Significant pairwise differences were between *B. hortorum/ruderatus* and *B. pascuorum* ($p<0.050$)
1945 and *B. terrestris/lucorum* ($p<0.050$). Also *B. humilis* differed significantly from
1946 *B. hortorum/ruderatus* ($p<0.050$), *B. lapidarius* ($p<0.050$), *B. pascuorum* ($p<0.050$) and
1947 *B. terrestris/lucorum* ($p<0.050$). Therefore, *B. hortorum/ruderatus* and *B. humilis* appear the most
1948 distinct from other well-sampled species (Figure 3.3).

1949 **Bumble bee body tagmata (head, thorax, abdomen) sterol profiles**

1950 The body tagmata of bees are dominated by different organs and therefore tissue types. As a result,
1951 these differences may translate into distinct sterol profiles as the primary bulk usage of sterols is in
1952 membranes where they can affect properties such as fluidity. Figure 3.4 shows the NMDS
1953 comparing sterol profiles between tagmata (stress=0.155). Results from the PERMANOVA
1954 showed that sterol profile differed significantly between tagma ($F_{2,181}=4.530, p=0.001$) and as a
1955 result of species identity ($F_{5,181}=2.294, p=0.001$). However, the variance explained by both was
1956 very low (Tagmata: $R^2=0.045$, Species: $R^2=0.057$). There was no significant difference in variance
1957 between tagmata groups ($F_{2,186}=2.871, p>0.050$) but there was between species groups
1958 ($F_{5,183}=2.718, p<0.050$). The results of pairwise comparisons showed that the sterol profile of the
1959 head differed significantly from the abdomen ($p<0.010$) and thorax ($p<0.005$) but there was no
1960 difference between the abdomen and thorax ($p>0.100$) (Figure 3.4). Investigating the effect of
1961 species using pairwise comparisons showed that the sterol profile of *B. hortorum/ruderatus*
1962 samples differed significantly from *B. terrestris/lucorum* and that *B. hyponorum* differed
1963 significantly from *B. terrestris/lucorum* and *B. pascuorum* (all $p<0.050$) (Figure 3.4).

1964 **Sex differences in sterol profile**

1965 As stated above, it is possible that differences in the sterol composition of organs can impact sterol
1966 profile more widely. Differences in reproductive tissues may therefore lead to sex differences
1967 within species. As it was not possible to collect female samples for some species, this analysis is
1968 also key to determining if substituting male samples is acceptable. There was no significant
1969 difference between male and female specimens of *Bombus vestalis* (NMDS stress= 0.129,
1970 PERMANOVA: $F_{1,17}= 1.802$, $p>0.100$, dispersion: $F_{1,17}=0.480$, $p>0.100$). Across the eight species
1971 for which there were male and female specimens available, there was no effect of sex
1972 (PERMANOVA: $F_{1,7}= 1.270$, $p>0.100$, dispersion: $F_{1,14}=0.010$, $p>0.500$) or species identity
1973 (PERMANOVA: $F_{7,7}= 1.064$, $p>0.100$, dispersion: $F_{7,8}= 1.384 \times 10^{29}$, $p<0.001$) on sterol profile
1974 (NMDS stress= 0.151). NMDS biplots are shown in supplementary figures S3.1 and S3.2.

1975 **Intra-species sterol profile across different foraging environments**

1976 To understand whether the availability of different forage plants impacted sterol profile, a set of
1977 widely distributed and common species were sampled from at least three floristically distinct sites.
1978 Significant intraspecies variation between sites would demonstrate plasticity in sterol profile, even
1979 in the absence of experimental conditions. The results of PERMANOVAs on the species collected
1980 at different sites showed a significant difference between sites for *Bombus pascuorum* (NMDS
1981 stress = 0.183, PERMANOVA: $F_{3,22}=1.811$, $p<0.050$, dispersion: $F_{3,22}=0.523$, $p>0.500$). However,
1982 this was not strongly significant ($p=0.048$), and the variance explained by collection site was low
1983 ($R^2=0.198$). Both *B. lapidarius* and *B. terrestris/lucorum* showed no significant difference in
1984 composition between sites (*B. lapidarius*: NMDS stress = 0.130, PERMANOVA: $F_{2,13}=1.453$,
1985 $p>0.100$, dispersion: $F_{2,13}=1.069$, $p>0.100$. *B. terrestris/lucorum*: NMDS stress = 0.186,
1986 PERMANOVA: $F_{3,35}=1.093$, $p>0.100$, dispersion: $F_{3,35}=0.609$, $p>0.500$). All NMDS biplots are
1987 shown in Figure 3.5.

1988 **Sterol requirements of cuckoo bumble bees and their host(s)**

1989 Figure 3.6 shows a comparison between the whole-body sterol profile of cuckoo species,
1990 highlighted in bold at the top of each graph, and their respective host species. The PERMANOVA
1991 comparing *Bombus vestalis* with its host and non-host species showed there was no significant
1992 difference between groups (NMDS stress value = 0.127, PERMANOVA: $F_{2,199}=1.762$, $p>0.050$,
1993 dispersion: $F_{2,199}=10.844$, $p<0.001$). Cuckoo bumble bees also did not show a sterol profile distinct
1994 from non-cuckoo species (NMDS stress value = 0.099, PERMANOVA: $F_{1,16}=0.816$, $p>0.500$,
1995 dispersion: $F_{1,16}=1.345$, $p>0.100$) (Figure 3.7). However ISA showed the unidentified sterol
1996 ST(28:2)C (IV=0.810, $p=0.050$) was significantly associated with the cuckoo group. For this
1997 analysis, a mean value was used for each species to prevent groups being skewed by more sampled
1998 species.

1999 **Sterol profiles of bumble bee macro-parasites**

2000 The sterol profiles of parasites removed from the bodies and guts of bumble bees are shown in
2001 Figure 3.8 next to a mean of the samples they were extracted from. The conopid fly larvae and
2002 mites show a profile dominated by 24-methylenecholesterol, β -sitosterol, isofucosterol and
2003 campesterol, similar to that shown across *Bombus* spp.. However, *Sphaerularia bombi* was
2004 dominated by isofucosterol and has comparatively lower levels of campesterol and 24-
2005 methylenecholesterol. The fly larvae could not be identified to species-level and therefore it is
2006 unknown whether the differences in sterol profile among samples, namely differences in β -
2007 sitosterol and isofucosterol, were due to differences in developmental stage or species
2008 requirements.

2009 **Comparison between bumble bee collected pollen and whole-body tissue sterol**
2010 **profiles**

2011 The three most dominant sterols in bumble bees, isofucoesterol, 24-methylenecholesterol and β -
2012 sitosterol are also the most dominant in the corbiculate pollen they collected (Figure 3.9). The
2013 PERMANOVA showed significant differences between bumble bees, corbiculate pollen and hand
2014 collected pollen samples (taken from Chapter 2) (NMDS stress value= 0.179, PERMANOVA:
2015 $F_{2,350}=13.408$, $p=0.001$). Corbiculate pollen and bumble bees showed higher 24-
2016 methylenecholesterol than hand collected pollen which had higher cycloartenol. There was also a
2017 significant difference in the variance of the samples types ($F_{2,350}=28.066$, $p < 0.001$). However, as
2018 the pollen sample types both covered a much wider taxonomic breadth than the bumble bees, this
2019 variation is to be expected. ISA showed corbiculate pollen, bumble bees and hand collected pollen
2020 were all associated with at least one sterol each. Hand collected pollen was strongly associated
2021 with the $\Delta 0$ sterols cyclolaudenol and cycloartenol whereas corbiculate pollen was strongly
2022 associated with the $\Delta 5$ sterols desmosterol and cholesterol (Table 3.2).

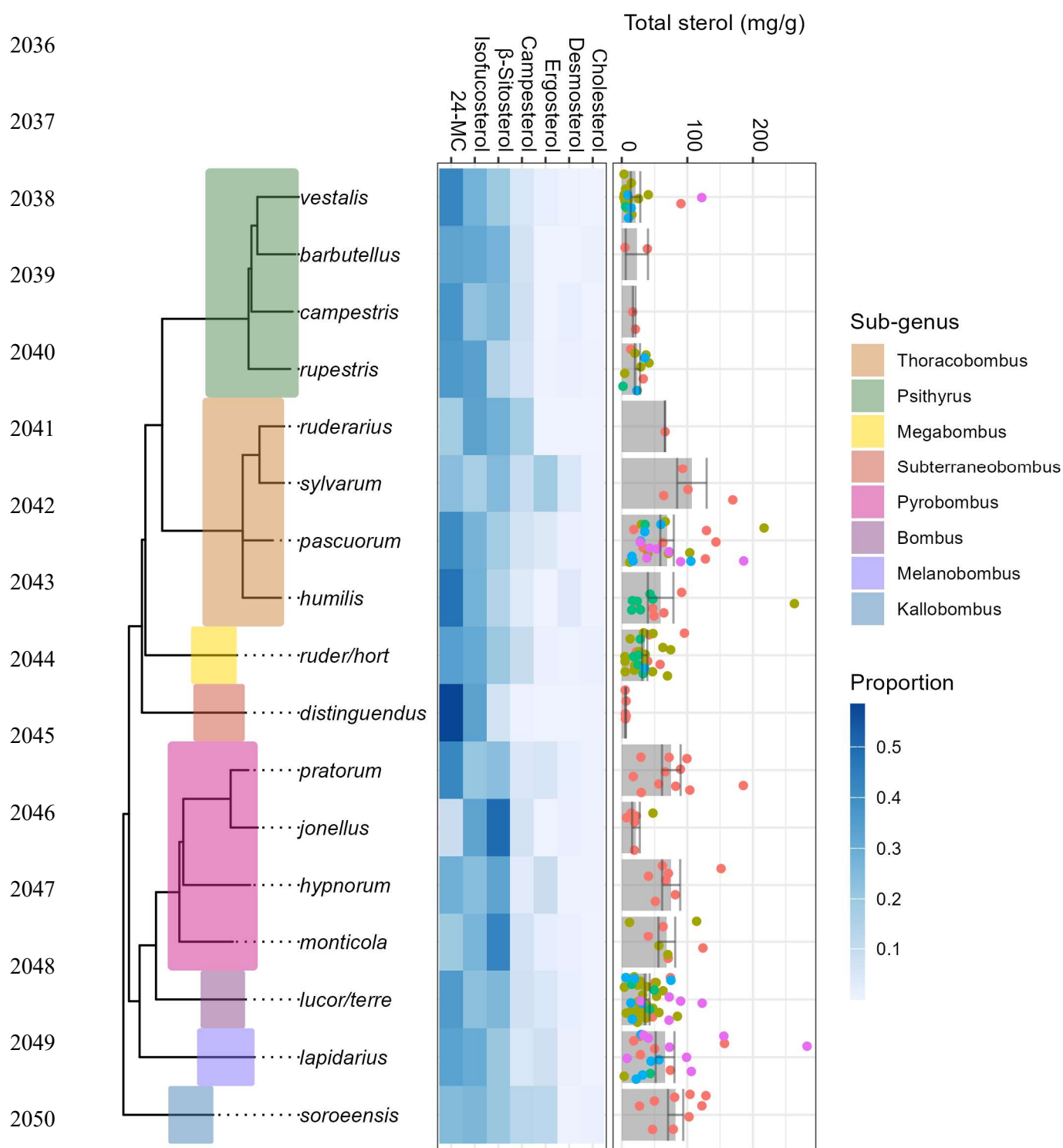
2023 **Comparison between bumble bee favoured pollen and the sterol profiles of other**
2024 **pollen**

2025 Plant species with pollen frequently collected by bumble bees, shown in Figure 3.10A, were used
2026 in an NMDS and PERMANOVA to compare their composition with all other species of hand
2027 collected pollen analysed in Chapter 2. Results did not show a significant difference between
2028 groups ($F_{1,290}=1.083$, $p > 0.100$) and there was also a significant difference in variance between
2029 groups ($F_{1,290}=8.780$, $p < 0.005$). The first two dimensions of the 3D NMDS of the data (stress=
2030 0.165) is plotted in Figure 3.10B.

2031 **Table 3.1.** Median, inter-quartile range (IQR), maximum and minimum of all sterols detected in
 2032 whole *Bombus* samples. Number of species each sterol was found in also listed (maximum 18).
 2033 Arranged by median value. Un-named sterols are labelled with carbon chain length (CC) and
 2034 double bond count (DB) as (CC:DB).

Sterol	Median	IQR	Max	Min	Species detected in
24MC	34.653	14.575	58.459	8.775	18
Isofucosterol	27.401	7.725	33.613	16.797	18
β -Sitosterol	22.406	7.309	49.724	6.666	18
Campesterol	6.635	2.583	17.836	0.918	18
Ergosterol	2.078	7.578	18.921	0.033	18
Desmosterol	1.171	0.905	5.374	0.092	18
ST(28:2)C	0.507	0.495	4.363	0.045	18
Cycloartenol	0.491	0.242	0.896	0.021	18
Cholesterol	0.349	0.350	1.249	0.037	18
Cyclolaudenol	0.137	0.123	0.837	0.015	18
ST(31:2)A	0.048	0.042	0.164	0.004	18

2035



2051 **Figure 3.2.** Species phylogeny adapted with permission from Hines (2008). Heatmap shows mean
 2052 species proportion for the most commonly dominant six sterols plus cholesterol. Bar graph shows
 2053 mean species total sterol production \pm SEM. Points show individual samples of each species with
 2054 different colours for each county the species was sampled from. The eight subgenera represented
 2055 by species analysed are highlighted.

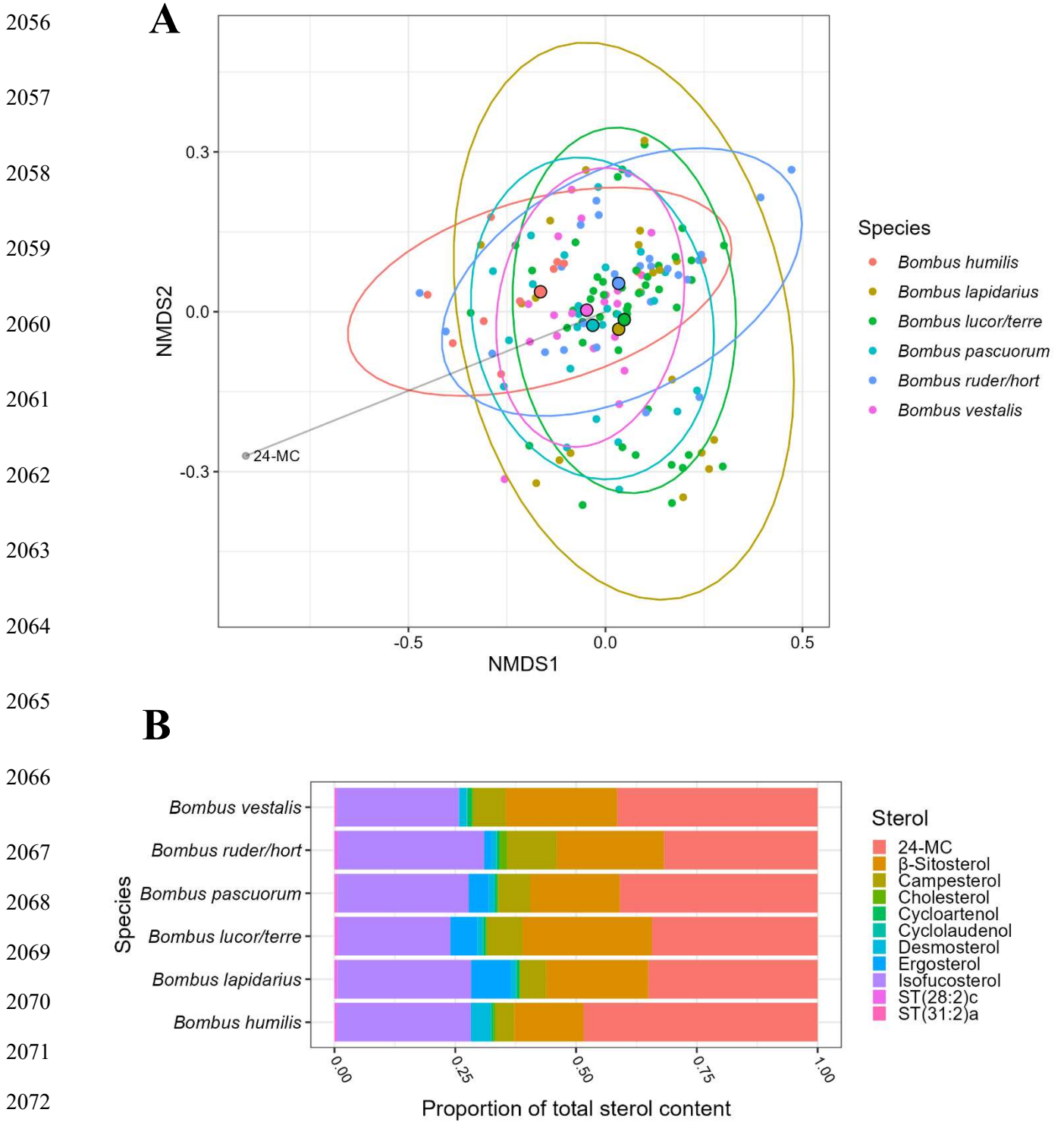


Figure 3.3.

A) The first two dimensions of a 3D NMDS carried out using the six most replicated species in the dataset (stress =0.135). Subsequent analysis showed significant inter-species differences.

B) Bar graph of species means shown in Figure 3.3A.

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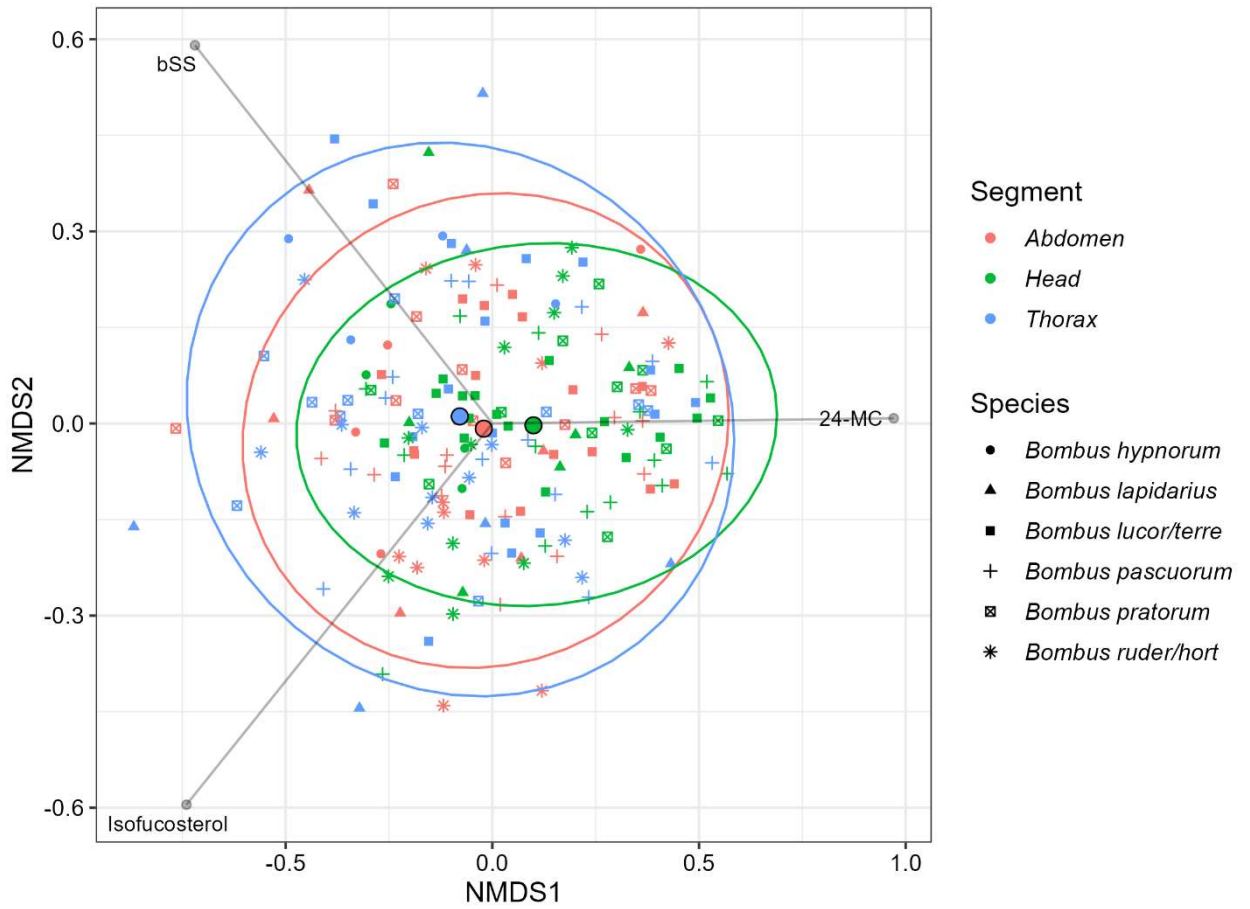
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Figure 3.4. The first two dimensions of 3D NMDS (stress value 0.155) comparing sterol profile of body tagmata. Pairwise comparisons showed the composition of the head to be significantly different from the other two tagmata.

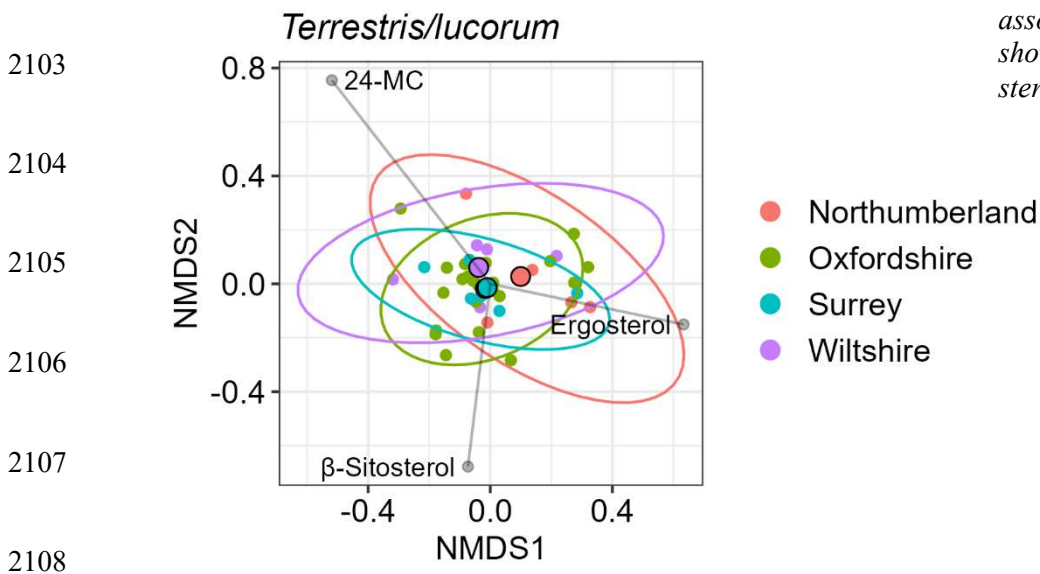
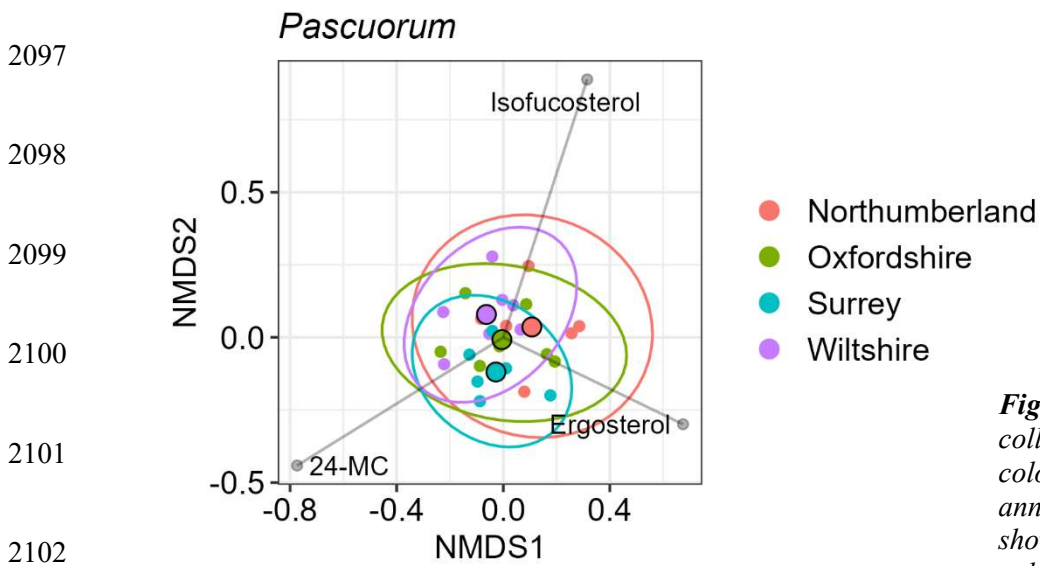
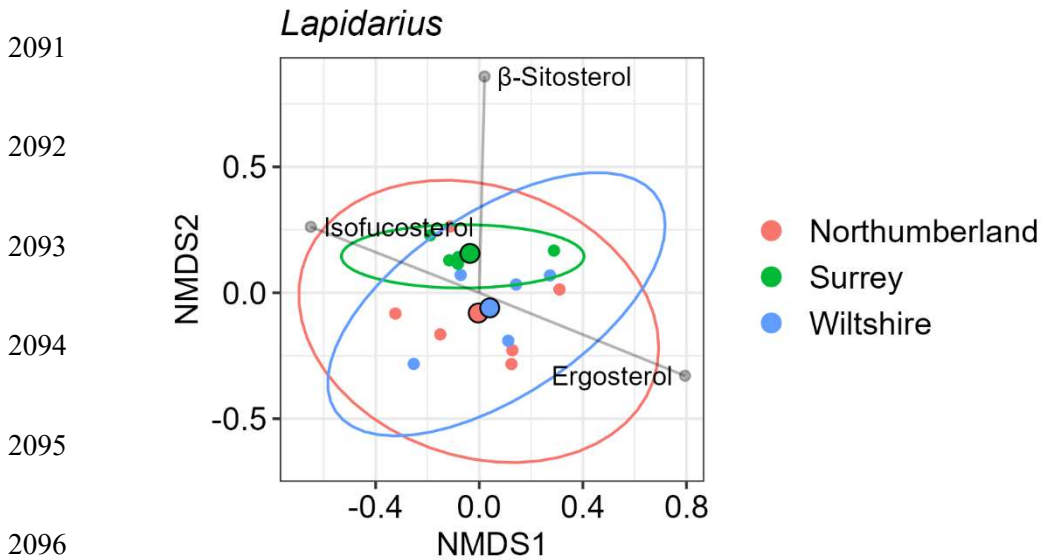
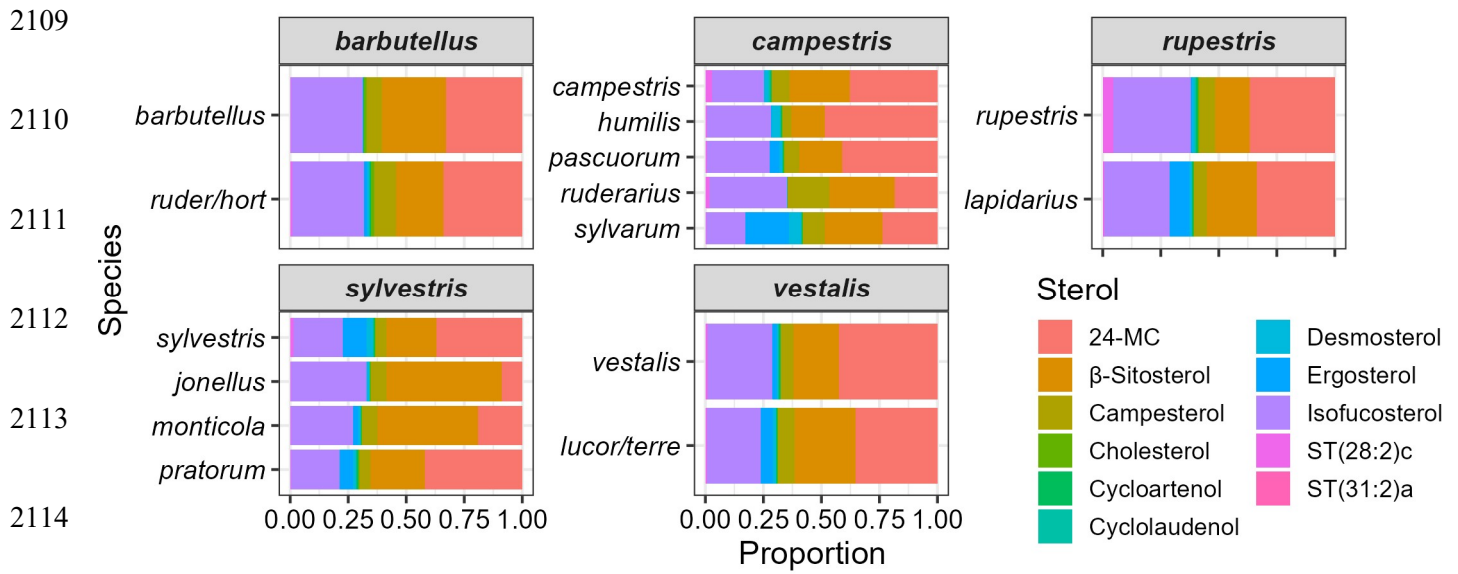


Figure 3.5. NMDS of the species collected from multiple counties, coloured by collection sites and annotated with sterols which showed strong (>0.6 absolute value) and significant ($p < 0.010$) associations. Only *B. pascuorum* showed significant differences in sterol profile among sites.



2115 **Figure 3.6.** Bar graphs comparing the mean sterol profiles of cuckoo bumble bees and their
 2116 associated host species. Cuckoo species are shown in the header of each graph.

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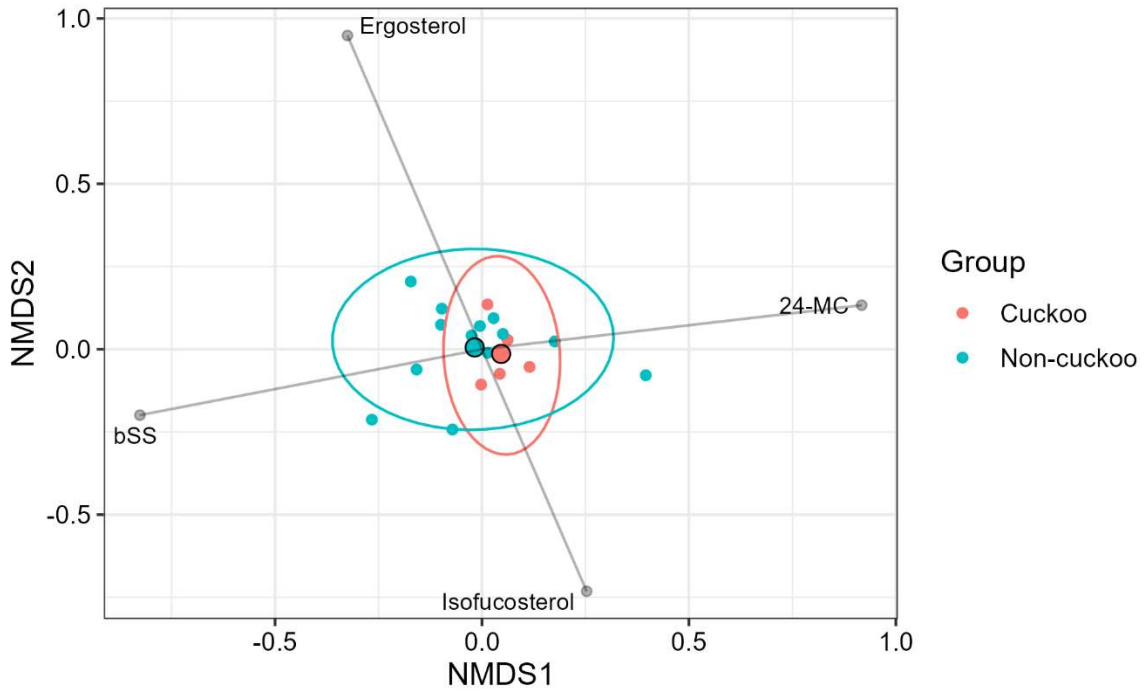
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2126 **Figure 3.7.** 2D NMDS comparing the sterol profiles of cuckoo and non-cuckoo species
 2127 (stress=0.099). Mean values are used for each species to prevent sampling bias. Grey lines
 2128 indicate strong (>0.7 absolute value) and significant ($p<0.0100$) correlations. Groups were shown
 2129 to not differ significantly by PERMANOVA.

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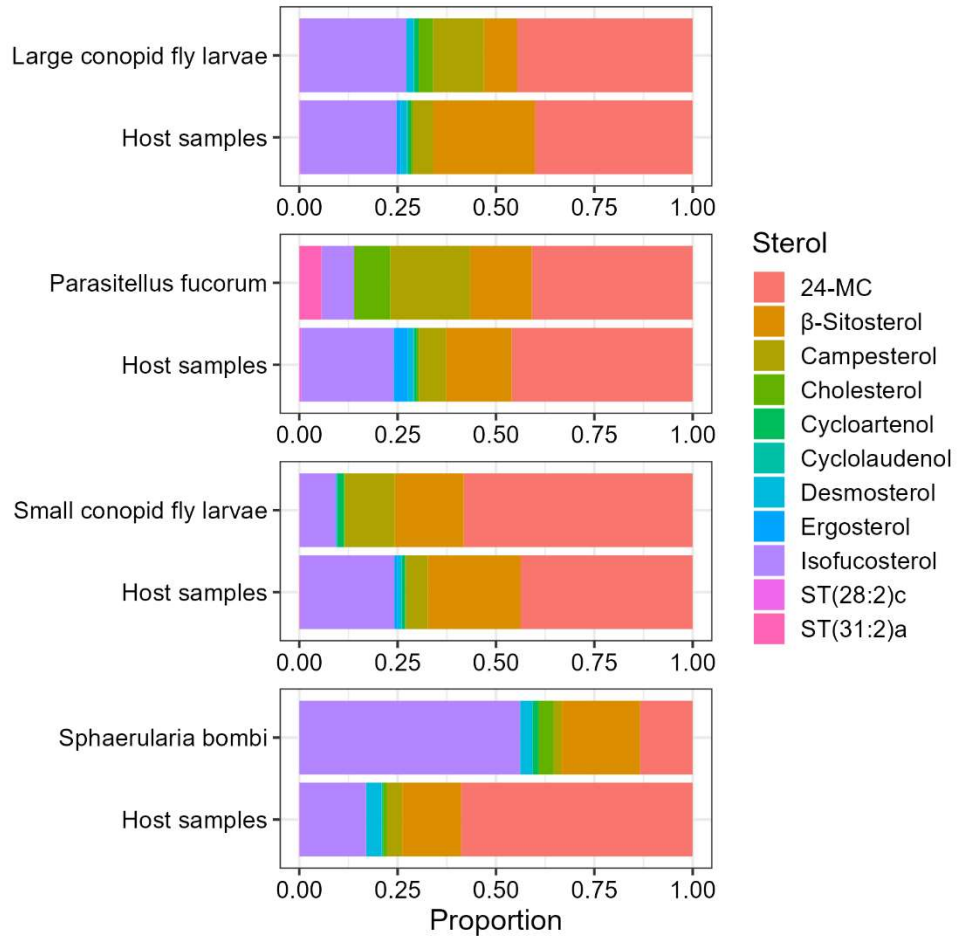
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2141 **Figure 3.8.** The sterol profiles of parasites removed from bumble bee abdominal cavities and outer
2142 bodies during dissection. Parasites are plotted alongside a mean sterol profile of the bumble bees
2143 they were removed from. Number of hosts from which each parasite was removed is as follows:
2144 Large conopid fly larvae (4), Parasitellus fucorum (12), small conopid fly larvae (4), Sphaerularia
2145 bombi (3).

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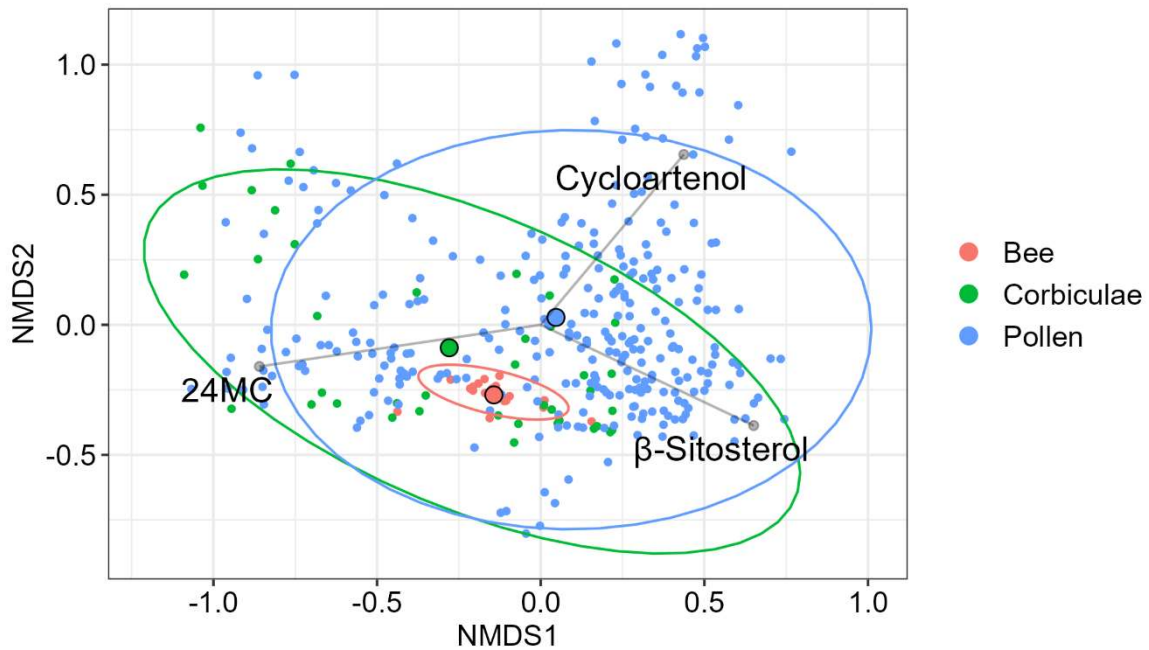
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2154 **Figure 3.9.** Comparison of whole-body bumble bee sterols with corbiculate and hand collected
 2155 pollens. Only sterols present in both datasets were used for analysis (24-methylenecholesterol, β -
 2156 sitosterol, campesterol, cholesterol, cycloartenol, cyclolaudenol, desmosterol, ergosterol and
 2157 isofucosterol). Bumble bee tissue sterols show a composition shared by a small proportion of hand
 2158 collected pollens but a more similar mean profile to corbiculate pollen. Both bumble bees and
 2159 corbiculate pollen showed higher levels of 24-methylenecholesterol. Hand collected pollen was
 2160 more associated with increased cycloartenol. Grey lines show strong (>0.6 absolute value) and
 2161 significant ($p < 0.010$) correlations. Stress value for plot = 0.179.

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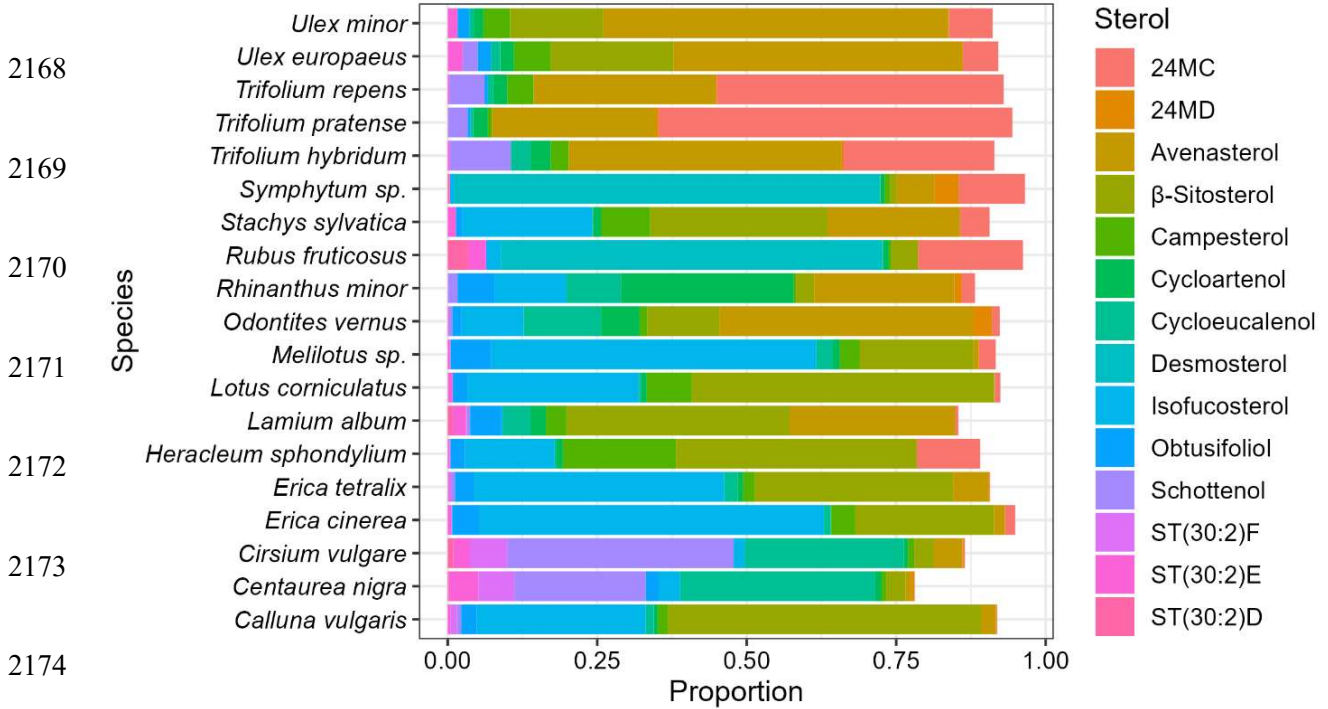
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2164 **Table 3.2.** Summary of Indicator Species Analysis results showing associations between groups in
 2165 Figure 3.9 and individual sterols. Table reports Indicator Values (IV) and p-values.

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Sterol	IV	P-value	Sterol	IV	P-value	Sterol	IV	P-value
<i>Bumble bee whole bodies</i>			<i>Corbiculate pollen</i>			<i>Hand collected pollen</i>		
Ergosterol	0.795	0.005	Desmosterol	0.753	0.020	Cyclolaudenol	0.835	0.005
			Cholesterol	0.730	0.005	Cycloartenol	0.780	0.005

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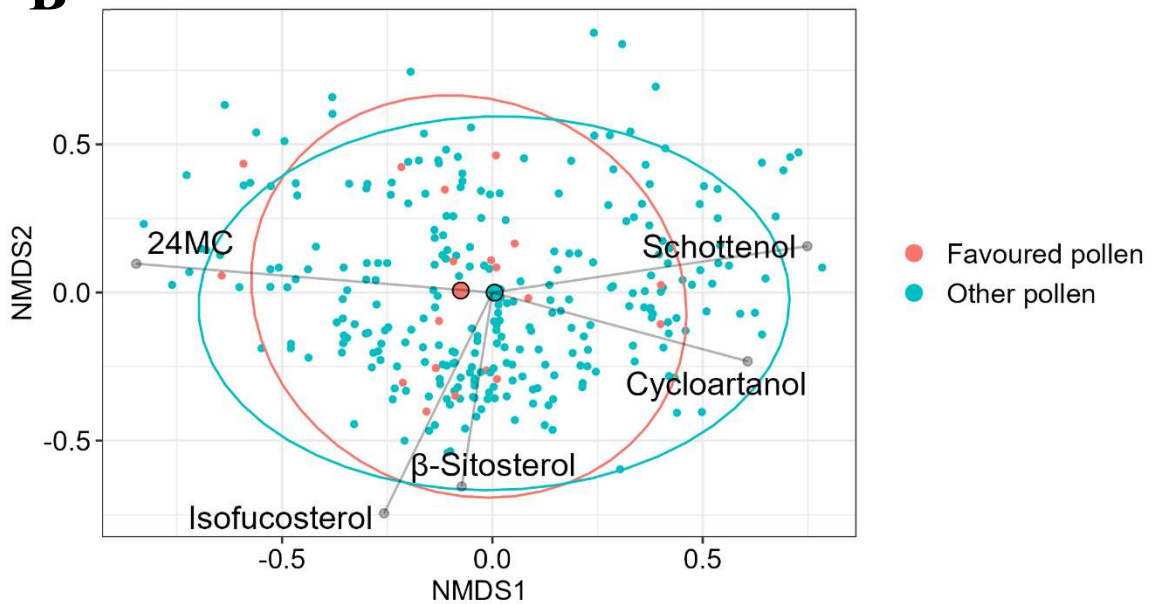
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Figure 3.10.

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A) The sterol profile of pollens frequently collected by *Bombus* spp. according to the Database of Pollinator Interactions (DoPI). Sterols displayed are limited to those which occur as one of the most dominant four sterols in at least one species.

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B) NMDS (stress=0.165) of pollens shown in Figure 3.10A compared with all other available pollen species from Chapter 2 (species means). Groups were not significantly different.

2187 **Discussion**

2188 By analysing the whole-body sterol contents of 18 *Bombus* species, this work demonstrates that the
2189 sterolome of UK bumble bees is dominated by 24-methylenecholesterol, isofucoesterol and β -
2190 sitosterol. The relative proportions of these sterols are comparable to that reported in honey bees
2191 (Svoboda *et al.*, 1980). Given the range of species and habitats sampled, the consistency of this
2192 profile suggests it fulfils physiological requirements which are shared by *Bombus* spp..
2193 Isofucoesterol, 24-methylenecholesterol and β -sitosterol are, however, not ubiquitous in floral
2194 pollens as shown in Chapter 2. Therefore, bumble bees must selectively forage for desirable pollen
2195 sterols in different habitats. The popularity of *Trifolium* species across all *Bombus* spp. may in part
2196 be explained by the high proportion of 24-methylencholesterol they provide compared to other
2197 popular plants for pollen collection, and also by their abundance and long flowering period, despite
2198 their relatively low pollen production. However, there are several Rosaceae species which display
2199 a higher proportion of 24-methylenecholesterol and were not among the most popular pollen plants
2200 for *Bombus* spp. Sterol content is therefore only motivating foraging in combination with other
2201 nutritional needs.

2202 There was no phylogenetic signal in sterol profile across the species sampled, therefore *Bombus*
2203 subgenera do not appear to have developed distinct sterol profiles. By extension, this would mean
2204 cuckoo bumble bees, which form their own subgenus (*Psithyrus*), do not differ in sterol profile
2205 from other bumble bee species. This does not mean *Bombus* spp. do not have a distinct and
2206 phylogenetically constrained sterol profile, but that it may only be apparent in comparison with
2207 more ecologically and phylogenetically diverse bee species. Variation in total sterol content across
2208 species may have been the result of samples being collected throughout the flight season,
2209 impacting the fat stores present.

2210 All bumble bee species analysed showed a consistent dominance of 24-methylenecholesterol,
2211 isofucoesterol and β -sitosterol. The low proportion of cholesterol (median = 0.4% of total sterols)
2212 demonstrates that all species are not converting dietary sterols into cholesterol but are still
2213 potentially utilising small quantities of cholesterol for use in synthesising moulting hormones and
2214 egg production. The presence of ergosterol across all species suggests it is often consumed by
2215 bumble bees, perhaps as a result of fungal growth on pollen in the nest or directly on flowers, as
2216 ergosterol cannot be synthesised by animals or plants (Weete, 1973; Desmond and Gribaldo,
2217 2009).

2218 The significance of interspecies differences and species-sterol associations shown here reflect
2219 small variations in proportions of different sterols rather than the presence/absence of certain
2220 sterols. Many species were collected at single sites or a single time of year and therefore may not
2221 reflect the true variation present within the species. As such, the biological relevance of these
2222 differences may be limited. Only two *Bombus* species showed a significant association with an
2223 individual sterol, likely due to the overall similarity in sterol profile across the dataset. The
2224 associations between *Bombus distinguendus* and *Bombus ruderarius* with 24-methylenecholesterol
2225 and campesterol respectively is potentially interesting as these sterols were present in all other
2226 species analysed. *B. distinguendus* has declined greatly in the UK and is now only found in flower
2227 rich machair and has a strong preference for *Trifolium pratense*. However, it is important to note
2228 that in this dataset, both species were represented by only a single collection site, and *Bombus*
2229 *ruderarius* by a single sample. Therefore, the higher proportions of these sterols may be site
2230 specific in this species, as opposed to a universal trend. Both of these associations were also weak,
2231 likely due to the commonality and abundance of these sterols across other species. Similarly, the
2232 interspecies differences between *Bombus hortorum/ruderatus* and *B. humilis* compared to the other
2233 most sampled species in the dataset is dubious despite its statistical significance. These species are
2234 not closely related but with shared ecological similarities, being long-tongued and associated with

2235 Fabaceae. *Bombus hortorum* and *B. ruderatus* are, however, much more common and widespread
2236 than *B. humilis*.

2237 Despite differences in detection of minor sterols between runs, the decision was made to combine
2238 the two analysis runs of bumble bee samples. The principal aim of the analysis was to enable
2239 comparisons between species and the inclusion of the second run enabled the addition of more two
2240 species (*Bombus distinguendus* and *B. jonellus*), including an additional subgenus
2241 (*Subterraneobombus*). Inclusion of the second data set did not alter the significance of any findings
2242 regarding phylogenetic and interspecies analysis. Only the associations between species and
2243 individual sterols were altered by inclusion of this data. It is therefore difficult to disentangle the
2244 effect of separate analysis runs on this finding as both species, *Bombus distinguendus* and
2245 *B. jonellus*, are restricted in distribution and show strong foraging preferences. Therefore, they
2246 naturally may be expected to show different sterol associations to the rest of the species.

2247 The lack of significant differences between sexes demonstrates that reproductive organs do not
2248 lead to a significant difference in sterol profile. This could be due to either our analysis not having
2249 sufficient resolution to detect small differences in the context of all other abdominal tissues, or
2250 because similar sterols are indeed used in both organ systems. As both males and females are fed
2251 and reared by the same workers it follows that they would develop the same adult sterol
2252 composition unless they possessed different sterol uptake mechanisms. The major use of sterols in
2253 animals is for integration into cell membranes to maintain integrity and fluidity. The similarity in
2254 sterol profiles between tagmata may therefore represent an optimum ratio of sterols for the cell
2255 membranes in most tissues. Under sterol starvation conditions however, sterols may be selectively
2256 lost from tissues (Carvalho *et al.*, 2010); for example, studies in *Drosophila* have shown that
2257 central nervous system neurons and the gut apical membrane retained sterol content more than
2258 other tissues in sterol-limited conditions. Furthermore, previous work in honey bees has shown the
2259 hypopharyngeal and mandibular glands of nurse bees contained higher cholesterol than whole body

2260 samples (Svoboda, Herbert and Thompson, 1986). In support of this, in my data, only the head
2261 showed a sterol profile different to other tagmata, though this was with a weak effect size. Future
2262 studies where specific organs are dissected out for analysis may discover that there are subtle
2263 differences in the sterol profiles of these tissues.

2264 Cuckoos were not shown to have a significantly different sterol profile from non-cuckoo species,
2265 with *Bombus vestalis* also not showing a significant difference to its host or non-host species. The
2266 similarity of sterol profiles across different bumble bee species may potentially facilitate the
2267 socially parasitic life strategy of cuckoo bumble bees. For species which target a range of hosts,
2268 such as *Bombus campestris*, physiological similarity enables a broad target pool of visually similar
2269 bumble bees.

2270 As might be expected if sterol profile depended on specific uptake mechanisms designed to
2271 maintain an optimum sterol ratio in bumblebees, it was shown that collection site, and therefore
2272 available plant community, did not affect sterol profile in two out of three species which were
2273 compared between habitats. This suggests bumble bees are capable of obtaining the dietary sterols
2274 they need from different plant species and, where possible, will maintain an optimum sterol profile
2275 through foraging. Previous work has shown honey bees can survive and reproduce on a number of
2276 mono-sterol diets in the short term as sterols are secreted by nurse bees from endogenous pools and
2277 fed to larvae to maintain sterols ratios (Herbert *et al.*, 1980; Svoboda *et al.*, 1980). For bumble
2278 bees, a fully cholesterol or 24-methylenecholesterol based diet may be equally beneficial for
2279 producing brood, as shown in honey bees (Herbert *et al.*, 1980); however, this would be
2280 challenging to obtain reliably from wildflower pollens which are mostly very low in cholesterol.
2281 The seemingly stable ratio between sites may therefore represent a compromise between readily
2282 available dietary sterols and a physiological optimum (Knittelfelder *et al.*, 2020). This strategy may
2283 contribute to the ecological success of bumble bees, allowing them to forage in an extremely wide
2284 range of habitats, using pollen from many different plant species.

2285 Corbiculate pollen showed a profile distinct from other hand-collected pollens, correlating with
2286 higher isofucoesterol and 24-methylenecholesterol. Bumble bees can change their pollen foraging
2287 target during a single trip (Martínez-Bauer *et al.*, 2021). In support of this, some corbiculate pollen
2288 samples in this study were observed to contain a mixture of pollens. Bumblebees also add saliva to
2289 collected pollen to aid compaction into their corbiculae. Both of these may contribute to natural
2290 variation between hand-collected and bee collected pollens. It is also worth noting that due to
2291 turnover in available pollens throughout the season, the bees analysed in this study will be
2292 collecting different pollen to those they were raised on. Direct tracking of sterols consumed during
2293 development and the ones present in adulthood would require an experimental approach.

2294 Future work can build on these data by experimentally manipulating the diets of bumble bees to
2295 determine if they are fixed to a particular profile of specific sterols, like honey bees, or if they
2296 demonstrate flexibility in which sterols they use and the proportions of these sterols. Furthermore,
2297 the similarity in sterol profile among tagmata could also be tested experimentally to determine if
2298 this is maintained under nutritional stress. The ecological and economic importance of bumble bees
2299 makes understanding their nutrition an important step for future study of wild bee nutrition. These
2300 new data have shown that despite differences in conservation status and distribution, UK bumble
2301 bees show a conserved sterol profile which can be maintained by a generalist foraging strategy.
2302 This suggests that the same set of floral pollens would be capable of meeting the sterol nutritional
2303 requirements of all species and that conservation measures such as seed mixes could therefore be
2304 tailored to address this.

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2317 recommendations. Finally, thanks also to my colleagues Liam Crowley and Jonathan Patrick for
2318 providing samples and to Daniel Stabler and Jennifer Chennells for helping develop the analysis
2319 methodology.

2320 **Supplementary**

2321 **Table S3.1.** Summary of all bumble bee samples collected including species, sex, caste, county and
 2322 collection month.

Species	Sex	Caste	County	Month collected	Number of samples
<i>Bombus barbutellus</i>	F	Q	Oxfordshire	May-22	1
<i>Bombus barbutellus</i>	M	NA	Oxfordshire	Jun-21	1
<i>Bombus campestris</i>	M	NA	Oxfordshire	Jul-20	2
<i>Bombus distinguendus</i>	F	W	Outer Hebrides	Aug-23	5
<i>Bombus humilis</i>	F	W	Kent	Sep-21	5
<i>Bombus humilis</i>	F	W	Newport	Sep-21	1
<i>Bombus humilis</i>	F	W	Wiltshire	Jul-22	2
<i>Bombus humilis</i>	F	W	Wiltshire	Sep-21	4
<i>Bombus hypnorum</i>	F	Q	Oxfordshire	Feb-22	1
<i>Bombus hypnorum</i>	F	Q	Oxfordshire	Mar-22	1
<i>Bombus hypnorum</i>	F	Q	Wiltshire	Mar-22	1
<i>Bombus hypnorum</i>	F	Q	NA	Apr-21	1
<i>Bombus hypnorum</i>	F	W	Oxfordshire	Jun-21	1
<i>Bombus hypnorum</i>	F	W	Oxfordshire	May-21	6
<i>Bombus jonellus</i>	F	W	Berkshire	Aug-23	5
<i>Bombus jonellus</i>	F	W	Shropshire	Aug-21	1
<i>Bombus lapidarius</i>	F	Q	Oxfordshire	Apr-21	3
<i>Bombus lapidarius</i>	F	Q	Oxfordshire	Apr-22	1
<i>Bombus lapidarius</i>	F	Q	Oxfordshire	May-21	2
<i>Bombus lapidarius</i>	F	W	Northumberland	Aug-22	6
<i>Bombus lapidarius</i>	F	W	Shropshire	Aug-21	1
<i>Bombus lapidarius</i>	F	W	Surrey	Jun-22	5
<i>Bombus lapidarius</i>	F	W	Wiltshire	Jul-22	5
<i>Bombus lapidarius</i>	M	NA	Wiltshire	Jul-22	3
<i>Bombus lucor/terre</i>	F	Q	Oxfordshire	Apr-21	1
<i>Bombus lucor/terre</i>	F	Q	Oxfordshire	Apr-22	1
<i>Bombus lucor/terre</i>	F	Q	Oxfordshire	Feb-22	2
<i>Bombus lucor/terre</i>	F	Q	Oxfordshire	Mar-22	4
<i>Bombus lucor/terre</i>	F	Q	Oxfordshire	May-21	1
<i>Bombus lucor/terre</i>	F	Q	West Yorkshire	Apr-21	4
<i>Bombus lucor/terre</i>	F	Q	Wiltshire	Mar-22	4
<i>Bombus lucor/terre</i>	F	W	Northumberland	Aug-22	6
<i>Bombus lucor/terre</i>	F	W	Oxfordshire	Jun-21	6
<i>Bombus lucor/terre</i>	F	W	Oxfordshire	May-21	15

Species	Sex	Caste	County	Month collected	Number of samples
<i>Bombus lucor/terre</i>	F	W	Oxfordshire	May-22	1
<i>Bombus lucor/terre</i>	F	W	Shropshire	Aug-21	1
<i>Bombus lucor/terre</i>	F	W	Shropshire	Sep-21	2
<i>Bombus lucor/terre</i>	F	W	Surrey	Jun-22	6
<i>Bombus lucor/terre</i>	F	W	Wiltshire	Jul-22	5
<i>Bombus lucor/terre</i>	M	NA	Oxfordshire	Jun-22	3
<i>Bombus monticola</i>	F	W	Northumberland	Aug-22	4
<i>Bombus monticola</i>	F	W	Shropshire	Aug-21	1
<i>Bombus monticola</i>	F	W	Shropshire	Sep-21	1
<i>Bombus monticola</i>	F	W/Q	Shropshire	Aug-21	1
<i>Bombus monticola</i>	M	NA	Shropshire	Sep-21	1
<i>Bombus pascuorum</i>	F	Q	Oxfordshire	Apr-21	3
<i>Bombus pascuorum</i>	F	Q	Oxfordshire	Mar-22	1
<i>Bombus pascuorum</i>	F	Q	Oxfordshire	May-21	6
<i>Bombus pascuorum</i>	F	Q	Wiltshire	Mar-22	3
<i>Bombus pascuorum</i>	F	Q	NA	Apr-21	1
<i>Bombus pascuorum</i>	F	W	Northumberland	Aug-22	6
<i>Bombus pascuorum</i>	F	W	Oxfordshire	Apr-21	1
<i>Bombus pascuorum</i>	F	W	Oxfordshire	Jun-21	5
<i>Bombus pascuorum</i>	F	W	Oxfordshire	May-21	1
<i>Bombus pascuorum</i>	F	W	Shropshire	Aug-21	1
<i>Bombus pascuorum</i>	F	W	Surrey	Jun-22	6
<i>Bombus pascuorum</i>	F	W	Wiltshire	Jul-22	6
<i>Bombus pascuorum</i>	F	W/Q	Wiltshire	May-20	1
<i>Bombus pratorum</i>	F	Q	Oxfordshire	Apr-22	1
<i>Bombus pratorum</i>	F	Q	Oxfordshire	Mar-22	6
<i>Bombus pratorum</i>	F	Q	Oxfordshire	May-21	1
<i>Bombus pratorum</i>	F	Q	Wiltshire	Apr-22	1
<i>Bombus pratorum</i>	F	Q	Wiltshire	Mar-22	2
<i>Bombus pratorum</i>	F	W	Oxfordshire	Jun-21	3
<i>Bombus pratorum</i>	F	W	Oxfordshire	May-21	8
<i>Bombus ruder/hort</i>	F	Q	Oxfordshire	2021	1
<i>Bombus ruder/hort</i>	F	Q	Oxfordshire	Apr-21	4
<i>Bombus ruder/hort</i>	F	Q	Oxfordshire	Apr-22	2
<i>Bombus ruder/hort</i>	F	Q	Oxfordshire	Jun-22	1
<i>Bombus ruder/hort</i>	F	Q	Oxfordshire	Mar-22	1
<i>Bombus ruder/hort</i>	F	Q	Oxfordshire	May-21	2
<i>Bombus ruder/hort</i>	F	Q	Wiltshire	Mar-22	1
<i>Bombus ruder/hort</i>	F	W	Northumberland	Aug-22	5
<i>Bombus ruder/hort</i>	F	W	Oxfordshire	Aug-21	3

Species	Sex	Caste	County	Month collected	Number of samples
<i>Bombus ruder/hort</i>	F	W	Oxfordshire	Jul-20	3
<i>Bombus ruder/hort</i>	F	W	Oxfordshire	Jun-22	1
<i>Bombus ruder/hort</i>	F	W	Oxfordshire	May-21	4
<i>Bombus ruder/hort</i>	F	W	Surrey	Jun-22	5
<i>Bombus ruder/hort</i>	F	W	Wiltshire	Jul-22	1
<i>Bombus ruder/hort</i>	M	NA	Oxfordshire	Jul-20	2
<i>Bombus ruder/hort</i>	M	NA	Oxfordshire	Jun-21	2
<i>Bombus ruder/hort</i>	M	NA	Oxfordshire	Jun-22	1
<i>Bombus ruderarius</i>	M	NA	Wiltshire	Jul-22	1
<i>Bombus rupestris</i>	F	Q	Oxfordshire	Sep-22	1
<i>Bombus rupestris</i>	F	Q	Shropshire	Sep-21	1
<i>Bombus rupestris</i>	M	NA	Gloucestershire	Jul-22	2
<i>Bombus rupestris</i>	M	NA	Oxfordshire	Jul-20	5
<i>Bombus rupestris</i>	M	NA	Wiltshire	Jul-22	2
<i>Bombus soroeensis</i>	F	W	Wiltshire	Sep-21	9
<i>Bombus sylvarum</i>	F	W	Newport	Sep-21	4
<i>Bombus sylvestris</i>	F	Q	Oxfordshire	Sep-21	1
<i>Bombus sylvestris</i>	M	NA	Gloucestershire	Jun-22	1
<i>Bombus sylvestris</i>	M	NA	Oxfordshire	Jul-20	1
<i>Bombus sylvestris</i>	M	NA	Oxfordshire	Jun-22	3
<i>Bombus vestalis</i>	F	Q	Oxfordshire	Aug-21	1
<i>Bombus vestalis</i>	F	Q	Oxfordshire	May-21	1
<i>Bombus vestalis</i>	F	Q	Oxfordshire	May-22	5
<i>Bombus vestalis</i>	F	Q	Surrey	Jun-22	1
<i>Bombus vestalis</i>	M	NA	Gloucestershire	Jun-22	1
<i>Bombus vestalis</i>	M	NA	Oxfordshire	Jun-21	2
<i>Bombus vestalis</i>	M	NA	Oxfordshire	Jun-22	4
<i>Bombus vestalis</i>	M	NA	Warwickshire	Jun-20	3
<i>Bombus vestalis</i>	M	NA	Wiltshire	Jul-22	1

2323

2324 **Table S3.2.** Retention time, mass to charge ratio (*m/z*) and structure description of sterols (carbon
 2325 count (*CC*): double bond equivalents (*DB*)) used in Quality Assurance (*QA*) mixture.

Sterol	Structure	<i>m/z</i>	Retention time (min)
24-methylenecholesterol	(28:2)	398.3549	5.9
24-methylenecycloartenol	(31:2)	440.4018	7.5
Anthelsterol	(29:3)	410.3549	5.3
Avenasterol	(29:2)	412.3705	6.9
β-sitosterol	(29:1)	414.3862	9.7
Brassicasterol	(28:2)	398.3549	6.6
Campesterol	(28:1)	400.3705	8.2
Cholesterol	(27:1)	386.3549	7.0
Cycloartenol	(30:2)	426.3862	8.3
Cycloeucalenol	(30:2)	426.3862	7.7
Cyclolaudenol	(31:2)	440.4018	9.5
Desmosterol	(27:2)	384.3392	5.1
Episterol	(28:2)	398.3549	5.7
Ergosterol	(28:3)	396.3392	5.5
Isofucosterol	(29:2)	412.3705	7.5
Schottenol	(29:1)	414.3862	9.0
Sitostanol	(29:0)	416.4018	11.1
Spinasterol	(29:2)	412.3705	8.0
Stigmasterol	(29:2)	412.3705	8.5

2326

2327

Bombus vestalis

2328

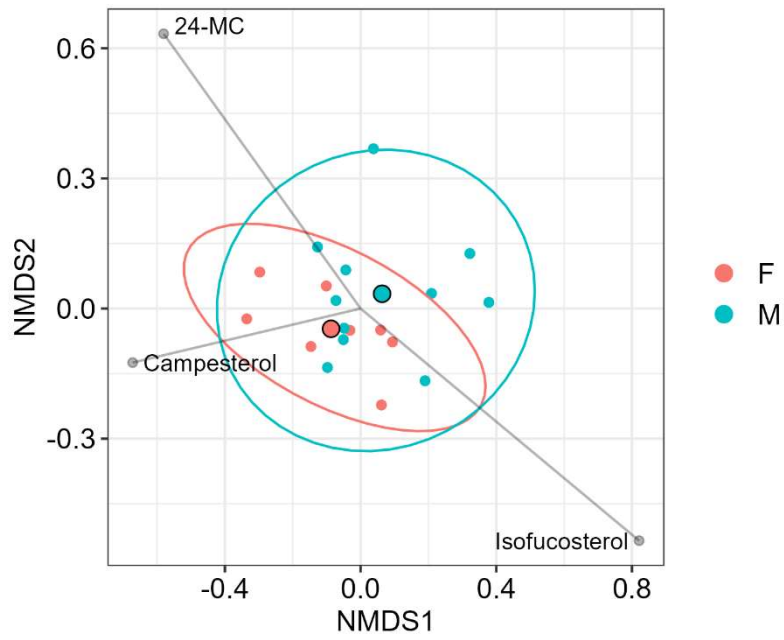
2329

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2334

Figure S3.1. NMDS comparison of male and female *B. vestalis* specimens showing no significant difference between sexes.

2335

2336

2337

Sex matched species

2338

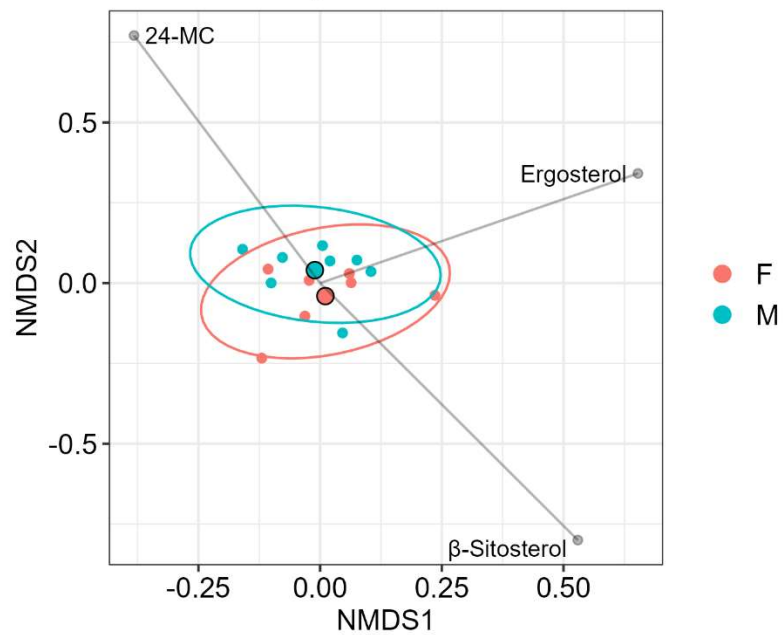
2339

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2343



2344

Figure S3.2. NMDS comparison of mean values of male and female specimens of *B. barbutellus*, *B. hortorum/ruderatus*, *B. lapidarius*, *B. monticola*, *B. rupestris*, *B. sylvestris*, *B. terrestris/lucorum* and *B. vestalis* showing no significant difference between sexes. Stress value is 0.151.

2345

2346

2347 **Chapter 4: Sterol profile diversity in solitary bees**

2348 Contributors: Ellen Baker (sample collection, sample processing, data analysis and writing),
2349 Samuel Furse (sterolomics analysis and signal processing), Hauke Koch (study design and sample
2350 collection), Katie Berry (sample processing and identification), Ellen Lamborn (sample collection),
2351 Owen Lewis (study design), Phil Stevenson (study design), Geraldine Wright (study design).

2352 **Abstract**

2353 Solitary bees display a wide range of foraging strategies, from those that collect pollen from
2354 multiple plant families to others that collect from only a single plant species. However, all are
2355 constrained by their need to fulfil certain nutritional requirements. One group of nutrients, sterols,
2356 is essential for all insects but cannot be synthesised *de novo*. Previous work has shown honeybees
2357 acquire sterols from pollen and do not de-alkylate these phytosterols to produce cholesterol. The
2358 extent to which this is true of other bee species is unknown, but it is reasonable to assume that
2359 most, if not all, bee species are constrained by what is available in the pollen they consume. Given
2360 the diversity of foraging strategies present in solitary bees, this could produce a wide range of
2361 tissue sterol profiles between even closely related species. This chapter reports the sterol profiles of
2362 56 bee species in the UK. It demonstrates there is no phylogenetic signal for the majority of sterols
2363 across the dataset but there are clear inter-species differences. Bee sterol profiles are distinct from
2364 pollen, even for monoleptic species, indicating the selective use of certain sterols such as 24-
2365 methylenecholesterol, isofucosterol and β -sitosterol by bees. Some kleptoparasitic bee species
2366 showed similar sterol profiles to their hosts, despite being distantly related. These results build a
2367 complex picture of sterol use in solitary bees but one that appears driven largely by ecological
2368 adaptation rather than evolutionary constraints.

2369 **Introduction**

2370 Solitary bees account for most of the bee species in the UK and provide an important pollination
2371 service for many wildflower species as well as contributing to pollination of some crops
2372 (Hutchinson *et al.*, 2021). They differ from eusocial species such as bumble bees (*Bombus* spp.)
2373 and the European honey bee (*Apis mellifera*) by a lack of colony structure and caste system.
2374 Females of solitary species forage only for their own offspring and provision a single bulk food
2375 source for larvae rather than continually feeding offspring throughout larval development as is the
2376 case with eusocial species. However, under certain conditions a minority of species can form social
2377 structures in parts of the UK, such as some *Halictus* and *Lasioglossum* species (Falk and
2378 Lewington, 2015). Solitary bees also exhibit a range of dietary specialisations in pollen foraging
2379 (hereafter ‘lecty’); monoleptic species are dependent on a single plant species for pollen intake, a
2380 strategy which is rare and only demonstrated by a small number of UK species (including *Andrena*
2381 *florea*, *Macropis europaea*, *Melitta dimidiata* and *Melitta tricincta*) (Falk and Lewington, 2015).
2382 Oligolectic species will collect pollen from multiple, closely related species which belong to a
2383 single genus or family. As a result of their specialist foraging, these species can be limited in
2384 distribution and emergence time. For instance *Melitta dimidiata* is confined to Salisbury plain in
2385 the south of England due to its need for *Onobrychis viciifolia* (Falk and Lewington, 2015) , and
2386 *Colletes hederæ* is one of the latest emerging solitary bees to match the flowering of its major
2387 pollen source, *Hedera helix* (Falk and Lewington, 2015). Asteraceae pollen is associated with a
2388 number of oligolectic bee species in the UK but is rarely gathered by polylectic species such as
2389 honey bees. This is referred to as the ‘Asteraceae paradox’ (Müller and Kuhlmann, 2008; Praz,
2390 Müller and Dorn, 2008; Vanderplanck, Gilles, *et al.*, 2020). In Chapter 2 Asteraceae pollen was
2391 shown to have a lower proportion of $\Delta 5$ sterols compared to other pollen, therefore solitary bees
2392 specialising on Asteraceae may show the same trend. Polylectic bee species collect pollen from

2393 multiple families and are considered generalist foragers. In the UK all bumble bee species, honey
2394 bees and some solitary bee species are polylectic (Falk and Lewington, 2015).

2395 There are also a number of bee species in the UK which are socially parasitic (kleptoparasitic) on
2396 other solitary bees. They lay an egg alongside the host's before the cell is sealed and once the
2397 kleptoparasite larvae emerges it will kill the host's offspring and consume the provisions (Falk and
2398 Lewington, 2015). As a result, these kleptoparasitic bees do not have any physiological adaptations
2399 for collecting pollen on their bodies. They can target either single or multiple host species. For
2400 instance, *Nomada fucata* parasitises only *Andrena flavipes* but *N. rufipes* targets multiple *Andrena*
2401 species (Falk and Lewington, 2015). These species differ in their parasitic strategy from cuckoo
2402 bumble bee species (subgenus *Psithyrus*), discussed in Chapter 3, which usurp the queen of an
2403 existing colony and manipulate the workers to raise their own young.

2404 As a result of their diverse relationships with floral pollen, solitary bees present a useful
2405 opportunity to examine the effects of sterols in diet on body composition. As covered in the
2406 introductory chapter, sterols are a subgroup of lipids defined by the presence of hydroxyl group,
2407 steroid nucleus and alkane side chain (Jing and Behmer, 2020). The bulk of their use is in
2408 membrane structure but they also serve important roles in the production of hormones, such as
2409 moulting hormones in insects (Behmer and Nes, 2003; Jing and Behmer, 2020). Insects are not
2410 able to synthesise these essential compounds *de novo* and the majority therefore convert dietary
2411 sterols into cholesterol via dealkylation, creating a whole-body tissue sterol profile dominated by
2412 cholesterol (Svoboda and Thompson, 1987). Some insects however have adapted to using other
2413 sterols from fungal or plant matter (Janson *et al.*, 2009). The extent to which this is true in the
2414 Apidae is presently unknown. Pollen contains a diverse profile of sterols, phytosterols, distinct
2415 from other floral tissues which vary in their carbon chain length, double bond positions and
2416 attached methyl group (Ischebeck, 2016; Zu *et al.*, 2021; Furse, Martel, *et al.*, 2023). Sterol
2417 structure impacts suitability for incorporation into physiological functions, it is therefore

2418 reasonable to expect that pollen of particular plant species is considered more or less desirable
2419 based on the needs of the foraging insect (Herbert *et al.*, 1980). Honey bees are unable to convert
2420 dietary sterols into cholesterol and instead incorporate a subset of specific pollen sterols into their
2421 tissues (Herbert *et al.*, 1980). The same state is inferred to be present in solitary bees (Feldlaufer *et*
2422 *al.*, 1993; Jing and Behmer, 2020; Vanderplanck, Zerck, *et al.*, 2020a).

2423 Solitary bees have received considerably less research attention than the model eusocial species
2424 *Apis mellifera* and *Bombus terrestris*. As an ecologically and taxonomically diverse group,
2425 individual studies may not be applicable to solitary bees in other families. Research on the sterol
2426 requirements of solitary bees has shown that they contain a range of phytosterols and in varying
2427 proportions, distinct from honey bees and bumble bees. Examples include an almost complete
2428 dominance of 24-methylenecholesterol in *Diadasia rinconis*, isofucoesterol proportions three times
2429 higher in *Megachile rotundata* than honey bees and β -sitosterol accounting for over 50% of total
2430 sterolic content in *Colletes cunicularius* (Svoboda and Lusby, 1986; Feldlaufer *et al.*, 1993;
2431 Vanderplanck, Zerck, *et al.*, 2020a). It is also likely that solitary bee species produce different
2432 moulting hormones, for example 20-hydroxyecdysone from cholesterol (Feldlaufer *et al.*, 1993) or
2433 makisterone A from campesterol (Feldlaufer *et al.*, 1986; Vanderplanck, Zerck, *et al.*, 2020a).

2434 This study sought to analyse the sterol profiles of a diverse set of UK solitary bees alongside a
2435 subset of bumble bee species (*Bombus* spp.) to address the following hypotheses: 1) Solitary bee
2436 species comprise significantly different sterol profiles, 2) Closely related solitary bees with
2437 different foraging preference (specialist versus generalist) have more divergent sterol profiles than
2438 expected based on their evolutionary relatedness, 3) Kleptoparasitic bees have sterol profiles more
2439 similar to their host than expected from their evolutionary relatedness, 4) Bees specialising on
2440 Asteraceae have a sterol profile similar to Asteraceae pollen that differs from non-Asteraceae
2441 specialists and generalist bees, and 5) Monolectic bee species have sterol profiles similar to their
2442 host pollen.

2443 **Methods**

2444 **Sample collection and preparation**

2445 Species were chosen so as to cover a wide phylogenetic range and encompassed all foraging
2446 strategies (monolectic, oligolectic, polylectic) as well as kleptoparasitic species. Close relatives
2447 that had different foraging strategies were prioritised to understand the effects of phylogeny and
2448 ecology. Seven wild bumble bee species (*Bombus*), including the cuckoo bumble bee *Bombus*
2449 *vestalis*, were included in the dataset for comparison with the solitary species. Two wasp species,
2450 *Philanthus triangulum* and *Mellinus arvensis*, were also collected but later removed from data
2451 analysis. A replication of three samples for each species was targeted initially but this was not
2452 possible for some species. Very common species were collected from different sites to best capture
2453 intra-species variation. In total, 56 bee species were collected across 2021 to 2023, with ten of
2454 these represented by a single sample. All samples were female except for *Melecta albifrons* which
2455 was represented by two male specimens. Collection was primarily focused in the South and
2456 Southeast of England, from Royal Botanic Gardens (RBG), Kew and Wakehurst by HK. Other
2457 species procured from specific sites were done so with the permission of landowners. Available
2458 details for sample collection are summarised in supplementary Table S4.1. Bees were collected
2459 with a net then euthanised and stored at -20°C. Pollen was brushed from the outside of the body
2460 and removed from any pollen-collecting structures such as corbiculae or scopa. Guts were retained
2461 within specimens as removal would have likely damaged other internal tissues for the smallest
2462 specimens and so processing was kept consistent.

2463 Sample processing was carried out by KB at RBG Kew. Specimens were covered in deionised
2464 water, frozen at -80°C and freeze dried until all water had been removed. Samples were then stored
2465 at -80°C before being homogenised. Specimens were homogenised in GCTU using a BioSpec
2466 Tissue Tearor with a 14mm head attachment. GCTU was made from Guanidine (6 M guanidinium

2467 chloride) and thiourea (1.5 M) dissolved in deionised H₂O together and stored at room temperature
2468 out of direct sunlight.

2469 **Sample run**

2470 Samples were analysed in collaboration with staff at RBG Kew. Sample run was prepared by EB,
2471 KB and SF. For each sample, 60µl of homogenate was transferred to a 96-well plate for sterol
2472 extraction.

2473 Samples were run across two 384-well plates comprising eight individual 96-well plates. Sample
2474 order was randomised within each 96-well plate. Each plate also contained intermittent samples
2475 that monitored instrument functioning:

2476 All runs began with a blank (60µl GCTU) to ensure the instrument was not detecting peaks where
2477 none should be present. A stock solution of reference materials (QA) verified by multi-nuclear
2478 NMR were used to calculate coefficient of variance. Reference materials were available for: 24-
2479 methylenecholesterol, 24-methylenecycloartenol, anthelsterol (a sterol found in *C. anthelmintica*,
2480 ST(28:3), that is active under 330 nm UV but whose structure has not been determined using
2481 NMR), avenasterol, β-sitosterol, brassicasterol, campesterol, cholesterol, cycloartenol,
2482 cycloeucalenol, cyclolaudenol, desmosterol, episterol, ergosterol, isofucosterol, schottenol,
2483 sitostanol, spinasterol and stigmasterol. The *m/z* and retention time for each sterol is shown in
2484 Table S4.2. Aliquots of 40 µl were used in the run. Finally, QC solutions of homogenised
2485 commercially available honeybee collected pollen (*Helianthus annuus*, *Nymphaea* sp., *Fagopyrum*
2486 *esculentum*) and bumble bee adults, larvae and pupae in GCTU (6M guanidine + 1.5M thiourea to
2487 make 1L dissolved in deionised water) were run at a series of concentrations to test the range of
2488 linearity of the instrument. Three concentrations of QC were used: 100%, 50%, 25%,
2489 corresponding to 40 µl, 20 µl and 10 µl aliquots of the QC stock solution. These were analysed in

2490 the same way as the samples to calculate the correlation between analyte concentration and signal
2491 size. Variables whose ratio was <0.75 were discarded.

2492 All samples were then extracted by SF as 96-well plates (96-well plate, Esslab Plate+™,
2493 2.4 ml/well, glass-coated) using a 96-channel pipette (liquid-handling robot, Integra Viaflow
2494 96/384 channel pipette) as follows. First, 150 µl of internal standard (*d*₇-cholesterol) was added to
2495 all samples. The concentration of *d*₇-cholesterol was 5 mg/l (1.99098e⁻⁹ Moles/sample). Then 500
2496 µl DMT (250 µl ×2) was added to all samples. DMT was made from dichloromethane (DCM) (3
2497 parts), methanol (1 part) and triethylammonium chloride (0.0005 parts, i.e. 500 mg/l), mixed and
2498 stored at room temperature out of direct sunlight, followed by 500 µl deionised water before
2499 agitating using the multi-channel pipette. Layers were separated by spinning for 2 min (methanol +
2500 water, Solid, DMT + sterol) and 50 µl of extract (DMT & sterol) was transferred to a 384-well
2501 plate and left for 30 min for the DMT to evaporate. Once the 384-well plate was full, 150 µl of
2502 LCMS quality methanol was added and the plate sealed with foil.

2503 **Sample analysis and signal processing**

2504 The sample run was completed under the supervision of SF following the protocol established in
2505 Furse, Martel, *et al.* (2023). Instrument output was also processed by SF as established in Furse,
2506 Martel, *et al.* (2023). Relative abundance was calculated by dividing the signal area for each
2507 metabolite by the signal of internal standard (*d*₇-cholesterol).

2508 These values were used for the majority of data analysis. For total sterol analysis, instrument
2509 output (*m/z*) was converted to mg/g first by dividing the signal area for each metabolite by the
2510 signal of internal standard (*d*₇-cholesterol) then using the following formula:

$$2511 \quad \frac{\text{Sterol (mg)}}{\text{Tissue (g)}} = \frac{\text{sterol molecular mass} \left(\frac{\text{g}}{\text{mol}} \right) \times (\text{signal} \times \text{mol of } d7cho .) \times 1,000}{\text{Starting weight (g)}}$$

2512 mol of *d*₇-cholesterol = 1.99098e⁻⁹ Moles/sample.

2513 **Phylogeny and phylogenetic analysis**

2514 The supermatrix phylogeny produced by Henríquez-Piskulich, Hugall and Stuart-Fox (2024) was
2515 used to assess phylogenetic signal in the sterol dataset for the bee species tested here. Two
2516 outgroup wasp species in our dataset, *Philanthus triangulum* and *Mellinus arvensis*, were only
2517 represented by genus composites (*Philanthus*) or not at all (*Mellinus arvensis*) in the bootstrap
2518 phylogeny. Therefore, the 4,586 bee species dated chronogram with outgroups removed was
2519 selected for analysis and the two wasp species dropped from data analysis. The tree was
2520 downloaded from Dryad and imported into R using `read.tree()` from APE (Paradis and Schliep,
2521 2019). The tree was pruned to 56 species using `drop.tip()` from APE (Paradis and Schliep, 2019). A
2522 composite phylogeny showing phylogeny, foraging strategy, sterol proportions and total sterols
2523 (mg/g) was plotted using `ggtree()` (Yu *et al.*, 2017, 2018; Yu, 2020), `ggplot()` (Wickham, 2016) and
2524 the APLOT package (Yu, 2023) (Figure 4.1).

2525 Nodes for family groupings were identified with `getMRCA()` from the APE package (Paradis and
2526 Schliep, 2019). Our dataset contained six out of the seven currently recognised families within the
2527 clade of Anthophila (bees): Andrenidae, Apidae, Colletidae, Halictidae, Megachilidae and
2528 Melittidae. Supplementary taxonomic data from Henríquez-Piskulich, Hugall and Stuart-Fox
2529 (2024) for all 56 species is available in the supplementary resources (Table S4.3).

2530 Phylogenetic signal was calculated following Zu *et al.* (2021). Phylo4d objects were created using
2531 `phylo4d()` from PHYLOBASE (R Hackathon *et al.*, 2020). `Phylosignal(reps=999)` from
2532 PHYLOSIGNAL (Keck *et al.*, 2016) was used to calculate Pagel's λ and Blomberg's K. These
2533 measures of phylogenetic signal were calculated for the individual proportions of all 19 sterols,
2534 total sterol (mg/g) and proportion of sterols belonging to different carbon chain lengths (27, 28, 29,
2535 30, 31) and B-ring double bond position categories (0, 5, 7, NA). Ergosterol was classed as NA for

2536 double bond position as it has double bonds at both 5 and 7. All sterols with a cyclopropane ring
2537 were classed as Δ^0 alongside stanols which have a saturated B-ring. All unidentified sterols were
2538 classed as NA.

2539 **Data analysis**

2540 All data analysis was carried out in R version 4.2.3 (2023-03-15 ucrt) -- "Shortstop Beagle" in
2541 RStudio 2023.06.1+524 "Mountain Hydrangea" Release. All data manipulation was done using
2542 functions from TIDYVERSE (Wickham *et al.*, 2019).

2543 For each species with at least three replicates, a Bray-Curtis dissimilarity matrix was used to
2544 identify outliers using `disana()` from the LABDSV package (Roberts, 2023). Any data point with a
2545 minimum dissimilarity over 0.5 compared to all other points for that species was identified as an
2546 outlier, as per author guidance, and so was removed. This led to the removal of five samples in
2547 total: One *Andrena flavipes*, two *Andrena florea*, one *Anthophora plumipes* and one *Colletes*
2548 *hederae*. Seven specimens (*Andrena bicolor*, *Andrena florea*, *Anthophora furcata*, *Nomada fucata*,
2549 *Osmia leaiana*, *Mellinus arvensis*, *Epeolus cruciger*) were removed from total sterol analysis as
2550 their post-freeze drying weight was zero or negative; meaning their signals data could not be
2551 converted into mg/g units. This was likely due to incorrectly calibrated weighing equipment.

2552 Summary statistics were calculated from species means using the STATS package from R (R Core
2553 Team, 2023). Proportion data (0-1) was used for data analysis, where zero denotes a sterol is
2554 absent from a sample, and one denotes it is completely dominant. All NMDS analyses were carried
2555 out as follows: data were arcsine square root transformed and the result was used to calculate a
2556 Bray-Curtis dissimilarity matrix using `vegdist(method="bray")`. NMDS was plotted using
2557 `metaMDS(trymax=200, k=2, autotransform=FALSE)`. The lowest number of axes was selected
2558 where stress was ≤ 0.2 (established cut off, (Bakker, 2024)). Stress values reflect how well the
2559 ordination represents the original data. PERMANOVA was used to assess statistical difference

2560 between groups using `adonis2()`. `Anova()` and `betadisper()` were used to test for significant
2561 differences in variance between groups which may invalidate comparisons. Following Bakker
2562 (2024), pairwise comparisons were made using `adonis2()` and a Benjamini-Hochberg correction.
2563 All functions were from VEGAN (Oksanen *et al.*, 2022) except `anova()` from the STATS package
2564 (R Core Team, 2023). Where NMDS stress value was <0.2 , a 2D biplot was created from the
2565 NMDS with strong (>0.7 absolute value) and significant ($p < 0.010$) sterol associations plotted over
2566 the datapoints using `ggplot()` and `ggrepel()` from GGLOT2 (Wickham, 2016) and GGREPEL
2567 (Slowikowski, 2023). Indicator Species Analysis (ISA) was carried out for all NMDS plotted data
2568 using `multipatt()` from the INDICSPECIES package (De Cáceres and Legendre, 2009) to determine
2569 significant associations between groups and individual sterols. ISA was done at the level of
2570 individual groups only and not for combinations of groups.

2571 ***Differences in sterol profile across bee species***

2572 Species which were represented by at least 10 samples in the dataset were used for an NMDS
2573 comparison of inter-species differences. This included nine species from seven genera. An NMDS
2574 was calculated from proportion data using all available samples for these species.

2575 *Colletes hederæ* and *C. succinctus* both had at least 10 replicates and have different foraging
2576 preferences. They were therefore used in an NMDS to compare intra-species variability between
2577 two closely related species.

2578 ***Similarities in sterol profile between kleptoparasitic bees and their host(s)***

2579 For all kleptoparasitic bees, hosts were identified using Falk and Lewington (2015). All species
2580 except *Coelioxys inermis* had at least one host in the dataset. Sterol proportions for each host
2581 species were plotted alongside their kleptoparasite for comparison. To determine whether the sterol

2582 profile of kleptoparasitic species grouped more closely with their host than their co-genera species,
2583 *Epeolus* and *Nomada* species were used for separate NMDS analyses with their host species.

2584 ***Differences in sterol profile between foraging type groups (specialist versus generalist)***

2585 The sterol profiles of all oligolectic and polylectic species in the dataset were compared by NMDS.
2586 Mean proportions were used for each species (18 oligolectic, 26 polylectic). *Andrena* was the most
2587 speciose genus in the dataset and contained a similar number of oligolectic (5) and polylectic (7)
2588 species which do not form monophyletic groups. *Andrena* species were therefore used in a NMDS
2589 to calculate if the two lecty groups had significantly different sterol profiles. The monolectic
2590 species *Andrena florea* was removed from this analysis.

2591 ***Asteraceae specialist bees***

2592 An NMDS was calculated to compare the sterol profiles of bees specialising on Asteraceae, non-
2593 Asteraceae specialists, polylectic bees and pollen from three subfamilies of Asteraceae
2594 (Carduoideae, Cichorioideae, Asteroideae). When comparing pollen and bee sterol profiles, only
2595 sterols that had been detected in both datasets were used and proportions re-calculated using the
2596 remaining sterols. This led to a total of 13 sterols being used (24-methylenecholesterol,
2597 avenasterol, β -sitosterol, campesterol, cholesterol, cycloartanol, cycloartenol, cyclolaudenol,
2598 desmosterol, ergosterol, isofucosterol, salisterol and sitostanol). Aligning data in this way will have
2599 altered the proportions of the remaining sterols in both datasets. However, to retain them would
2600 have made it impossible to fairly compare in an analysis.

2601 Sterol data were then also grouped by B-ring double bond position (absent/ Δ 0, Δ 5, Δ 7, Δ 8, NA)
2602 with ergosterol and all unidentified sterols classed as NA (ergosterol is a Δ 5 and Δ 7 sterol). All
2603 sterols with a cyclopropane ring were also classed as absent/ Δ 0 for B-ring double bond. In order to
2604 compare the proportions of different B-ring double bond groups between Asteraceae and non-

2605 Asteraceae specialist bees, a Kruskal-Wallis rank sum test was carried out using `kruskal.test()`
2606 followed by post-hoc Dunn tests using `dunnTest()`, from the STATS package (R Core Team, 2023)
2607 and FSA package (Ogle *et al.*, 2023) respectively. Both analyses used a Benjamini-Hochberg
2608 correction for multiple testing.

2609 ***Sterol profiles of monolectic and specialist bee species compared with their pollens***

2610 The dataset contained four monolectic bee species and one species which is strongly specialised in
2611 the UK (*Colletes hederæ*). These bee species were plotted in an NMDS alongside samples of their
2612 pollens from Chapter 2 to compare sterol profiles between bee-pollen groups. The variance in
2613 sterol profile between samples of *Andrena florea* was considerably greater than in all other groups
2614 and so this species was removed from the analysis to enable more robust comparison among the
2615 other groups.

2616 **Results**

2617 A total of 19 sterols were detected across the 56 bee species analysed, covering 21 genera in 7
2618 families. The dataset included seven eusocial *Bombus* species and 49 solitary bee species. Two
2619 wasp species were also analysed but excluded from later analysis as they are ecologically and
2620 phylogenetically distinct from the other species in the dataset.

2621 Table 4.1 shows that the sterols with the highest median value across all species were
2622 isofucosterol, β -sitosterol and 24-methylenecholesterol. These sterols had a median percentage of
2623 >10% and were also the only sterols present in all species. However, the high inter-quartile range
2624 shows they are by no means abundant in all species. β -Sitosterol has the highest minimum value
2625 and only it and 24-methylenecholesterol ever accounted for >60% of a species' sterol profile.
2626 Thirteen sterols detected did not have a median >1%. Eleven of the 19 sterols were found in at
2627 least 50/56 species. Ergosterol was only detected in 39 species and salisterol in 26, making them
2628 the rarest sterols in the dataset (Table 4.2). Of the 56 bee species analysed, 17 contained all 19
2629 sterols. A further 27 contained at least 16. Only three species contained <10 sterols, *Halictus*
2630 *rubicundus* (9), *Ceratina cyanea* (8), *Nomada flavopicta* (8). Five species contained no cholesterol:
2631 *Andrena wilkella*, *Nomada flavopicta*, *Halictus rubicundus*, *Lasioglossum smeathmanellum* and
2632 *Coelioxys inermis*. Furthermore, *Halictus rubicundus* and *Ceratina cyanea* contained no
2633 campesterol.

2634 **Phylogenetic signal**

2635 Only three sterols showed significant ($p<0.050$) phylogenetic signal for both metrics: desmosterol
2636 ($K=0.327$, $p<0.050$, $\lambda=0.790$, $p<0.005$), campesterol ($K=0.525$, $p=0.001$, $\lambda=1.012$, $p=0.001$) and
2637 ST(28:1)B, an unidentified sterol ($K=0.479$, $p<0.050$, $\lambda=0.758$, $p=0.001$). The mean proportions of
2638 these sterols for each species are plotted in Figure 4.2. Only five other sterols showed significance

2639 (i.e. $p < 0.05$) for at least one metric; ST(28:1)A, 24-methylenecholesterol, sitostanol, salisterol and
 2640 the 28 carbon chain sterols group. For all except sitostanol and salisterol, this significance was in
 2641 Blomberg's K.

2642 As shown in Figure 4.1, ST(28:1)B is higher in *Anthophora* species compared to the rest of the
 2643 dataset. Campesterol percentage is higher in Megachillidae (mean=17.32%) and lower in
 2644 Halictidae (mean=5.15%) and Andrenidae (mean=4.45%). The percentage of desmosterol is
 2645 consistently low (<8%) and more variable across the entire dataset but is lowest within the
 2646 Melittidae (mean=0.26%) and Halictidae (mean=0.32%).

2647 **Table 4.1.** Median, interquartile range, minimum and maximum percentages for all 19 sterols
 2648 detected, including six unidentified sterols. Isofucosterol, β -sitosterol and 24-methylenecholesterol
 2649 show the highest median values and are the only sterols common to all species. Data was
 2650 calculated from mean species proportions.

Sterol	Median	IQR	Max	Min
Isofucosterol	21.797	13.293	38.151	0.164
β -Sitosterol	20.581	19.168	74.291	2.307
24MC	12.633	36.718	88.009	0.378
ST(29:2)A	7.928	8.575	23.566	0.000
Campesterol	4.802	5.029	31.174	0.000
ST(30:1)A	1.255	0.876	4.295	0.000
ST(28:1)B	0.639	1.456	43.893	0.000
ST(29:1)A	0.541	0.756	4.502	0.000
Avenasterol	0.382	0.977	35.653	0.000
Desmosterol	0.382	0.751	6.201	0.000
Cholesterol	0.312	1.717	20.012	0.000
Cycloartenol	0.252	0.513	4.221	0.000
Cycloartanol	0.218	3.193	38.288	0.000
ST(28:1)A	0.212	0.431	2.773	0.000
Sitostanol	0.175	0.208	3.845	0.000
ST(30:1)B	0.165	0.511	51.694	0.000
Cyclolaudenol	0.145	0.294	6.344	0.000
Ergosterol	0.027	0.072	12.697	0.000
Salisterol	0.000	0.022	1.719	0.000

2651

2652 **Table 4.2.** Number of species each sterol was detected in arranged from most to least common.
 2653 Only three sterols occurred in all species, highlighted in grey.

Sterol	Number of species	Sterol	Number of species
24MC	56	ST(29:1)A	50
Isofucosterol	56	Avenasterol	49
β -Sitosterol	56	Cyclolaudenol	49
ST(30:1)A	55	ST(30:1)B	49
Campesterol	54	Cycloartenol	46
ST(29:2)A	54	ST(28:1)A	46
Desmosterol	53	Cycloartanol	43
ST(28:1)B	53	Ergosterol	39
Cholesterol	51	Salisterol	26
Sitostanol	51		

2654

2655 **Differences in sterol profile across bee species**

2656 As seen in Figure 4.1, most species have relatively high percentages of 24-methylenecholesterol, β -
 2657 sitosterol and isofucosterol. These proportions sometimes vary in closely related species, for
 2658 instance 24-methylenecholesterol levels in *Halictus* versus some *Lasioglossum* spp..

2659 Total sterol content varied both within and between species (Figure 4.1). For example,
 2660 *Lasioglossum villosulum* showed the widest range with a maximum of 1143.065 mg/g and a
 2661 minimum of 86.958 mg/g. The species with the highest mean values (*Nomada rufipes* = 550.599
 2662 mg/g and *Lasioglossum villosulum* = 489.054 mg/g) were both driven by high total sterol outliers.
 2663 For *Lasioglossum villosulum*, the two highest total sterol samples also had ST(28:1)B levels and
 2664 were collected from a different site than the other four samples. *Epeolus cruciger* appeared to have
 2665 a less variable but high total sterol content (293.196 mg/g) and was also better sampled.

2666 The sterol profiles of the nine most sampled species were compared by PERMANOVA and plotted
 2667 in an NMDS (Figure 4.3, stress value = 0.151). This showed that species do have distinct sterol
 2668 profiles, but with considerable overlap between some species. For instance, *Epeolus cruciger* and

2669 *Colletes succinctus* had low variation and are extremely similar due to their parasite-host
2670 relationship. Similarly, *Andrena cineraria* and *Nomada fucata* had much more variable sterol
2671 profiles than the other species plotted but showed similar sterol profiles. The species groups
2672 showed significantly different sterol profiles ($F_{8,118}=25.306$, $p=0.001$) with species identity
2673 explaining over 50% of the variation in the data ($R^2=0.632$). However, species groups also
2674 displayed significantly different variances ($F_{8,118}=6.825$, $p<0.001$). Pairwise comparisons showed
2675 the only species which were not significantly different from each other ($p<0.050$) were *Andrena*
2676 *cineraria* and *Nomada fucata* ($p>0.100$). ISA showed all species except *Epeolus cruciger* were
2677 significantly associated with at least one sterol. This may be due to the strong similarity in sterol
2678 profile between *Epeolus cruciger* and *Colletes hederæ* which was associated with three sterols
2679 (Figure 4.3). *Dasygaster hirtipes* showed the highest number of associations, five, including strong
2680 associations with cycloartanol and cholesterol (Table 4.3).

2681 Comparing the sterol profiles of the ivy-foraging *Colletes hederæ* and heather-foraging
2682 *C. succinctus* showed consistency in sterol profile between replicates and distinct sterol profiles
2683 despite their relatedness (Figure 4.4, stress= 0.105). The species showed significantly different
2684 sterol profiles by PERMANOVA ($F_{1,31}=37.418$, $p=0.001$), with species identity accounting for
2685 over 50% of the variation in the data ($R^2=0.547$). However, dispersion was also significantly
2686 different between species ($F_{1,31}=27.761$, $p<0.001$). ISA showed *Colletes hederæ* was significantly
2687 associated with six sterols, including cycloartenol (IV=0.946, $p=0.005$), avenasterol (IV= 0.856,
2688 $p=0.005$) and cholesterol (IV= 0.769, $p<0.050$). *Colletes succinctus* was strongly associated with
2689 β -sitosterol (IV= 0.778, $p=0.005$), isofucosterol (IV= 0.757, $p=0.005$) and two un-identified
2690 sterols (Table 4.4).

2691 **Similarities in sterol profile between kleptoparasitic bees and their host(s)**

2692 Kleptoparasitic species rely on the pollen foraging of their host to feed their young. As a result it
2693 may be expected that the sterol profiles of kleptoparasites resemble their hosts. Figure 4.5 shows
2694 the sterol profile of kleptoparasitic bee species plotted alongside their host(s) where available.
2695 Three of the parasites, *Bombus vestalis*, *Sphcodes monilicornis* and *Melecta albifrons* were in the
2696 same family as their host. However, *Epeolus cruciger*, *E. variegatus*, *Nomada flavopicta*, *N. fucata*
2697 and *N. rufipes* were all in separate families from their hosts (*Colletes* spp., *Melitta leporina* and
2698 *Andrena* spp.).

2699 Figure 4.6A shows an NMDS (stress=0.142) containing *Epeolus cruciger* and *E. variegatus* with
2700 hosts *Colletes succinctus*, *C. halophilus* and *C. daviesanus*. The two kleptoparasite-host groups
2701 showed a significant difference in their sterol profiles ($F_{1,42}=19.217$, $p=0.001$); however the
2702 variance was significantly different between groups ($F_{1,42}=50.355$, $p<0.001$) and kleptoparasite-
2703 host group did not explain a high proportion of the variation in the data ($R^2=0.314$). ISA showed
2704 the *E. variegatus* group to be significantly associated with five sterols, most strongly cycloartanol
2705 and cholesterol. The *E. cruciger* group was only significantly associated with β -sitosterol (Table
2706 4.5). Figure 4.6B shows an NMDS (stress=0.128) of *Nomada flavopicta*, *N. fucata* and *N. rufipes*
2707 with hosts *Melitta leporina*, *Andrena flavipes*, *A. fuscipes* and *A. denticulata*. These groups did not
2708 have significantly different variances ($F_{2,53}=2.263$, $p>0.100$) and host-kleptoparasite groups were
2709 significantly different by PERMANOVA ($F_{2,53}=8.570$, $p=0.001$), including all pairwise
2710 comparisons between groups ($p<0.0100$). However, host-kleptoparasite group explained only a
2711 low proportion of the variation in the data ($R^2=0.244$). ISA showed all three *Nomada*
2712 kleptoparasite-host groups were associated with at least two sterols. The *N. rufipes* group was most
2713 strongly associated with avenasterol, *N. flavopicta* with ST(30:1)B and *N. fucata* with ST(28:1)B
2714 (Table 4.6).

2715 **Differences in sterol profile between foraging type groups (specialist versus**
2716 **generalist)**

2717 Due to their differences in pollen foraging breadth, sterol profile was predicted to differ between
2718 generalist (polylectic) and specialist (oligolectic) bee species. Analysis by PERMANOVA showed
2719 there was a significant difference between lecty groups ($F_{1,41}=5.113, p<0.005$) (Figure 4.7, stress =
2720 0.135) however lecty only explained a small proportion of the variation in the dataset ($R^2=0.111$).
2721 Variances were not significantly different between lecty groups ($F_{1,41}=0.003, p>0.500$). ISA
2722 showed oligolectic species were strongly associated with cycloartanol (IV= 0.839, $p<0.050$),
2723 cholesterol (IV=0.833, $p<0.050$) and cyclolaudenol (IV=0.786, $p<0.05$) whereas polylectic species
2724 were strongly associated with 24-methylenecholesterol (IV=0.819, $p=0.005$) and two un-named
2725 sterols (Table 4.7).

2726 Another analysis focused on the genus *Andrena* was used to compare closely related species which
2727 differed in pollen foraging (Figure 4.8, stress value was 0.059). There was again a significant
2728 difference between lecty groups ($F_{1,10}=3.744, p<0.050$) and no significant difference in dispersion
2729 ($F_{1,10}=0.039, p>0.500$) but with lecty only explaining a small proportion of the variation in the data
2730 ($R^2=0.272$). Oligolectic species were correlated with higher cholesterol and cycloartenol, whereas
2731 polylectic species had higher levels of 24-methylenecholesterol and ST(28:1)A (Figure 4.8). These
2732 data are supported by findings in the wider dataset (Figure 4.7). Furthermore, an ISA showed that
2733 polylectic *Andrena* spp. were strongly and significantly associated with 24-methylenecholesterol
2734 (IV= 0.836, $p<0.050$) and the unidentified sterol ST(28:1)B (IV=0.884, $p<0.050$) whereas
2735 oligolectic species were associated with cholesterol (IV=0.931, $p<0.050$) and unidentified sterol
2736 ST(30:1)A (IV=0.747, $p=0.050$).

2737 **Asteraceae specialist bees**

2738 Eight solitary bee species in the dataset specialise on collecting pollen from Asteraceae flowers,
2739 which commonly have lower proportions of $\Delta 5$ sterols, as outlined in Chapter 2. A PERMANOVA
2740 comparing the sterol profiles of Asteraceae subfamily pollens to bees which do/do not forage on
2741 them showed a significant difference between groups (Figure 4.9A, stress=0.151; $F_{5,96}=16.041$,
2742 $p=0.001$). Group identity explained nearly 50% of the variation in the dataset ($R^2=0.455$) and all
2743 pairwise comparisons between groups were significant ($p<0.050$) except Asteroideae versus
2744 Carduoideae pollen ($p>0.100$). Variances were significantly different between groups ($F_{5,96}=2.362$,
2745 $p<0.050$). ISA showed generalist bee species were significantly associated with 24-
2746 methylenecholesterol, as per other polylectic species (Table 4.7, Figure 4.8). Non-Asteraceae
2747 specialist bees were significantly associated with β -sitosterol and Asteraceae specialists with
2748 cholesterol. The sterol profile of the Asteraceae specialist bees showed the most similarity to
2749 Cichorioideae pollen out of the three Asteraceae subfamilies. Different Asteraceae subfamily
2750 pollens were associated with at least one $\Delta 0$ sterol containing a cyclopropane ring each:
2751 Asteroideae with cycloartenol (IV=0.564, $p=0.005$), Carduoideae with cyclolaudenol (IV=0.568,
2752 $p=0.005$) and Cichorioideae with cycloartanol (IV=0.576, $p=0.010$). Carduoideae was associated
2753 with the most individual sterols (Table 4.8).

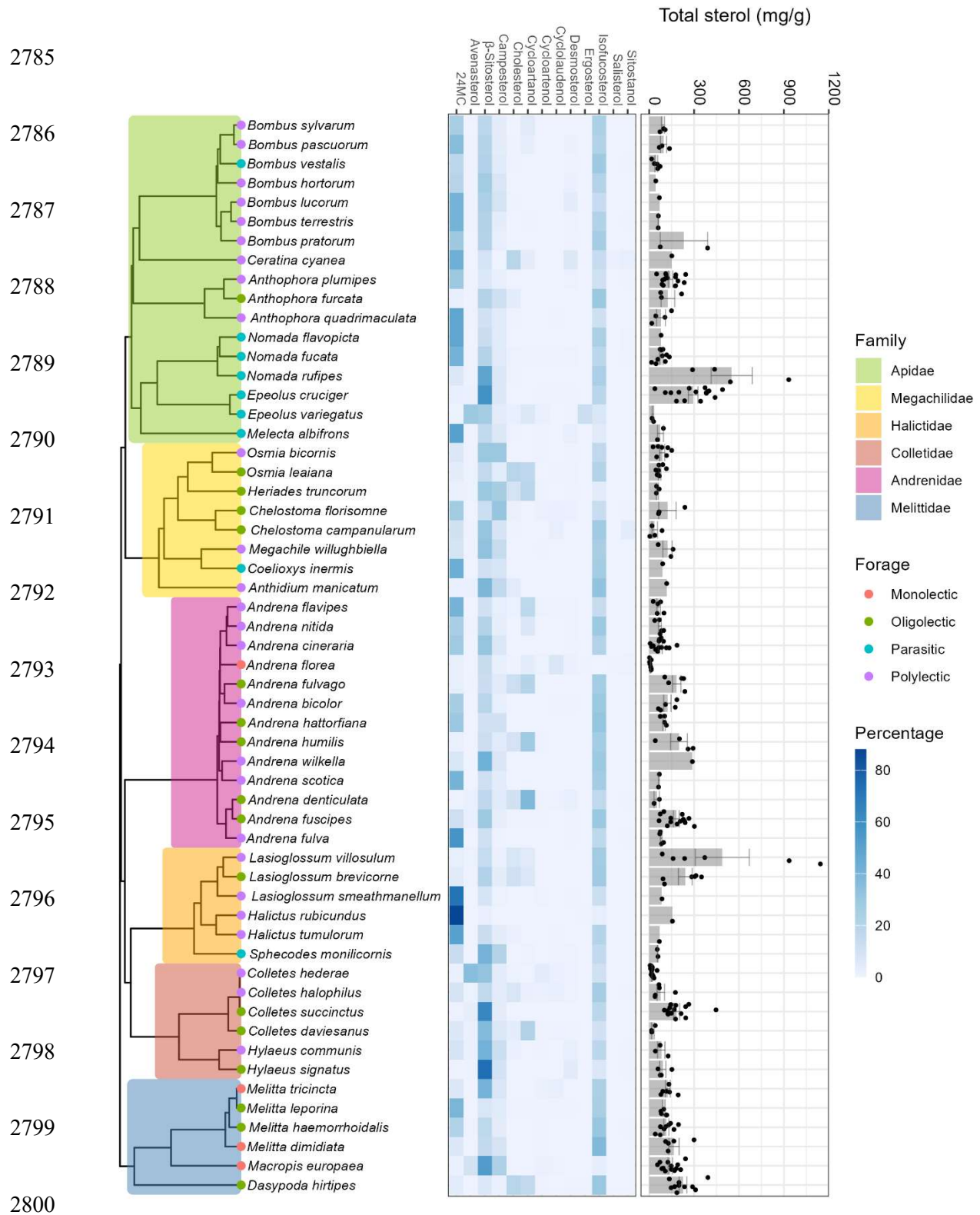
2754 To determine if Asteraceae specialist bees showed lower proportions of $\Delta 5$ sterols, as Asteraceae
2755 pollen does, sterol profile was summarised by the B-ring double bond position for all bee species.
2756 In this analysis, Asteraceae specialists showed significantly lower $\Delta 5$ and higher $\Delta 0$ sterol
2757 proportions than non-Asteraceae specialist, generalist and parasitic bee species ($\Delta 0$: $H(3)=18.695$,
2758 $p<0.005$, $\Delta 5$: $H(3)=12.743$, $p<0.050$) (Figure 4.9B). All post-hoc Dunn tests between Asteraceae
2759 specialists and the other groups were significant for $\Delta 0$ ($p<0.005$) and $\Delta 5$ ($p<0.050$). No other
2760 comparisons were significantly different.

2761 **Sterol profiles of monolectic and specialist bee species and their pollens**

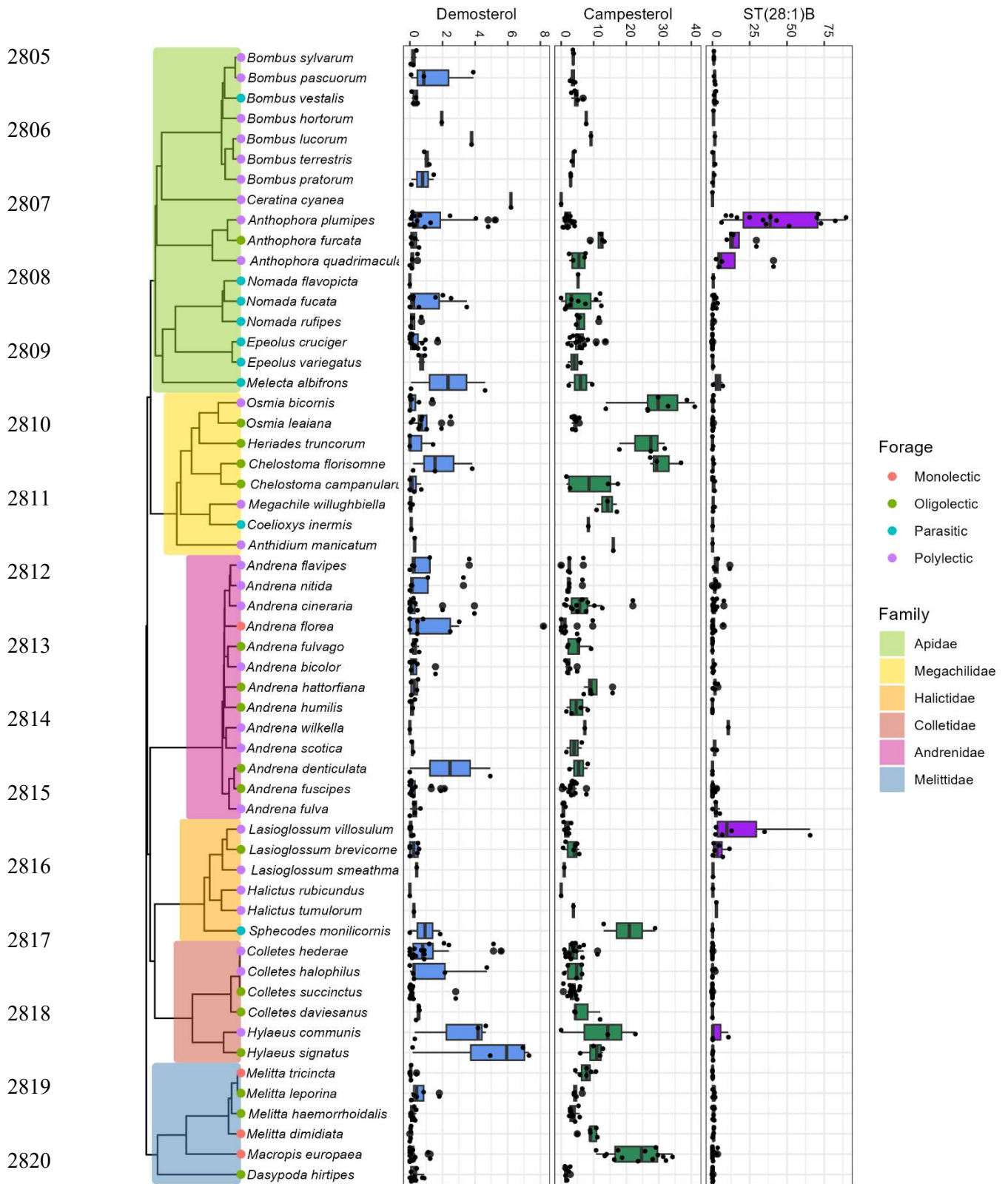
2762 Monolectic and other specialist bee species are limited in the pollen sterols available to them
2763 because of their restricted foraging. The sterol profiles of these bees and their pollen were therefore
2764 compared to determine if bee species were more similar to their preferred pollen than other bees or
2765 pollen. The results of the PERMANOVA show significant differences in sterol profile between
2766 bee-pollen groups ($F_{3,42}=24.123$, $R^2=0.379$, $p=0.001$) and between bees and pollen ($F_{1,42}=52.986$,
2767 $R^2=0.277$, $p=0.001$). The interaction between these effects was also significant ($F_{3,42}=7.893$,
2768 $R^2=0.124$, $p=0.001$) meaning the effect of sample type, bee or pollen, varied between groups
2769 (Figure 4.10, stress value of 0.119).

2770 Sterol correlations showed higher cycloartenol in all pollen samples and higher campesterol in all
2771 bee samples. ISA showed all bee-pollen groups were significantly associated with at least one
2772 sterol each: The sainfoin group (*Melitta dimidiata* and *Onobrychis viciifolia*) was most strongly
2773 associated with salisterol (IV=0.777, $p=0.005$) and cycloartenol (IV=0.652, $p=0.005$), the ivy
2774 group (*Colletes hederæ* and *Hedera helix*) with desmosterol (IV=0.656, $p<0.050$) and avenasterol
2775 (IV=0.651, $p=0.005$), the yellow loosestrife group (*Macropis europæa* and *Lysimachia vulgaris*)
2776 with campesterol (IV=0.631, $p=0.005$) and β -sitosterol (IV=0.570, $p=0.005$) and the red bartsia
2777 group (*Melitta tricincta* and *Odontites vernus*) with 24-methylenecholesterol (IV=0.602,
2778 $p<0.050$)(Table 4.9).

2779 Some of the specialist bee species contained sterols which were not detected in their pollen.
2780 *Andrena florea* and *Melitta dimidiata* both contained ergosterol while their pollens did not (0.706%
2781 and 0.012% of total sterols respectively). *Macropis europæa* and *Colletes hederæ* both contained
2782 cholesterol, which did not occur in their pollen (0.265% and 0.926% respectively). Desmosterol
2783 was absent from *Hedera helix* but present in *Colletes hederæ* (1.299%). Only *Melitta tricincta*
2784 contained sterols that were totally represented in its pollen, *Odontites vernus*.



2801 **Figure 4.1.** Phylogeny of 56 bee species subset from the supermatrix phylogeny produced by
 2802 *Henríquez-Piskulich, Hugall and Stuart-Fox (2024)*, showing family clades and foraging status.
 2803 Species mean sterol percentages for all identified sterols are plotted along with total sterols (mg/g)
 2804 for all samples. Species means are shown by grey bars, error bars show \pm standard error.



2821 **Figure 4.2.** Phylogeny as shown in Figure 4.1, based on *Henríquez-Piskulich, Hugall and Stuart-*
 2822 *Fox (2024)* chronogram. Showing percentage of the three sterols found to have phylogenetic signal
 2823 across the dataset for both *Blomberg's K* and *Pagel's λ* . Boxplots show median, 25th and 75th
 2824 percentiles and $\pm 1.5x$ interquartile ranges.

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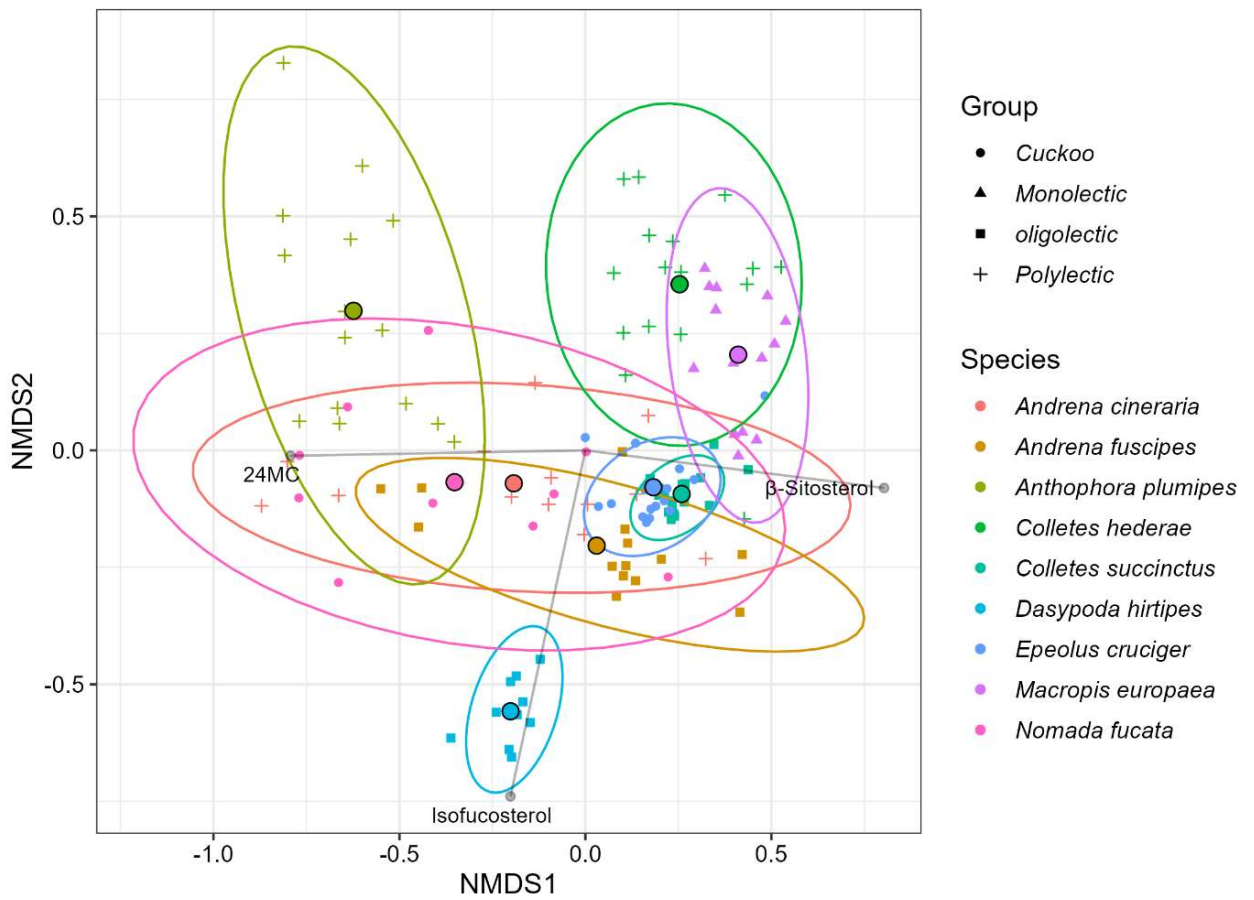
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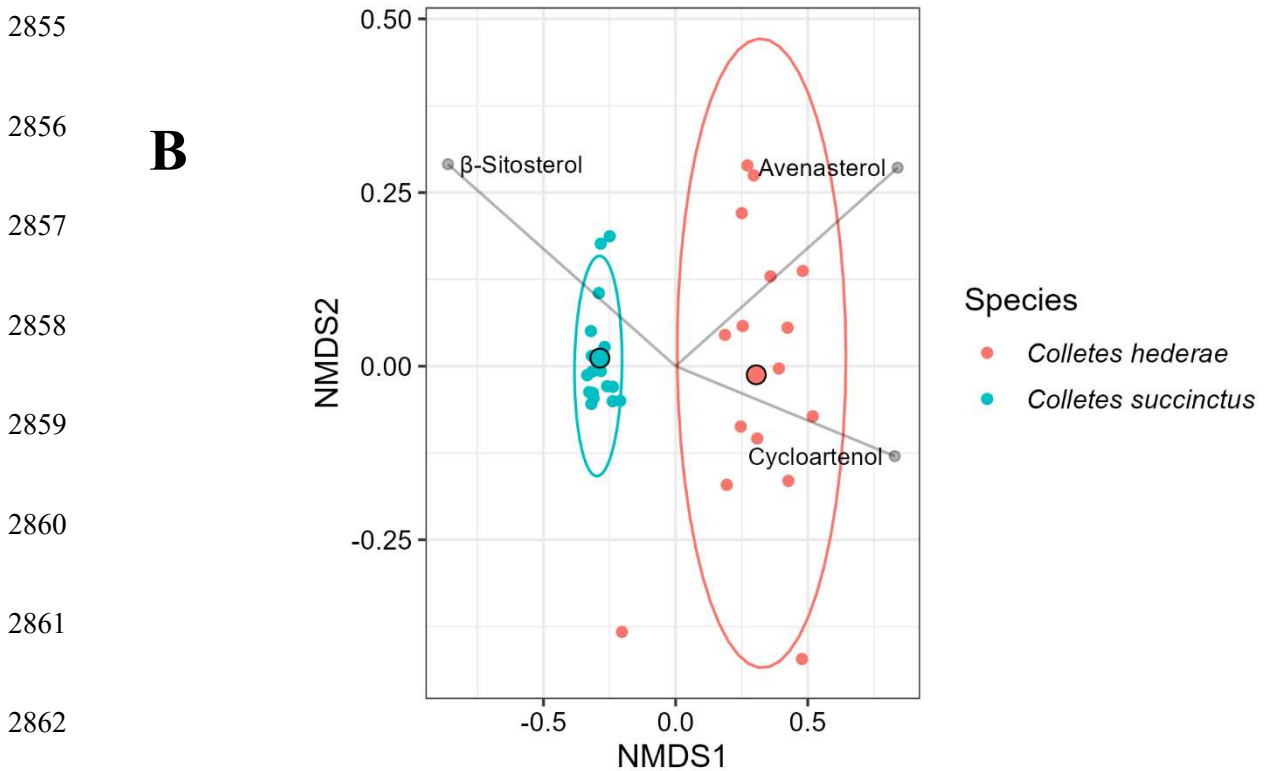
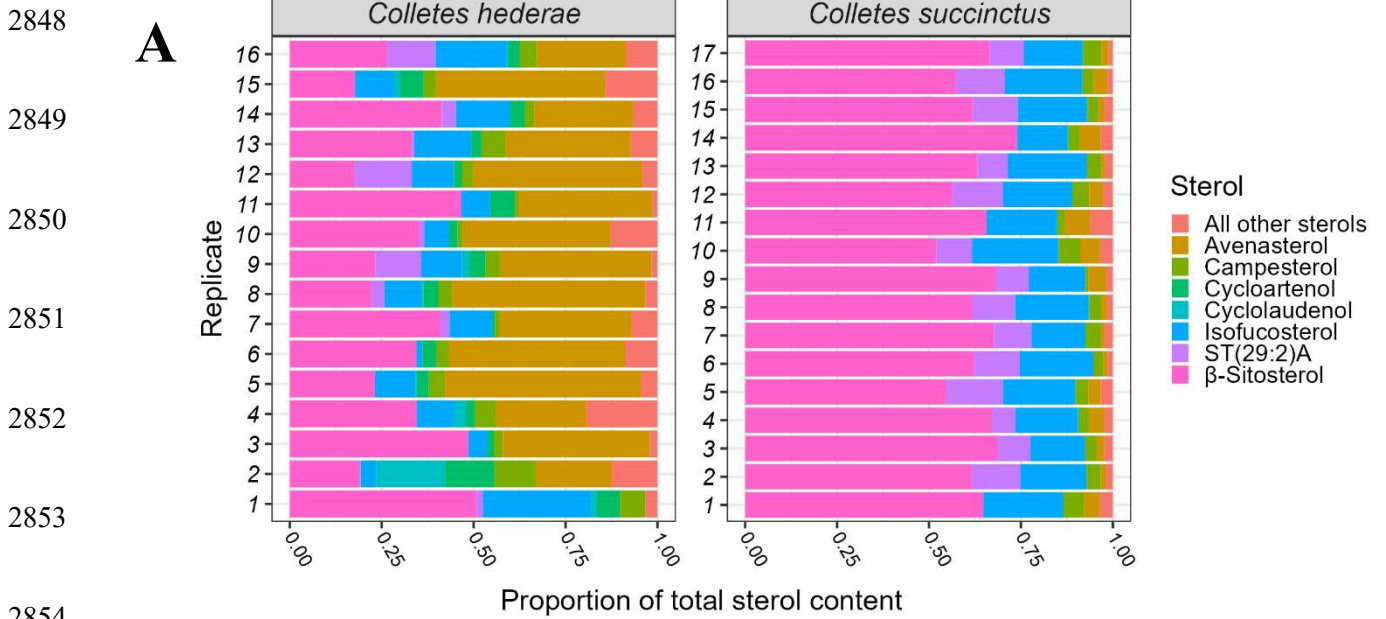
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Figure 4.3. Interspecies comparison of sterol profile among species with ≥ 10 samples. NMDS stress value = 0.151. Grey lines indicate significant ($p < 0.010$) and strong (absolute value > 0.7) correlations. There are clear similarities in the sterol profile of ecologically linked species such as *Epeolus cruciger* with *Colletes succinctus* and *Nomada fucata* with *Andrena cineraria* which are both kleptoparasite-host pairs.

2844 **Table 4.3.** Indicator Species Analysis of species with ≥ 10 samples. Of the 19 sterols tested, 15
 2845 were indicators of individual species groups. Indicator values (IV) and p-values are shown for
 2846 significant sterols.

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

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2863 **Figure 4.4.**

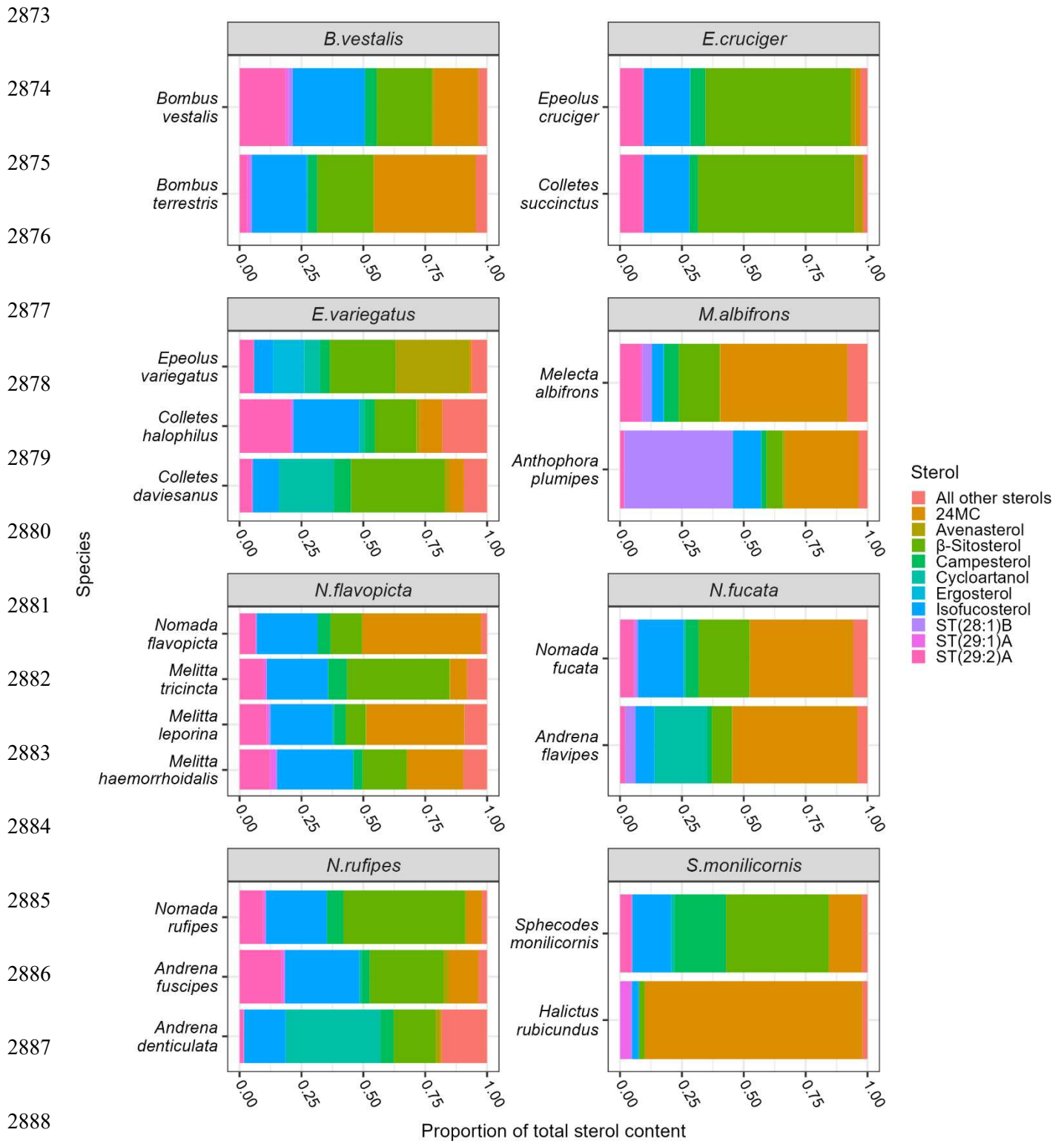
2864 **A)** Sterol composition of all replicates of *Colletes hederæ* and *C. succinctus*. Only major sterols
 2865 named, remainder collapsed into single group.

2866 **B)** NMDS of *Colletes hederæ* and *C. succinctus* (stress= 0.105). There is clear separation between
 2867 the two species despite their relatedness. *C. hederæ* is associated with higher avenasterol and
 2868 cycloartenol whereas *C. succinctus* is associated with higher β -sitosterol.

2869 **Table 4.4.** Results of Indicator Species Analysis of the sterol profiles of *Colletes hederæ* and
 2870 *Colletes succinctus*. Of the 19 sterols tested, 10 were indicators of individual groups. Indicator
 2871 values (IV) and p-values are shown for significant sterols.

Sterol	IV	P-value	Sterol	IV	P-value
<i>Colletes hederæ</i>			<i>Colletes succinctus</i>		
Cycloartenol	0.946	0.005	ST(29:1)A	0.853	0.005
Avenasterol	0.856	0.005	β -Sitosterol	0.778	0.005
ST(30:1)B	0.772	0.005	Isofucosterol	0.757	0.005
Cholesterol	0.769	0.045	ST(29:2)A	0.749	0.050
Cyclolaudenol	0.697	0.050			
Cycloartanol	0.603	0.020			

2872



2889 **Figure 4.5.** Sterol profiles of kleptoparasitic bees and their associated hosts. Kleptoparasitic
 2890 species are shown in the header of each subplot. Some species show greater similarities with their
 2891 host (*Epeolus cruciger*) than others (*Sphecodes monilicornis*). Only major sterol components are
 2892 labelled, the remainder are collapsed into a single group.

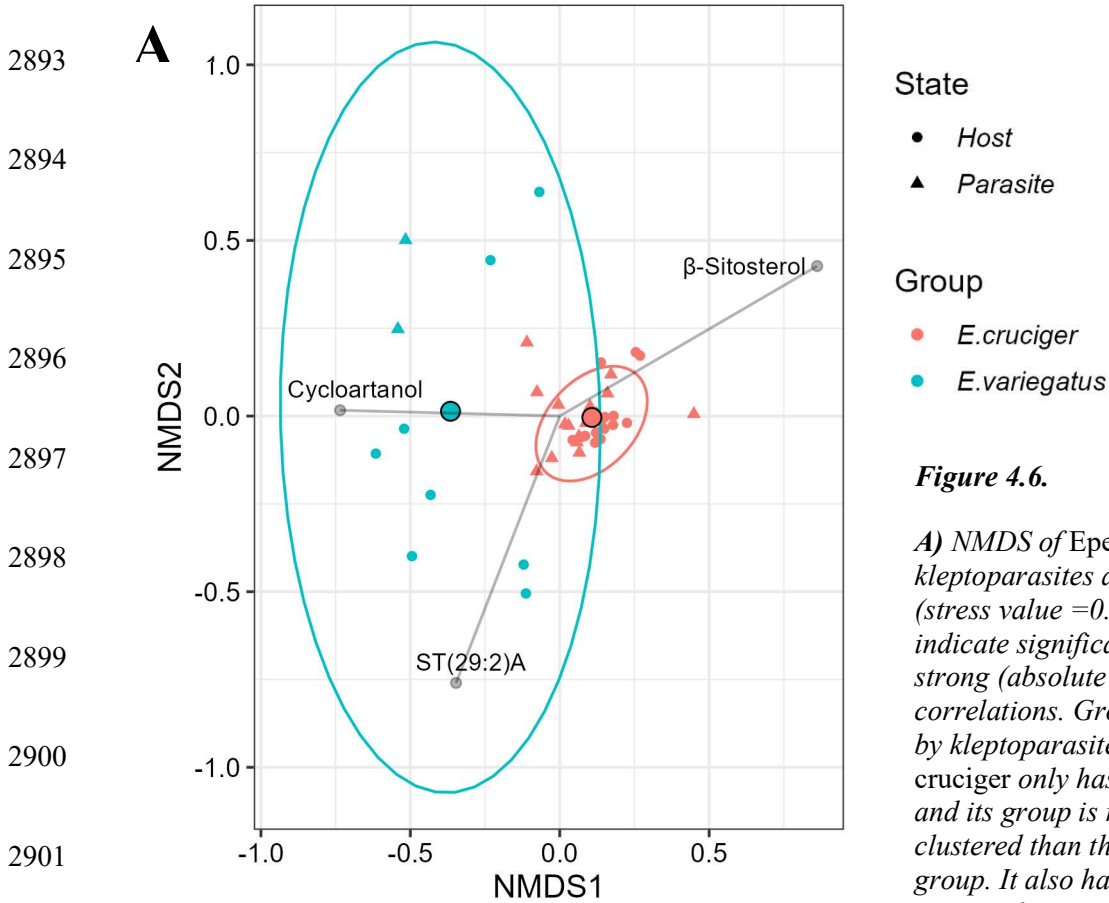
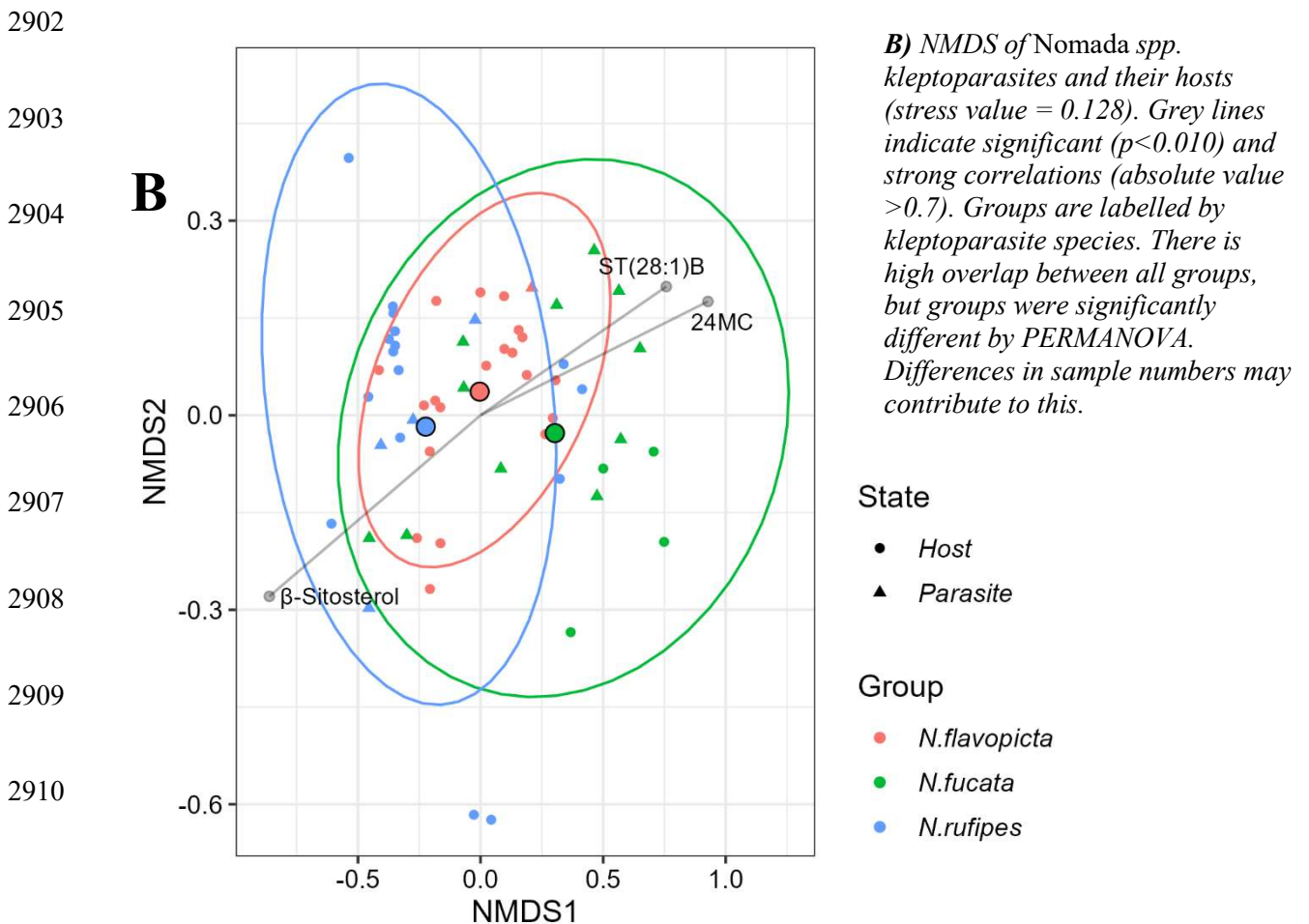


Figure 4.6.

A) NMDS of *Epeolus* spp. kleptoparasites and their hosts (stress value = 0.142). Grey lines indicate significant ($p < 0.010$) and strong (absolute value > 0.7) correlations. Groups are labelled by kleptoparasite species. *Epeolus cruciger* only has one host species and its group is much more tightly clustered than the *E. variegatus* group. It also has higher β -sitosterol proportions.



B) NMDS of *Nomada* spp. kleptoparasites and their hosts (stress value = 0.128). Grey lines indicate significant ($p < 0.010$) and strong correlations (absolute value > 0.7). Groups are labelled by kleptoparasite species. There is high overlap between all groups, but groups were significantly different by PERMANOVA. Differences in sample numbers may contribute to this.

2911 **Table 4.5.** Indicator Species Analysis of sterol profiles of the kleptoparasites *Epeolus cruciger* and
 2912 *E. variegatus* and their associated host species. Of the 19 sterols tested, 6 were indicators of
 2913 kleptoparasite-host groups. Indicator values (IV) and p-values are shown for significant sterols.

Sterol	IV	P-value	Sterol	IV	P-value
<i>E. variegatus</i>			<i>E. cruciger</i>		
Cycloartanol	0.968	0.005	β -Sitosterol	0.798	0.005
Cholesterol	0.933	0.005			
ST(30:1)A	0.748	0.035			
Cyclolaudenol	0.746	0.015			
ST(30:1)B	0.716	0.025			

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2917 **Table 4.6.** Indicator Species Analysis of the sterol profiles of kleptoparasites *Nomada flavopicta*,
 2918 *N. fucata* and *N. rufipes* and their associated host species. Of the 19 sterols tested, 8 were
 2919 indicators of kleptoparasite-host groups. Indicator values (IV) and p-values are shown for
 2920 significant sterols.

2921

Sterol	IV	P-value	Sterol	IV	P-value	Sterol	IV	P-value
<i>N. flavopicta</i>			<i>N. fucata</i>			<i>N. rufipes</i>		
ST(30:1)B	0.818	0.005	ST(28:1)B	0.717	0.005	Avenasterol	0.768	0.005
ST(29:1)A	0.659	0.005	24MC	0.711	0.010	β -Sitosterol	0.638	0.020
Isofucosterol	0.610	0.050	Ergosterol	0.654	0.010			

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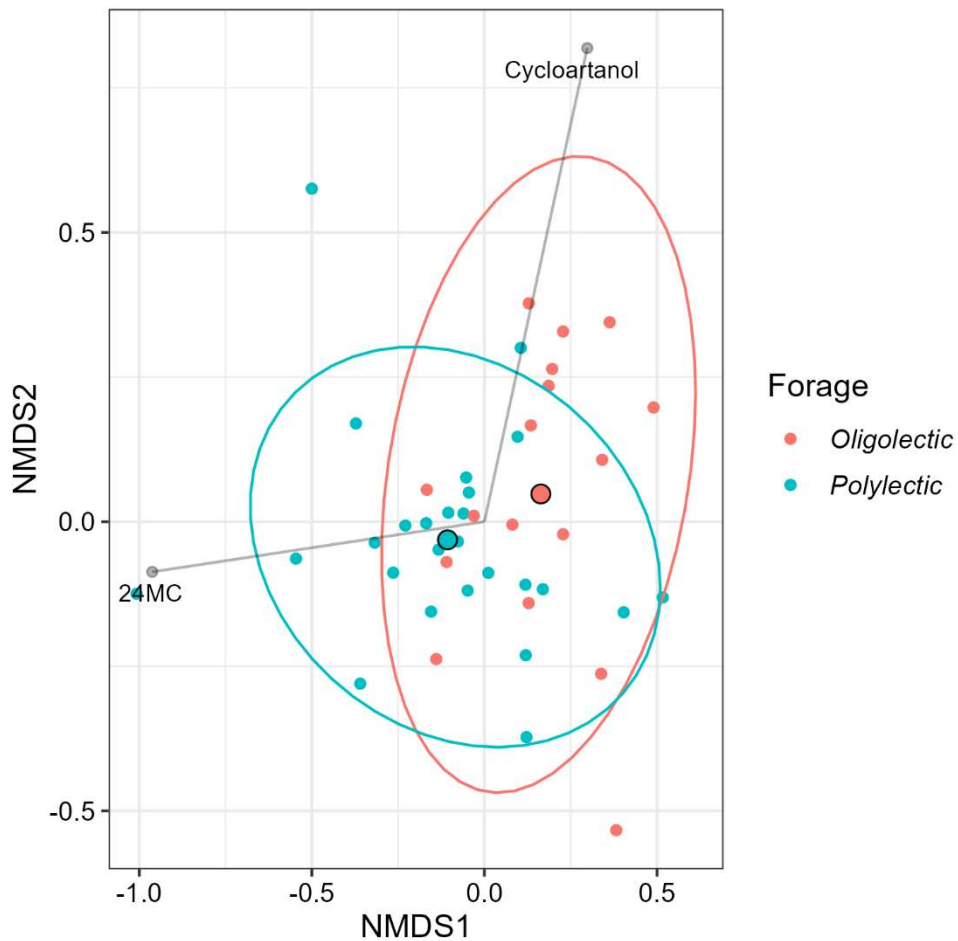
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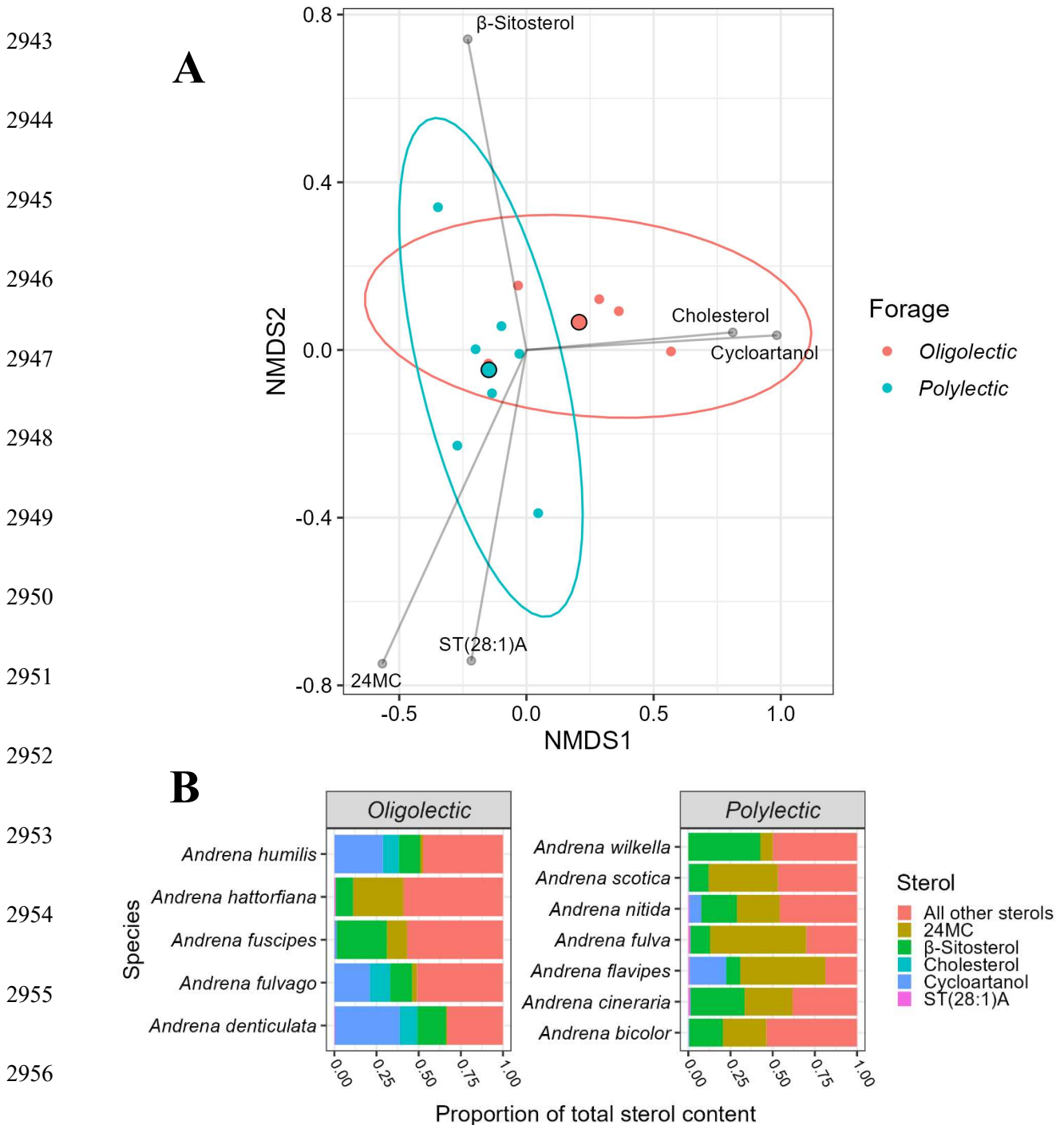
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2933 **Figure 4.7.** NMDS of all oligolectic and polylectic species in the dataset (stress=0.135). Points
 2934 represent species means. Grey lines indicate significant ($p < 0.010$) and strong correlations
 2935 (absolute value > 0.7). Groups were significantly different by PERMANOVA, polylectic species
 2936 were associated with higher proportions of 24-methylenecholesterol and oligolectic was associated
 2937 with higher levels of cycloartanol.

2938 **Table 4.7.** Indicator Species Analysis of the sterol profiles of all oligolectic and polylectic bee
 2939 species in the dataset. Of the 19 sterols tested, 6 were indicators of individual groups. Indicator
 2940 values (IV), p-values and significance levels are shown for significant sterols. Proportions were
 2941 averaged by species.

Sterol	IV	P-value	Sterol	IV	P-value
Oligolectic			Polylectic		
Cycloartanol	0.839	0.015	24MC	0.819	0.005
Cholesterol	0.833	0.020	ST(28:1)B	0.794	0.050
Cyclolaudenol	0.786	0.030	ST(28:1)A	0.751	0.030

2942



2957 **Figure 4.8.**

2958 **A)** NMDS of polylectic and oligolectic *Andrena* species (stress value = 0.059) which showed a
 2959 significant difference between groups. Grey lines indicate significant ($p < 0.010$) and strong
 2960 correlations (absolute value > 0.7). Polylectic species were correlated with higher 24-
 2961 methylenecholesterol and oligolectic species are associated with higher cholesterol.

2962 **B)** Proportions of sterols shown to have significant ($p < 0.010$) and strong correlations (absolute
 2963 value > 0.7) in Figure 4.8A. All remaining sterols are collapsed into a single group.

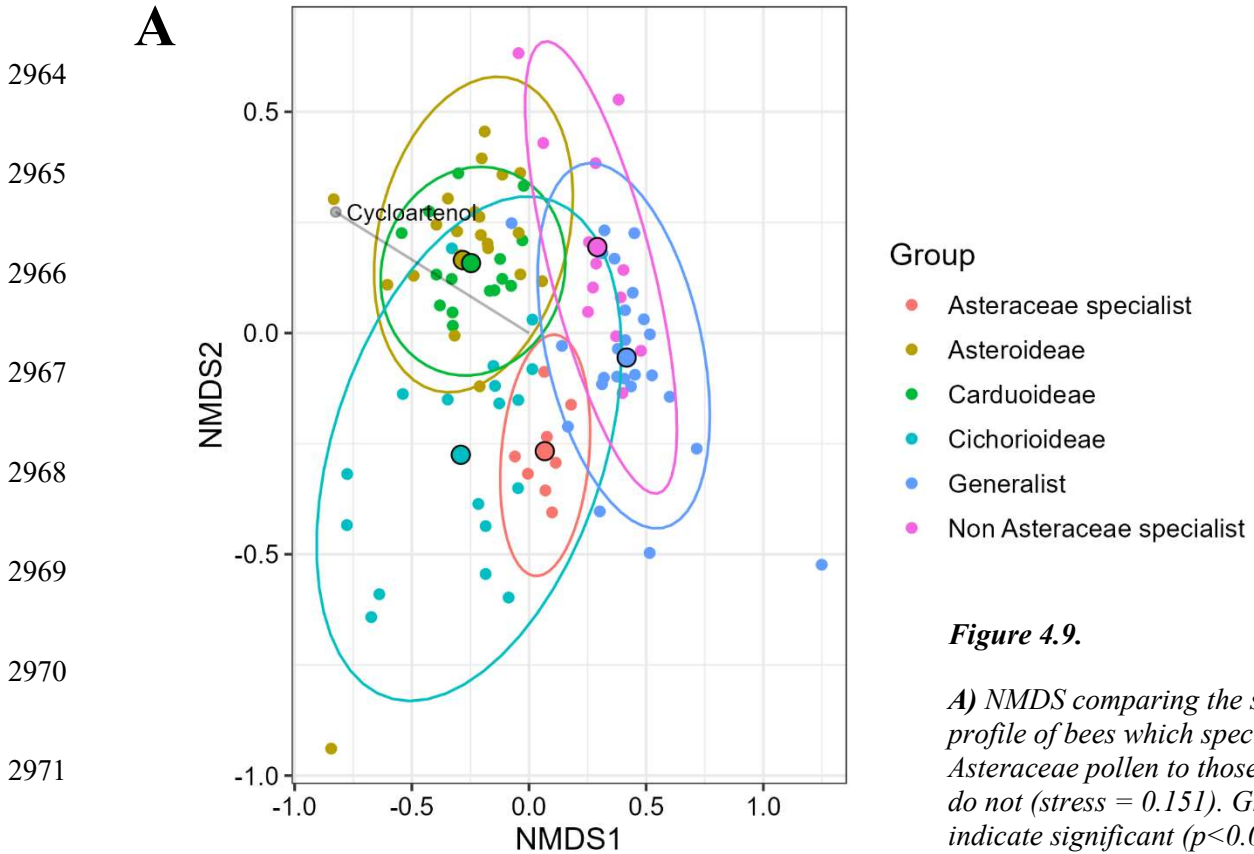
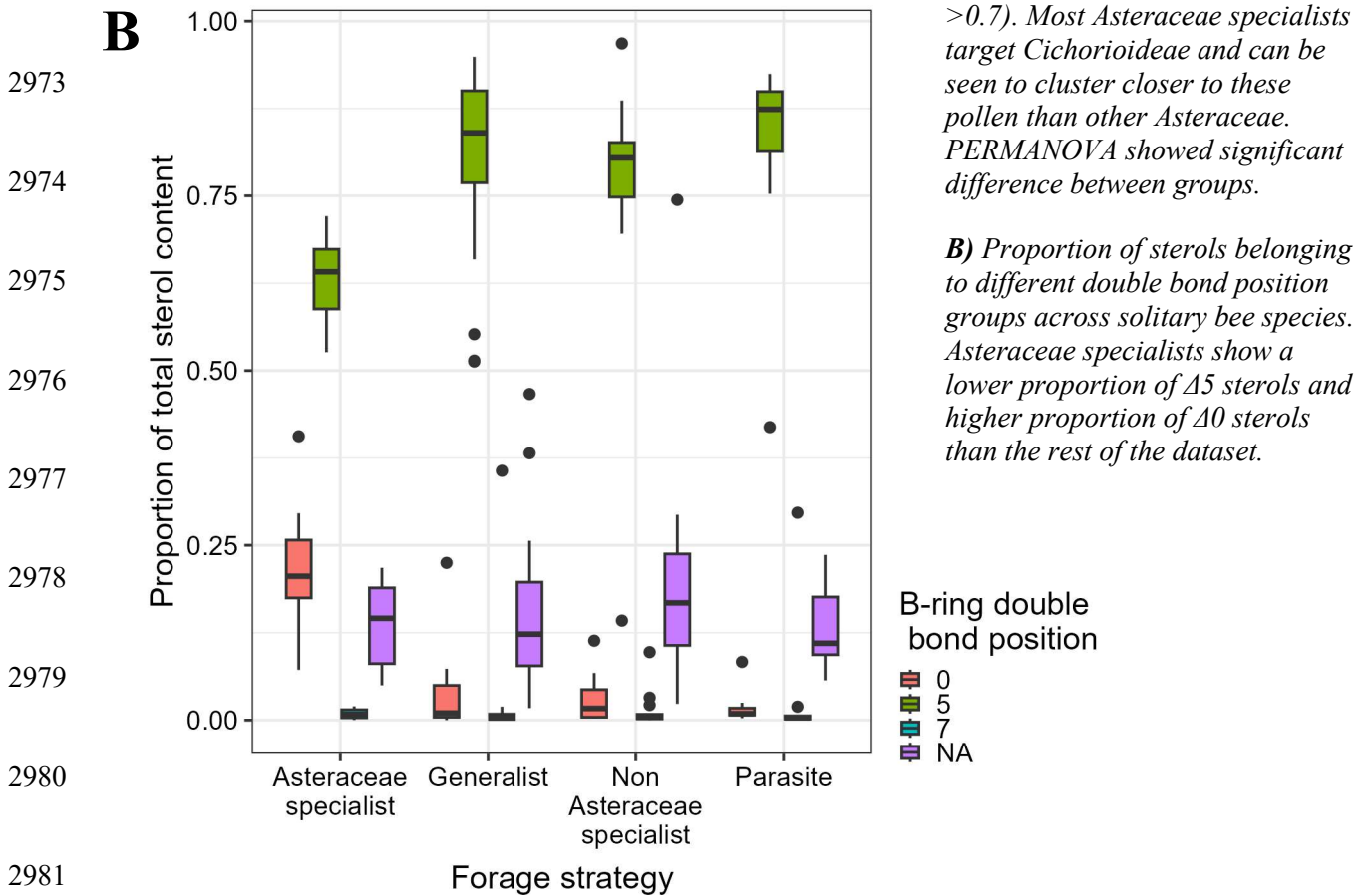


Figure 4.9.

A) NMDS comparing the sterol profile of bees which specialise on Asteraceae pollen to those which do not (stress = 0.151). Grey lines indicate significant ($p < 0.010$) and strong correlations (absolute value > 0.7). Most Asteraceae specialists target Cichorioideae and can be seen to cluster closer to these pollen than other Asteraceae. PERMANOVA showed significant difference between groups.



B) Proportion of sterols belonging to different double bond position groups across solitary bee species. Asteraceae specialists show a lower proportion of $\Delta 5$ sterols and higher proportion of $\Delta 0$ sterols than the rest of the dataset.

2982 **Table 4.8.** Results of Indicator Species Analysis of the sterol profiles of all bees and Asteraceae
 2983 pollens. Of the 13 sterols tested, 9 were indicators of individual groups. Indicator values (IV) and
 2984 p-values are shown for significant sterols. Proportions were averaged by species.

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

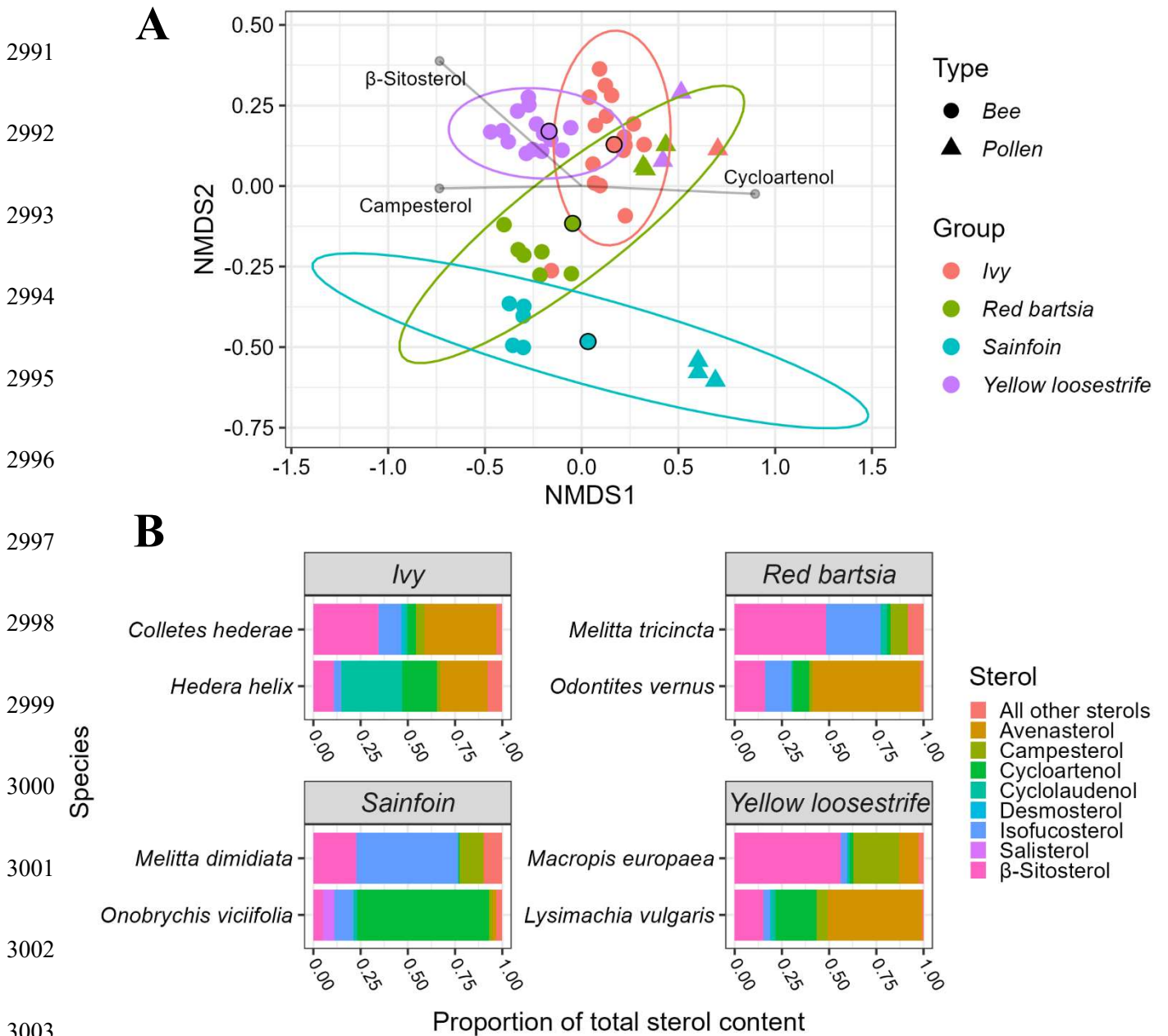
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2987 **Table 4.9.** Results of Indicator Species Analysis of interspecies sterol profile for specialist
 2988 oligolectic bee-pollen pairings. Of the 13 sterols tested, 9 were indicators of individual groups.
 2989 Indicator values (IV) and p-values are shown for significant sterols.

Sterol	IV	P-value
Ivy		
Desmosterol	0.656	0.025
Avenasterol	0.651	0.005
Cholesterol	0.636	0.015
Red bartisa		
24MC	0.602	0.045
Sainfoin		
Salisterol	0.777	0.005
Cycloartenol	0.652	0.005
Isofucosterol	0.622	0.005
Yellow loosestrife		
Campesterol	0.631	0.005
β -Sitosterol	0.570	0.005

2990



3004 **Figure 4.10.**

3005 **A)** NMDS showing monolectic and pollen specialist bees with their associated pollen (stress value
 3006 = 0.119). Bee-pollen groups were significantly different. Grey lines indicate significant ($p < 0.010$)
 3007 and strong correlations (absolute value > 0.7). Pollen contains more cycloartenol whereas bees
 3008 contain more campesterol.

3009 **B)** Mean sterol profile of species in Figure 4.10A. Only sterols which form part of the top three
 3010 sterols by proportion in one of the species are shown. Bee species are listed first in each plot. Bars
 3011 demonstrate that an abundance of a sterol in the pollen source does not guarantee its dominance
 3012 in bee tissues.

3013 Discussion

3014 While the dataset displayed a wide range in bee sterol profiles, there was an overall trend for high
3015 levels of 24-methylenecholesterol, β -sitosterol and isofucoesterol, confirming previous work on
3016 sterols in solitary bee species (Svoboda and Lusby, 1986; Vanderplanck, Zerck, *et al.*, 2020a).
3017 Combined with their known importance for *Apis mellifera* and *Bombus* species (Furse, Koch, *et al.*,
3018 2023), this suggests these sterols are universally important to most UK bee species and are
3019 frequently acquired from their diet and incorporated into tissues without modification. As shown
3020 previously (Zu *et al.*, 2021), these sterols can occur in high proportions across a wide range of
3021 pollen and therefore represent widely available sterols for foraging bees. They also share some
3022 structural similarity with cholesterol, the ancestral state of sterol usage in animals, by having a
3023 double bond at position five in the steroid nucleus B-ring. However, the consistently low
3024 proportion of desmosterol, also a Δ^5 sterol, across the bee species compared with the high
3025 proportions available in some flowering plants, suggests that sterol abundance alone does not lead
3026 to their use by bees. This is supported by the high availability of cycloartenol, cycloeucalenol and
3027 obtusifoliol which have a median of >1% across all plants (Chapter 2) but are largely absent or
3028 present in low levels in bees.

3029 The phylogenetic trend seen in campesterol, where closely related species showed more similar
3030 proportions, may be due to its importance in the production of the moulting hormone makisterone
3031 A in some bee species. The absence of cholesterol or campesterol from some species (*Andrena*
3032 *wilkella*, *Nomada flavopicta*, *Halictus rubicundus*, *Lasioglossum smeathmanellum*, *Coelioxys*
3033 *inermis* and *Ceratina cyanea*.) is also interesting as these sterols are thought to be essential to the
3034 production of hormones (Feldlaufer *et al.*, 1986; Furse, Koch, *et al.*, 2023). There is no clear
3035 phylogenetic or ecological link between these species, and the hosts of the kleptoparasitic *Nomada*
3036 *flavopicta* do contain cholesterol. It is important to note that all species were represented by one

3037 sample in the dataset therefore it is possible cholesterol and campesterol were not detectable or
3038 were present in low amounts earlier in development but metabolised for the biosynthesis of steroid
3039 hormones.

3040 The very low level of cholesterol present across the dataset suggests that all the bee species
3041 analysed are not converting dietary phytosterols into cholesterol and are instead relying upon
3042 pollen for their sterol intake. In two bee species, cholesterol was present in the body tissues, though
3043 at low levels (<1%), but not in its primary pollen host. One of these species, *Colletes hederæ*
3044 collects pollen from flowers other than *Hedera helix* and so may be obtaining cholesterol from
3045 these less dominant pollen sources.

3046 Unlike bumble bees (*Bombus* spp.), solitary bee genera seem to display larger intra-genus
3047 variation, potentially as a result of more diverse intrageneric life histories. The genus *Andrena* for
3048 example includes polylectic, oligolectic and monolectic species, the former of which was
3049 associated with higher levels of 24-methylenecholesterol and oligolectic species with higher
3050 cycloartanol. However, there were also differences in the level of intra-species variation, such as
3051 between *Colletes hederæ* and *C. succinctus*, the latter of which showed much lower variation than
3052 its congener. This may reflect either differences in the plasticity of sterol requirements between
3053 species or sampling bias as collection was not targeted to capture intra-species variation. This
3054 plasticity in sterol requirements may have enabled bees to adapt to different pollen diets. Feeding
3055 studies to test the limits of sterol requirements in individual species may reveal that even polylectic
3056 species are constrained by sterol requirements. This would be consistent with the ‘covert specialist’
3057 hypothesis put forward by Vanderplanck *et al.* (2020) which suggests polylectic foraging can
3058 conceal specialist nutritional requirements. Alternatively, monolectic species may have more
3059 flexible requirements than could be assumed from their foraging, with other drivers causing their
3060 specialist behaviour.

3061 There was a strong similarity between the sterol profile of some kleptoparasitic species and their
3062 hosts. Many kleptoparasitic species are unrelated to their host, therefore shared ancestry is unlikely
3063 to be the cause of these similarities. This suggests there is an adaptive ecological benefit and
3064 supports the idea of plasticity in sterol usage. As kleptoparasites are dependent on the pollen
3065 foraging of a host species, adapting to use the same sterols as the host may enable them to exploit
3066 their provisions most efficiently. However, by aligning their sterol profile with their hosts,
3067 kleptoparasites may limit their ability to use alternative host species if sterol requirements are rigid.
3068 This study did not include all host species of some kleptoparasites or indeed any preferences
3069 kleptoparasitic bees may have for one host over another. Absence of a preferred host species may
3070 be why some kleptoparasites showed no obvious similarity to the host species included. For these
3071 species which display different sterol profiles from their host; it is interesting that their larvae must
3072 therefore be selectively utilising sterols differently from the host larvae, despite having access to
3073 the same food.

3074 Polylectic *Andrena* species contained higher levels of 24-methylenecholesterol on average than
3075 oligolectic ones. Their ability to forage from a wider range of plants may facilitate reliable access
3076 to this desirable sterol, though they may be faced with more competition for these resources. The
3077 higher cycloartanol and cholesterol proportions seen in the oligolectic group are largely driven by
3078 the three *Andrena* species (*A. humilis*, *A. fulvago* and *A. denticulata*) which specialise on
3079 Asteraceae flowers.

3080 Asteraceae flowers, particularly subfamily Cichorioideae, are associated with specialist bees and
3081 their pollen is rarely collected by generalist bee species despite their frequent availability. Previous
3082 work has speculated on the cause of this by suggesting defence mechanisms and nutritional
3083 deficiencies in the pollen. Solitary bees which are specialist on flowers other than Asteraceae are
3084 unable to develop on the diets of Asteraceae specialist bees (Praz, Müller and Dorn, 2008). When
3085 bumble bees were fed crushed *Taraxacum* pollen, they presented with digestive damage and

3086 reduced pollen collection. This supports the presence of chemical defences or nutritional
3087 deficiencies rather than physical defences in the pollen (Vanderplanck, Gilles, *et al.*, 2020). As
3088 stated in Chapter 2, the pollen sterols of Asteraceae flowers display lower levels of $\Delta 5$ sterols such
3089 as 24-methylenecholesterol, β -sitosterol, campesterol, desmosterol and isofucosterol. The data
3090 shows that this was mirrored in the bee species which specialise on them. These bees
3091 predominantly target yellow-flowered Asteraceae and display lower $\Delta 5$ sterol levels and higher $\Delta 0$
3092 than the rest of the dataset, suggesting they have adapted to the sterols available from
3093 Cichorioideae flowers.

3094 Given the strong phylogenetic signal seen in pollen sterol profile, specialising on a genus of plants
3095 can be functionally similar to specialising on a single species, in terms of available sterols. As a
3096 result, oligolectic species focusing on a single genus could be considered much more nutritionally
3097 specialised than bees which forage from a whole family.

3098 While sterol profiles were not completely aligned between monolectic bee species and their pollen,
3099 it is clear these bees were obtaining the sterols in their tissues from their pollen. It is important to
3100 note that the sterols present in the analysed bee tissues reflect pollen collected by the previous
3101 generation; therefore, it may not directly mirror the hand-collected pollen analysed here. This
3102 could be the result of intraspecies variation in pollen chemical composition or changes which have
3103 occurred due to microbes when pollen is stored in nest cells before consumption. Direct analysis of
3104 pollen loads and nest cell provisions would aid in understanding changes which may occur in sterol
3105 composition between collection and consumption (Vanderplanck, Zerck, *et al.*, 2020b). The
3106 differences in proportion may also be as a result of selective sterol transport across the bee gut. For
3107 example, the pollen of *Lysimachia vulgaris* has proportionally more avenasterol and lower β -
3108 sitosterol than *Macropis europea*, which is monolectic for *L. vulgaris*. A similar relationship
3109 between sterol proportions in pollen and those found in bees was also present in the plant, *Odonites*
3110 *vernus* and its pollinator, *Melitta tricincta*.

3111 Interestingly, all monoleptic bees had higher proportions of β -sitosterol and campesterol than their
3112 plants' pollen. Campesterol is used in the production of the moulting hormone makisterone A, a
3113 potential explanation for its selective uptake. These trends suggest certain sterols are commonly
3114 more important in bee physiology. It also suggests that bees do not necessarily incorporate sterols
3115 into their tissues that are not metabolically important, even if abundant in their food source. This is
3116 particularly clear with the high cycloartenol levels in *Onobrychis vicifolia* and its extremely low
3117 levels in *Melitta dimidiata*. The mechanism for these variations is unknown. Earlier work on
3118 honeybees reported that they did not selectively excrete sterols in order to increase the relative
3119 concentration of 24-methylenecholesterol (Feldlaufer and Harrison, 2020).

3120 The major sterols in each of the specialist bee species (β -sitosterol, isofucosterol, campesterol,
3121 ST(29:2)A and avenasterol) are common in flowering plants and could be obtained from a range of
3122 other species (Vanderplanck, Zerck, *et al.*, 2020b; Zu *et al.*, 2021). Therefore, it is likely that other
3123 nutritional or ecological factors are influencing these bees to rely on a single pollen host, a strategy
3124 which makes them highly vulnerable to changes in flowering times and distribution. For example,
3125 *Macropis europaea* also collects fatty floral oils from its main pollen plant *Lysimachia vulgaris* to
3126 waterproof its nests in damp soils (Falk and Lewington, 2015). Similarly, *Colletes hederæ* is a late
3127 emerging species and may therefore use *Hedera helix* as a reliable autumn food source.

3128 The importance of diverse floral communities has been highlighted for supporting diverse bee
3129 communities (Potts *et al.*, 2003). However, more recent work has emphasised the importance of
3130 nutritional diversity for supporting bees with different nutritional niches (Vaudo, Dyer and
3131 Leonard, 2023). Floral diversity also facilitates generalist foraging in order to fulfil dietary needs in
3132 the absence of any one 'perfect' resource (Vaudo, Dyer and Leonard, 2023). These authors
3133 similarly postulate the concept of 'cryptic nutritional specialists' which fulfil strict nutritional
3134 needs through a generalist foraging strategy. However, the challenge of rearing solitary bees in
3135 experimental conditions means there is an absence of data on their nutritional requirements. The

3136 diversity of sterol profiles reported here also demonstrates the importance of not extending
3137 findings from single species such as *Osmia bicornis* to be representative of solitary bees as a
3138 group.

3139 The results of this analysis do not reveal a singular clear trend in the sterol profile of solitary bees.
3140 There is no evidence for a strong phylogenetic signal in most sterols, therefore, the drivers of sterol
3141 profile in solitary bees would appear to be ecologically, rather than phylogenetically, constrained
3142 but with some plasticity. This can be seen where the sterol profiles of some kleptoparasitic species
3143 closely resemble those of their hosts, despite phylogenetic distance. However, there are multiple
3144 instances of sterol profiles differing from those expected by ecological drivers, for instance where
3145 monolectic bee species sterol profiles do not match the sterol proportions of their pollen host. It
3146 may be that selective uptake and sequestration means proportional differences are present between
3147 pollen and bee sterol profiles and there are other nutritional or ecological factors which strongly
3148 influence pollen foraging choices.

3149 **Acknowledgements**

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3155 **Supplementary**3156 **Table S4.1.** Summary of all wild bee samples including collection county and date.

Species	County collected	Month collected	Number of samples
<i>Andrena bicolor</i>	Greater London	Jul-21	2
<i>Andrena bicolor</i>	Greater London	Jul-22	2
<i>Andrena bicolor</i>	Greater London	Jun-22	2
<i>Andrena cineraria</i>	Greater London	Apr-21	3
<i>Andrena cineraria</i>	Greater London	Apr-22	2
<i>Andrena cineraria</i>	Greater London	May-22	4
<i>Andrena cineraria</i>	Oxfordshire	Apr-21	2
<i>Andrena cineraria</i>	Oxfordshire	Jun-21	1
<i>Andrena cineraria</i>	Oxfordshire	NA	1
<i>Andrena cineraria</i>	Sussex	Sep-22	1
<i>Andrena denticulata</i>	Greater London	Aug-21	2
<i>Andrena flavipes</i>	Greater London	Apr-21	2
<i>Andrena flavipes</i>	Sussex	Apr-21	2
<i>Andrena florea</i>	Essex	Jun-22	1
<i>Andrena florea</i>	Greater London	Jul-22	1
<i>Andrena florea</i>	Greater London	Jun-21	1
<i>Andrena florea</i>	Greater London	Jun-22	4
<i>Andrena fulva</i>	Oxfordshire	Apr-22	3
<i>Andrena fulva</i>	Oxfordshire	May-21	1
<i>Andrena fulvago</i>	Greater London	Jul-21	1
<i>Andrena fulvago</i>	Greater London	May-22	2
<i>Andrena fulvago</i>	NA	Jul-22	1
<i>Andrena fulvago</i>	NA	May-22	1
<i>Andrena fuscipes</i>	NA	Aug-22	6
<i>Andrena fuscipes</i>	NA	Aug-23	1
<i>Andrena fuscipes</i>	Sussex	Aug-22	2
<i>Andrena fuscipes</i>	Sussex	Jul-22	5
<i>Andrena hattorfiana</i>	Oxfordshire	Jul-21	4
<i>Andrena humilis</i>	Greater London	May-22	1
<i>Andrena humilis</i>	Sussex	Jun-22	3
<i>Andrena nitida</i>	Oxfordshire	Apr-21	4
<i>Andrena nitida</i>	Wiltshire	May-21	1
<i>Andrena scotica</i>	Greater London	Apr-22	1
<i>Andrena scotica</i>	Sussex	Apr-22	1

Species	County collected	Month collected	Number of samples
<i>Andrena wilkella</i>	Sussex	Jun-22	1
<i>Anthidium manicatum</i>	Greater London	Jul-21	1
<i>Anthophora furcata</i>	Greater London	Jul-21	2
<i>Anthophora furcata</i>	Greater London	Jun-22	1
<i>Anthophora furcata</i>	Sussex	Jul-22	1
<i>Anthophora plumipes</i>	Greater London	Apr-22	4
<i>Anthophora plumipes</i>	Greater London	Mar-22	8
<i>Anthophora plumipes</i>	Wiltshire	May-21	3
<i>Anthophora quadrimaculata</i>	Greater London	Jul-21	3
<i>Anthophora quadrimaculata</i>	Sussex	Jun-22	1
<i>Bombus hortorum</i>	Greater London	Mar-22	1
<i>Bombus lucorum</i>	Sussex	Mar-22	1
<i>Bombus pascuorum</i>	Greater London	Mar-22	3
<i>Bombus pratorum</i>	Greater London	Mar-22	2
<i>Bombus sylvarum</i>	Sussex	Apr-21	1
<i>Bombus sylvarum</i>	Sussex	Mar-22	1
<i>Bombus sylvarum</i>	Sussex	May-21	1
<i>Bombus terrestris</i>	Sussex	Mar-22	2
<i>Bombus vestalis</i>	Greater London	Apr-22	2
<i>Bombus vestalis</i>	Greater London	Mar-22	3
<i>Ceratina cyanea</i>	NA	Aug-22	1
<i>Chelostoma campanularum</i>	Greater London	Jul-21	3
<i>Chelostoma campanularum</i>	Sussex	Jun-21	1
<i>Chelostoma florissomne</i>	Greater London	May-22	3
<i>Coelioxys inermis</i>	Greater London	May-23	1
<i>Colletes daviesanus</i>	Greater London	Jun-21	3
<i>Colletes halophilus</i>	Essex	Sep-22	5
<i>Colletes hederæ</i>	Essex	Sep-22	10
<i>Colletes hederæ</i>	Greater London	Sep-21	4
<i>Colletes hederæ</i>	Greater London	Sep-22	1
<i>Colletes hederæ</i>	Kent	Sep-22	1
<i>Colletes succinctus</i>	NA	Aug-22	3
<i>Colletes succinctus</i>	NA	NA	1
<i>Colletes succinctus</i>	North Yorkshire	NA	13
<i>Dasypoda hirtipes</i>	Essex	Aug-22	5
<i>Dasypoda hirtipes</i>	NA	Aug-22	5
<i>Epeolus cruciger</i>	NA	Aug-22	2
<i>Epeolus cruciger</i>	North Yorkshire	NA	7
<i>Epeolus cruciger</i>	Sussex	Aug-22	2
<i>Epeolus cruciger</i>	Sussex	Jul-22	6

Species	County collected	Month collected	Number of samples
<i>Epeolus variegatus</i>	Kent	Sep-22	2
<i>Halictus rubicundus</i>	Sussex	Sep-22	1
<i>Halictus tumulorum</i>	Greater London	NA	1
<i>Heriades truncorum</i>	Greater London	Aug-21	2
<i>Heriades truncorum</i>	Greater London	Sep-21	1
<i>Hylaeus communis</i>	Greater London	Jul-21	2
<i>Hylaeus communis</i>	Sussex	Jul-22	1
<i>Hylaeus signatus</i>	Greater London	Jul-21	4
<i>Lasioglossum brevicorne</i>	Greater London	Jun-22	2
<i>Lasioglossum brevicorne</i>	NA	Jul-22	2
<i>Lasioglossum brevicorne</i>	NA	NA	2
<i>Lasioglossum smeathmanellum</i>	Greater London	Jul-21	1
<i>Lasioglossum villosulum</i>	NA	NA	4
<i>Lasioglossum villosulum</i>	Sussex	Jun-23	2
<i>Macropis europaea</i>	Greater London	Jul-21	4
<i>Macropis europaea</i>	Greater London	Jul-22	3
<i>Macropis europaea</i>	Oxfordshire	Jul-21	5
<i>Macropis europaea</i>	Oxfordshire	Mar-22	2
<i>Megachile willughbiella</i>	Greater London	Jul-21	3
<i>Melecta albifrons</i>	Greater London	Apr-22	2
<i>Melitta dimidiata</i>	Wiltshire	NA	5
<i>Melitta haemorrhoidalis</i>	Greater London	Jul-21	3
<i>Melitta haemorrhoidalis</i>	Greater London	Jul-22	3
<i>Melitta haemorrhoidalis</i>	Greater London	Jun-21	3
<i>Melitta leporina</i>	Greater London	Jul-21	1
<i>Melitta leporina</i>	Greater London	Jul-22	3
<i>Melitta leporina</i>	NA	Jul-22	1
<i>Melitta tricincta</i>	Kent	Aug-22	1
<i>Melitta tricincta</i>	Wiltshire	Aug-21	5
<i>Nomada flavopicta</i>	Greater London	Jul-21	1
<i>Nomada fucata</i>	Greater London	Apr-21	3
<i>Nomada fucata</i>	Greater London	May-21	1
<i>Nomada fucata</i>	Greater London	May-23	2
<i>Nomada fucata</i>	Oxfordshire	Apr-21	5
<i>Nomada rufipes</i>	Sussex	Aug-22	1
<i>Nomada rufipes</i>	Sussex	Jul-22	3
<i>Osmia bicornis</i>	Greater London	Apr-22	2
<i>Osmia bicornis</i>	Greater London	May-22	1
<i>Osmia bicornis</i>	Sussex	Jun-22	1

Species	County collected	Month collected	Number of samples
<i>Osmia bicornis</i>	Sussex	May-21	2
<i>Osmia bicornis</i>	Sussex	May-22	1
<i>Osmia leaiana</i>	Greater London	Apr-22	5
<i>Osmia leaiana</i>	Greater London	Jul-21	1
<i>Osmia leaiana</i>	Greater London	Jun-21	1
<i>Osmia leaiana</i>	Greater London	Jun-22	1
<i>Osmia leaiana</i>	Oxfordshire	Jun-22	1
<i>Sphecodes monilicornis</i>	Greater London	Apr-21	2

3157

3158 **Table S4.2.** Retention time, mass to charge ratio (m/z) and structure description of sterols (carbon
3159 count (CC): double bond equivalents (DB)) used in Quality Assurance (QA) mixture

Sterol	Structure	m/z	Retention time (min)
24-methylenecholesterol	(28:2)	398.3549	5.9
24-methylenecycloartenol	(31:2)	440.4018	7.5
Anthelsterol	(29:3)	410.3549	5.3
Avenasterol	(29:2)	412.3705	6.9
β -sitosterol	(29:1)	414.3862	9.7
Brassicasterol	(28:2)	398.3549	6.6
Campesterol	(28:1)	400.3705	8.2
Cholesterol	(27:1)	386.3549	7.0
Cycloartenol	(30:2)	426.3862	8.3
Cycloeucaleanol	(30:2)	426.3862	7.7
Cyclolaudenol	(31:2)	440.4018	9.5
Desmosterol	(27:2)	384.3392	5.1
Episterol	(28:2)	398.3549	5.7
Ergosterol	(28:3)	396.3392	5.5
Isofucosterol	(29:2)	412.3705	7.5
Schottenol	(29:1)	414.3862	9.0
Sitostanol	(29:0)	416.4018	11.1
Spinasterol	(29:2)	412.3705	8.0
Stigmasterol	(29:2)	412.3705	8.5

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Table S4.3. Taxonomic information for all 56 bee species analysed, taken from *Henríquez-Piskulich, Hugall and Stuart-Fox (2024) supplementary*.

Family	Subfamily	Tribe	Genus	Species
Andrenidae	Andreninae	Andrenini	<i>Andrena</i>	<i>Andrena bicolor</i>
Andrenidae	Andreninae	Andrenini	<i>Andrena</i>	<i>Andrena cineraria</i>
Andrenidae	Andreninae	Andrenini	<i>Andrena</i>	<i>Andrena denticulata</i>
Andrenidae	Andreninae	Andrenini	<i>Andrena</i>	<i>Andrena flavipes</i>
Andrenidae	Andreninae	Andrenini	<i>Andrena</i>	<i>Andrena florea</i>
Andrenidae	Andreninae	Andrenini	<i>Andrena</i>	<i>Andrena fulva</i>
Andrenidae	Andreninae	Andrenini	<i>Andrena</i>	<i>Andrena fulvago</i>
Andrenidae	Andreninae	Andrenini	<i>Andrena</i>	<i>Andrena fuscipes</i>
Andrenidae	Andreninae	Andrenini	<i>Andrena</i>	<i>Andrena hattorfiana</i>
Andrenidae	Andreninae	Andrenini	<i>Andrena</i>	<i>Andrena humilis</i>
Andrenidae	Andreninae	Andrenini	<i>Andrena</i>	<i>Andrena nitida</i>
Andrenidae	Andreninae	Andrenini	<i>Andrena</i>	<i>Andrena scotica</i>
Andrenidae	Andreninae	Andrenini	<i>Andrena</i>	<i>Andrena wilkella</i>
Megachilidae	Megachilinae	Anthidiini	<i>Anthidium</i>	<i>Anthidium manicatum</i>
Apidae	Anthophorinae	na	<i>Anthophora</i>	<i>Anthophora furcata</i>
Apidae	Anthophorinae	na	<i>Anthophora</i>	<i>Anthophora plumipes</i>
Apidae	Anthophorinae	na	<i>Anthophora</i>	<i>Anthophora quadrimaculata</i>
Apidae	Apinae	Bombini	<i>Bombus</i>	<i>Bombus hortorum</i>
Apidae	Apinae	Bombini	<i>Bombus</i>	<i>Bombus lucorum</i>
Apidae	Apinae	Bombini	<i>Bombus</i>	<i>Bombus pascuorum</i>
Apidae	Apinae	Bombini	<i>Bombus</i>	<i>Bombus pratorum</i>
Apidae	Apinae	Bombini	<i>Bombus</i>	<i>Bombus sylvarum</i>
Apidae	Apinae	Bombini	<i>Bombus</i>	<i>Bombus terrestris</i>
Apidae	Apinae	Bombini	<i>Bombus</i>	<i>Bombus vestalis</i>
Apidae	Xylocopinae	Ceratinini	<i>Ceratina</i>	<i>Ceratina cyanea</i>
Megachilidae	Megachilinae	Osmiini	<i>Chelostoma</i>	<i>Chelostoma campanularum</i>
Megachilidae	Megachilinae	Osmiini	<i>Chelostoma</i>	<i>Chelostoma florissomne</i>
Megachilidae	Megachilinae	Megachilini	<i>Coelioxys</i>	<i>Coelioxys inermis</i>
Colletidae	Colletinae	na	<i>Colletes</i>	<i>Colletes daviesanus</i>
Colletidae	Colletinae	na	<i>Colletes</i>	<i>Colletes halophilus</i>
Colletidae	Colletinae	na	<i>Colletes</i>	<i>Colletes hederæ</i>
Colletidae	Colletinae	na	<i>Colletes</i>	<i>Colletes succinctus</i>
Melittidae	Dasypodainae	Dasypodaini	<i>Dasypoda</i>	<i>Dasypoda hirtipes</i>
Apidae	Nomadinae	Epeolini	<i>Epeolus</i>	<i>Epeolus cruciger</i>
Apidae	Nomadinae	Epeolini	<i>Epeolus</i>	<i>Epeolus variegatus</i>
Halictidae	Halictinae	Halictini	<i>Halictus</i>	<i>Halictus rubicundus</i>
Halictidae	Halictinae	Halictini	<i>Halictus</i>	<i>Halictus tumulorum</i>
Megachilidae	Megachilinae	Osmiini	<i>Heriades</i>	<i>Heriades truncorum</i>
Colletidae	Hylaeinae	na	<i>Hylaeus</i>	<i>Hylaeus communis</i>

Family	Subfamily	Tribe	Genus	Species
Colletidae	Hylaeinae	na	<i>Hylaeus</i>	<i>Hylaeus signatus</i>
Halictidae	Halictinae	Halictini	<i>Lasioglossum</i>	<i>Lasioglossum brevicorne</i>
Halictidae	Halictinae	Halictini	<i>Lasioglossum</i>	<i>Lasioglossum smeathmanellum</i>
Halictidae	Halictinae	Halictini	<i>Lasioglossum</i>	<i>Lasioglossum villosulum</i>
Melittidae	Melittinae	Macropidini	<i>Macropis</i>	<i>Macropis europaea</i>
Megachilidae	Megachilinae	Megachilini	<i>Megachile</i>	<i>Megachile willughbiella</i>
Apidae	Nomadinae	Melectini	<i>Melecta</i>	<i>Melecta albifrons</i>
Melittidae	Melittinae	Melittini	<i>Melitta</i>	<i>Melitta dimidiata</i>
Melittidae	Melittinae	Melittini	<i>Melitta</i>	<i>Melitta haemorrhoidalis</i>
Melittidae	Melittinae	Melittini	<i>Melitta</i>	<i>Melitta leporina</i>
Melittidae	Melittinae	Melittini	<i>Melitta</i>	<i>Melitta tricincta</i>
Apidae	Nomadinae	Nomadini	<i>Nomada</i>	<i>Nomada flavopicta</i>
Apidae	Nomadinae	Nomadini	<i>Nomada</i>	<i>Nomada fucata</i>
Apidae	Nomadinae	Nomadini	<i>Nomada</i>	<i>Nomada rufipes</i>
Megachilidae	Megachilinae	Osmiini	<i>Osmia</i>	<i>Osmia bicornis</i>
Megachilidae	Megachilinae	Osmiini	<i>Osmia</i>	<i>Osmia leaiana</i>
Halictidae	Halictinae	Sphecodini	<i>Sphecodes</i>	<i>Sphecodes monilicornis</i>

3163

Chapter 5: Discussion

3164

3165 The aim of this thesis was to improve our understanding of the diversity of sterols present in wild
3166 bees and pollen. This was done by generating the largest dataset of its kind, covering over 200
3167 plant species and 60 bee species, and detecting a wide range of sterols. The data was analysed to
3168 answer questions about ecological and phylogenetic causes of sterol profile variation between
3169 species. The key results of this research, their broader implications and future work are discussed
3170 in order below.

3171 **Summary of results**

3172 **Pollen phytosterol content is driven by physiology and phylogeny**

3173 The pollen sterol analysis revealed a diverse range of sterol structures in pollen and that this was
3174 phylogenetically conserved among related plant species. The strong phylogenetic trend in sterol
3175 profile was clear at the genus level whereby plant species showed strongly similar pollen sterol
3176 profiles despite very distinct habitats. Many pollens were dominated by $\Delta 5$ sterols such as β -
3177 sitosterol, 24-methylenecholesterol and isofucosterol. The frequency and abundance of these
3178 sterols suggests a common function across a wide range of plant species. Most pollens were
3179 dominated by one to three sterols indicating different expression of the enzymes involved in sterol
3180 biosynthesis pathways among clades. Studies that have explicitly tested whether pollinators might
3181 exert selection pressure on the sterol composition of plants were unable to find a relationship
3182 between sterols and preferred pollens (Zu *et al.*, 2021). Furthermore, sterol profiles do not appear
3183 to differ between plant species that use pollen versus nectar as a reward (Furse, Martel, *et al.*,
3184 2023). The data gathered in this thesis further supports these findings.

3185 The similarity between closely related species has implications for the design of planting
3186 interventions such as seed mixes that are intended to benefit pollinators in amenity and agri-
3187 environment schemes. Commonly, several *Trifolium* spp. are used in a single mix (Nowakowski
3188 and Pywell, 2016). The evidence presented here suggests that including multiple *Trifolium* species
3189 does not increase sterol nutritional diversity. It may, however, provide an abundant and continual
3190 supply of sterols important for some bee species, such as bumble bees which are known to favour
3191 *Trifolium* spp. (Carvell, 2002).

3192 Similarly, where sterol profiles differ less among plant species within a genus, bees specialising on
3193 one or a few closely related genera, such as the harebell carpenter bee (*Chelostoma*
3194 *campanularum*), may have highly specific sterol requirements, obtaining the same sterols from all
3195 species. The covert specialist hypothesis reported by Vanderplanck *et al.* (2019) suggests even in
3196 polylectic species such as bumble bees, foraging may be influenced by strict sterol requirements.
3197 The diversity of sterol profiles present in plant pollens means it may be possible for bees to meet
3198 strict sterol requirements while visiting a taxonomically wide range of flowers, making them
3199 nutritional specialists.

3200 **Bees are reliant on dietary phytosterols, a subset of which are used by a wide range of**
3201 **bee species**

3202 The sterol profile of all bees species analysed contained very low cholesterol along with multiple
3203 phytosterols. This confirmed that bees directly utilise sterols acquired in pollen food and are thus
3204 dependent on pollen for all their sterol requirements.

3205 The whole-body sterol profiles of all bumble bee species analysed were dominated by 24-
3206 methylenecholesterol, isofucosterol and β -sitosterol. This corroborates the trend reported in honey
3207 bees and showed similar relative proportions (Svoboda *et al.*, 1980). Bumble bee and honey bee
3208 colonies also have high pollen volume requirements and long flight periods. Given the abundance

3209 of these sterols in many un-related flowers, a generalist foraging strategy targeting the most
3210 rewarding species could ensure the collection of high pollen volumes in different floral
3211 communities. The reliance of bumble bees on a relatively common suite of pollen phytosterols may
3212 have facilitated their ability to thrive in a wide range of floral landscapes. While solitary bee
3213 species showed more variation in sterol profile than bumble bees, high proportions of
3214 24-methylenecholesterol, β -sitosterol and isofucoesterol were also recorded frequently across the
3215 solitary bee dataset. As stated above, this may reflect the availability of these sterols within pollens
3216 or potentially point to sterols which can serve physiological functions in a wide range of species.

3217 One of the few sterols to be detected in the pollen of all plant species analysed was campesterol.
3218 This sterol is a known precursor to the synthesis of the steroid moulting hormone makisterone A in
3219 honey bees, bumble bees and solitary bees (Feldlaufer *et al.*, 1986, 1993). Campesterol proportion
3220 was also one of the few sterols across the bees in Chapter 4 which displayed a phylogenetic signal.
3221 A cause of this signal may be that certain groups of related bees rely on campesterol for
3222 synthesising moulting hormones. If species require campesterol as a steroid hormone precursor,
3223 they may have higher proportions of it in their tissues. Other species may be reliant on another
3224 sterol for this purpose, such as cholesterol which is used to make the moulting hormone
3225 20-hydroxyecdysone (Feldlaufer *et al.*, 1986). However, cholesterol did not show a phylogenetic
3226 signal, suggesting no pattern of relatedness in species with higher cholesterol proportions.
3227 Furthermore, six species analysed contained no cholesterol (5) or no campesterol (2), supporting
3228 the notion that alternative steroid hormone synthesis precursors may occur in solitary bees
3229 (Vanderplanck, Zerck, *et al.*, 2020a). However, all six species were ecologically and
3230 phylogenetically dissimilar, and represented only by a single sample. More thorough sampling is
3231 necessary to draw this conclusion.

3232 Across the bee species analysed in Chapter 4, cycloartenol had a median proportion of <0.5% of
3233 total sterols and was not detected in all bee species. In pollen however, cycloartenol has a median

3234 proportion greater than 24-methylenecholesterol (24MC: 1.51%, cycloartenol: 2.32%). This trend
3235 was repeated throughout the data; abundance of a sterol in pollen did not necessarily mean it was
3236 incorporated by bees into their tissues. Bees clearly assimilate fewer sterols in their tissues than are
3237 found in pollen. Notably, monolectic bee species did not reflect the proportions of sterols found in
3238 their host pollen, further indicating their capacity to control which sterols were assimilated or used.
3239 Some pollens favoured by bumble bees, such as *Cirsium vulgare* and *Centaurea nigra* showed
3240 high proportions of sterols not detected in bumble bee tissues, such as schottenol. This
3241 demonstrated the importance of other nutritional factors in pollen foraging decisions. Selective
3242 uptake of sterols has been suggested previously to account for differences between dietary and
3243 tissue sterols in honey bees, but no known mechanism has been confirmed. Honey bees were not
3244 found to selectively excrete certain sterols to account for the relatively higher proportion of 24-
3245 methylenecholesterol in their tissues (Feldlaufer and Harrison, 2020). Similarly, outside of honey
3246 bees which add sterols to larval food, no mechanism has been confirmed showing bees are capable
3247 of adding sterols to pollen from their own endogenous pools, which would account for these
3248 differences (Svoboda *et al.*, 1986; Vanderplanck, Zerck, *et al.*, 2020b).

3249 **Asteraceae specialist bees are adapted to Asteraceae pollen sterols**

3250 The higher proportion of $\Delta 7$ sterols in Asteraceae pollen has previously been highlighted as a
3251 reason for poor bumble bee development on thistle pollen (Vanderplanck *et al.*, 2018). Asteraceae
3252 species in this dataset showed lower proportions of $\Delta 5$ sterols than all other species. This sterol
3253 composition may therefore be the reason for the less frequent use of Asteraceae pollen by
3254 generalist bee species (Asteraceae paradox) (Müller and Kuhlmann, 2008; Vanderplanck, Gilles, *et*
3255 *al.*, 2020).

3256 The trend for lower $\Delta 5$ sterol quantities in Asteraceae pollen was reflected in the solitary bees
3257 associated with this family. Oligolectic species specialising on Asteraceae contained higher

3258 cycloartanol and cholesterol and lower 24-methylenecholesterol than polylectic species. For bees
3259 which consume Asteraceae pollen, it often represents a plentiful food source. In some bee lineages
3260 Asteraceae specialisation is suggested to be an ancestral state (Michez *et al.*, 2008) and bees more
3261 broadly are thought to have evolved polylecty from oligolecty multiple times. If this were the case
3262 it would reflect a transition from cholesterol containing pollens to more 24-methylenecholesterol,
3263 β -sitosterol and isofucoesterol containing pollens as bees adapted to using phytosterols in their
3264 physiology.

3265 Total pollen sterol production varied 100-fold across the dataset showing extremely variable bulk
3266 sterol requirements across plant species. Asteraceae contained some of the highest and lowest total
3267 sterol values with Cichorioideae species having the highest total production. It is however possible
3268 that total sterols may have been affected by growth conditions as previous work has shown lipid
3269 content to vary in response to temperature (Russo *et al.*, 2020). Cichorioideae also included species
3270 which contained the highest proportions of cholesterol: *Helminthotheca echioides*: 60.05%, *Crepis*
3271 *capillaris*: 44.21%, *Leontodon saxatilis*: 31.33%, *Crepis vesicaria*: 27.77%. This high cholesterol
3272 content of Asteraceae pollen again supports its potential importance as a transitional step in the
3273 evolution of bees from cholesterol-rich predatory diets of sphecid wasps to the phytosterols in
3274 pollen (Dötterl and Vereecken, 2010).

3275 **Future work**

3276 The drivers behind sterol diversity in pollen and the benefits it may confer to plants remain largely
3277 unexplained. The range of sterol compositions reported in this thesis invites further investigation
3278 into the sterol synthesis pathways in plants, how this is controlled and whether these pathways
3279 have become truncated differently among taxa. Furthermore, the question remains as to whether
3280 sterol composition and production are influenced by abiotic factors, and if greater intra-species

3281 variation may therefore be possible. Changes in pollen sterol composition during development and
3282 germination are also likely and may reveal more about the functional role of sterols in pollen.

3283 The results similarly present multiple avenues for future questions regarding sterol nutrition in wild
3284 bees. The diversity of sterol requirements shown by even closely related solitary bee species
3285 demonstrated the importance of ecological drivers in understanding these nutritional requirements.
3286 How different bees regulate their own sterol intake and use remains a largely unanswered question.
3287 Similarly, how bees detect and select for different sterol profiles is also poorly understood. The
3288 moulting hormones used by many solitary bee species are also unknown and could provide
3289 important context for their sterol requirements. Whether sterol limitation governs the distribution
3290 of bees at the landscape level is an important question that can only be addressed in the context of
3291 other nutritional information.

3292 **Pollen phytosterols in wildflowers**

3293 As all pollen samples were collected from wild plants there was no control for between-site and
3294 between-year variation. By opportunistically collecting samples from the same species in different
3295 areas, intra-species variation was shown to not exceed inter-species differences in sterol profile.
3296 However, this sampling strategy did not enable determination of the strength of spatial and
3297 temporal variation, or any contributing variables. Direct experiments to test the impact of abiotic
3298 conditions such as temperature, moisture and soil nutrients would inform how sterol profiles and
3299 total sterol production may differ outside of the UK or vary spatially within the UK. It would also
3300 inform what changes might be expected in pollen sterol content as these abiotic conditions, such as
3301 temperature and nitrogen deposition, change.

3302 The similarity in sterol profiles between closely related plant species warrants an evolutionary
3303 analysis of sterol biosynthesis across plant species. It is possible that the truncated sterol synthesis
3304 pathway in pollen evolved differently across clades and the genes responsible for sterol

3305 metabolism differ between species. Following Roulston, Cane and Buchmann (2000) it would be
3306 interesting to unpick the drivers behind sterol requirements in pollen as they appear to be
3307 physiologically driven. Membrane synthesis during pollen tube growth necessitates a ready
3308 availability of sterols, therefore stigma-ovule distance may be expected to influence total sterol
3309 content and profile. The potential for sterol content to determine membrane fluidity under different
3310 temperature conditions (Furse, Koch, *et al.*, 2023) may also mean composition is influenced by
3311 flowering season and associated abiotic conditions.

3312 The pollen collection methodology used was designed during the COVID-19 pandemic and
3313 intended to be practical without access to a laboratory. As a result, the sampling protocol could no
3314 doubt be improved by more controlled storage conditions and methods to control static. Samples
3315 were kept frozen as much as reasonably possible but sample transport between storage and analysis
3316 facilities and defrosting to partition and weigh samples may have impacted pollen quality. A
3317 baseline of 10 mg minimum analysis weight was set by analytical constraints. However, reliably
3318 weighing at this level is challenging as pollen can scatter during handling due to static. Therefore,
3319 total sterol production values were potentially influenced by variability between balances and
3320 abiotic factors such as relative humidity in extraction and processing steps. In comparing sterols
3321 between pollen and bees, several sterol compounds were removed from the pollen dataset. Though
3322 this was necessary, it removed information from the analysis and pollen sterol compositions should
3323 be viewed as even more dissimilar to those of bees than shown in this analysis. Finally, a high
3324 number of sterolic compounds were detected which could not be identified from reference
3325 material; six in bees and 51 in pollen. These compounds may be intermediaries in sterol synthesis
3326 pathways or occur at levels low enough to not previously be detected by other methods.
3327 Identification of these compounds in both bees and pollen will be an important area of future
3328 research for building a complete picture of sterol usage in pollinator nutrition.

3329 **Bumble bee sterol profiles**

3330 To assess the effect of differing floral communities on sterol profile, bumble bee specimens of the
3331 same species were collected from multiple florally distinct sites. However, this did not control for
3332 the effect of season, and samples were not collected at distances to guarantee non-relatedness
3333 between individuals. As a result, there is the potential for the dataset to include related individuals
3334 raised in the same colony and on the same diets. This would therefore not accurately represent the
3335 full range of potential variation between colonies in an area.

3336 Due to the continual turn-over in floral resources for the duration of bumble bee colony cycles, the
3337 pollen removed from corbiculae is unlikely to be the same as that on which the forager was raised.
3338 As a result, the body tissues of workers may more closely resemble the pollens collected by the
3339 previous generation. Tracking sterol collection, consumption and utilisation over a full generation
3340 would reveal greater detail about the fate of foraged pollen sterols throughout development.

3341 A small number of the pollen baskets analysed were clearly a mixture of multiple pollens and
3342 would therefore not resemble any hand collected pollens. This mixing may be a strategy by bumble
3343 bees to achieve their sterol optimum in a heterogeneous environment where no single pollen could
3344 fulfil their requirements. Conducting free flight experiments where bees are presented with
3345 multiple mono-sterol foraging options would reveal if they are intentionally mixing sterols in this
3346 way. It would also suggest a capacity to detect pollen sterol content. Furthermore, previous feeding
3347 experiments on honeybees have shown their sterol proportions to alter when fed mono-sterol diets
3348 (Herbert *et al.*, 1980). Given the similarity in sterol profile between honey bees and bumble bees,
3349 bumble bee sterol proportions may also be changeable in response to experimental diets.

3350 Analysis of bumble bee internal parasites was not an intended outcome of this study; however, the
3351 opportunistic results obtained show that the parasites do not appear to convert phytosterols into
3352 cholesterol. Instead, they have adapted to the phytosterols available from their hosts. *Parasitellus*

3353 *fucorum* is an external mite of bumble bees which feeds upon wax, pollen and debris within nests.
3354 Its usage of phytosterols is therefore less surprising as its diet includes pollen. A more targeted
3355 investigation of bumble bee parasites and potential impacts upon the host sterolome would
3356 improve our understanding of these relationships.

3357 **Solitary bee sterols**

3358 The decision to retain guts within the Chapter 4 bee specimens meant there is the potential for
3359 pollen phytosterols to be detected in the analysis of the whole-body tissues. However, given the
3360 small size of many of these species and the removal of pollen from the outside of bodies, even with
3361 a full gut of pollen this would be unlikely to contribute to the overall sterol profile.

3362 Unlike bumble bees and honey bees which pack pollen into their corbiculae, solitary bees do not
3363 moisten and compact pollen in the same way. As such it was not possible to easily recover enough
3364 from their bodies for analysis. However, other authors have reported differences between the
3365 sterols present in bee-collected and hand-collected collected pollens (Vanderplanck, Zerck, *et al.*,
3366 2020b), therefore the sterols of solitary bee collected pollens merits further analysis.

3367 Previous work has shown generalist bumble bees and non-Asteraceae specialist solitary bees
3368 develop poorly on the pollen of Asteraceae (Praz, Müller and Dorn, 2008; Vanderplanck *et al.*,
3369 2018; Vanderplanck, Gilles, *et al.*, 2020). However, this work was conducted using pollen rather
3370 than artificial diets and so development may have been inhibited by other components of the pollen
3371 such as defence compounds. Determining whether generalist bees could develop on the sterol
3372 profiles shown by Asteraceae pollens may confirm whether in fact sterol profile is a key
3373 contributor to the Asteraceae paradox (Praz, Müller and Dorn, 2008).

3374 The sterol plasticity of solitary bee sterol profiles could be tested by feeding mono-sterol artificial
3375 diets as has been done for honey bees (Svoboda *et al.*, 1980). It may reveal greater plasticity than

3376 might be assumed from the foraging habits of some species. This would be particularly interesting
3377 for monoleptic bee species, as these had sterol requirements that can be obtained from other floral
3378 pollens. These species may be behaviourally or otherwise nutritionally limited to their host. The
3379 challenge of developing artificial diets and carrying out feeding trials with solitary bees however
3380 means this will likely not be possible for many species.

3381 Previous work has demonstrated that sterol proportions change during development (Svoboda *et*
3382 *al.*, 1980; Vanderplanck, Zerck, *et al.*, 2020a). However, this dataset analysed only adult females
3383 and so lacks this temporal information on sterol profile. Therefore, we cannot infer which sterols
3384 different bees may be using for the synthesis of steroid moulting hormones by changes in sterol
3385 proportions between developmental stages. Given the essential nature of moulting hormones for
3386 insect development, understanding these requirements may better inform pollen foraging choices.

3387 Finally, kleptoparasitic bees showed interesting patterns of similarity and dissimilarity with their
3388 hosts. Kleptoparasite-host pairs with strong similarity despite phylogenetic distance suggest it is
3389 possible for kleptoparasitic species to adapt their sterol requirements to make optimum use of the
3390 pollen provisioned by their host. By including the full suite of hosts for each kleptoparasite and
3391 ranking them by preference, or likely host in the area the kleptoparasite was collected, patterns of
3392 similarity-dissimilarity could be better mapped. The presence of kleptoparasite-host pairs with
3393 dissimilar sterol profiles suggests there are differences in the physiology governing sterol usage
3394 between these species as both will have had access to the same food. Testing the ability of
3395 kleptoparasitic species to develop on a range of pollen provisions, not only those gathered by its
3396 host species, would demonstrate whether these kleptoparasitic bees are limited by pollen
3397 requirements or other factors in their host usage.

3398 **Conclusion**

3399 Pollen sterols appear to have a strong similarity between closely related species and are potentially
3400 driven by the physiological requirements of the plant rather than pollinator relationships. Pollen
3401 contains a wide range of sterol compounds and shows greater diversity and variation in sterol
3402 proportions than bees. All bees analysed showed an essential requirement for the phytosterols
3403 acquired from dietary consumption of pollen. However, there were clear preferences for certain
3404 phytosterols, which occurred in high proportion across a wide range of species. Bumble bees are a
3405 genus of ecologically similar species, being generalist foragers with a colony structure and caste
3406 system. Therefore, their similarity in sterol profile may be the result of both their relatedness and
3407 similar life histories, despite floral preferences existing in some species. The range in sterol profile
3408 of solitary bees shows plasticity in phytosterol usage as they have evolved and adapted to different
3409 pollen foraging strategies. In summary, sterol nutrition represents a key link between flowering
3410 plants and pollinivorous bees. This thesis has demonstrated that bees have differing essential sterol
3411 requirements and are foraging to fulfil them in a highly heterogeneous landscape of available
3412 pollen sterols. Sterol content should therefore be considered a key nutritional group when
3413 designing artificial diets and analysing the nutritional provision of floral landscapes.

References

- 3415 Arbetman, M. P. *et al.* (2017) ‘Global decline of bumblebees is phylogenetically structured and
3416 inversely related to species range size and pathogen incidence’, *Proceedings of the Royal Society*
3417 *B: Biological Sciences*, 284(1859). doi: 10.1098/rspb.2017.0204.
- 3418 Bakker, J. D. (2024) *Applied Multivariate Statistics in R*. University of Washington. Available at:
3419 <https://uw.pressbooks.pub/appliedmultivariatestatistics/>.
- 3420 Balfour, N. J. *et al.* (2022) ‘DoPI: The Database of Pollinator Interactions’, *Ecology*, 103(11), pp.
3421 10–11. doi: 10.1002/ecy.3801.
- 3422 Baude, M. *et al.* (2016) ‘Historical nectar assessment reveals the fall and rise of floral resources in
3423 Britain’, *Nature*, 530(7588), pp. 85–88. doi: 10.1038/nature16532.
- 3424 Behmer, S. T. (2005) ‘Nutrition in Insects’, in *Encyclopedia of Entomology*. Dordrecht: Springer
3425 Netherlands, pp. 1577–1582. doi: 10.1007/0-306-48380-7_2923.
- 3426 Behmer, S. T. and Nes, W. D. (2003) ‘Insect Sterol Nutrition and Physiology: A Global
3427 Overview’, in Stephen Simpson (ed.) *Advances in Insect Physiology, Volume 31*. Academic Press,
3428 pp. 1–72.
- 3429 Bernard, F. A., Bernard, J. M. and Denny, P. (1990) ‘Flower Structure , Anatomy and Life History
3430 of *Wolffia australiana* (Benth .) den Hartog & van der Plas’, *Bulletin of the Torrey Botanical Club*,
3431 117(1), pp. 18–26. Available at: <http://www.jstor.org/stable/2997125?origin=crossref>.
- 3432 Biesmeijer, J. C. *et al.* (2006) ‘Parallel declines in pollinators and insect-pollinated plants in Britain
3433 and the Netherlands’, *Science*, 313(5785), pp. 351–354. doi: 10.1126/science.1127863.
- 3434 Brian, A. D. (1951) ‘The Pollen Collected by Bumble-Bees’, *Journal of Animal Ecology*, 20(2),
3435 pp. 191–194.
- 3436 Brown, M. J. F., Loosli, R. and Schmid-Hempel, P. (2000) ‘Condition-dependent expression of
3437 virulence in a trypanosome infecting bumblebees’, *Oikos*, 91(3), pp. 421–427. doi: 10.1034/j.1600-
3438 0706.2000.910302.x.
- 3439 De Cáceres, M. and Legendre, P. (2009) ‘Associations between species and groups of sites: indices
3440 and statistical inference’, *Ecology*, 90, pp. 3566–3574. doi: 10.1890/08-1823.1.
- 3441 Carvalho, M. *et al.* (2010) ‘Survival strategies of a sterol auxotroph’, *Development*, 137(21), pp.
3442 3675–3685. doi: 10.1242/dev.044560.
- 3443 Carvell, C. (2002) ‘Habitat use and conservation of bumblebees (*Bombus* spp.) under different
3444 grassland management regimes’, *Biological Conservation*, 103(1), pp. 33–49. doi: 10.1016/S0006-
3445 3207(01)00114-8.
- 3446 Chapman, R. F. (2013) *The Insects Structure and Function*. 5th edn. Edited by S. J. Simpson and
3447 A. E. Douglas. Cambridge University Press.

- 3448 Clark, A. J. and Bloch, K. (1959) 'The absence of sterol synthesis in insects.', *The Journal of*
3449 *biological chemistry*, 234(10), pp. 2578–2582. doi: 10.1016/s0021-9258(18)69741-8.
- 3450 Comont, R. (2017) *RSPB Spotlight Bumblebees*. Bloomsbury Natural History.
- 3451 Cooke, J. and Sang, J. H. (1970) 'Utilization of sterols by larvae of *Drosophila melanogaster*',
3452 *Journal of Insect Physiology*, 16(5), pp. 801–812. doi: 10.1016/0022-1910(70)90214-3.
- 3453 Desmond, E. and Gribaldo, S. (2009) 'Phylogenomics of Sterol Synthesis: Insights into the Origin,
3454 Evolution, and Diversity of a Key Eukaryotic Feature', *Genome Biology and Evolution*, 1, pp.
3455 364–381. doi: 10.1093/gbe/evp036.
- 3456 Dötterl, S. and Vereecken, N. J. (2010) 'The chemical ecology and evolution of bee-flower
3457 interactions: A review and perspectives', *Canadian Journal of Zoology*, 88(7), pp. 668–697. doi:
3458 10.1139/Z10-031.
- 3459 Edlund, A. F., Swanson, R. and Preuss, D. (2004) 'Pollen and stigma structure and function: The
3460 role of diversity in pollination', *Plant Cell*, 16(Supplement 1), pp. 84–97. doi: 10.1105/tpc.015800.
- 3461 Edwards, M. and Jenner, M. (2018) *Field Guide to the Bumblebees of Great Britain and Ireland*.
3462 Revised ed. Ocelli.
- 3463 Evtyugin, D. D. *et al.* (2023) 'Advances and Challenges in Plant Sterol Research: Fundamentals,
3464 Analysis, Applications and Production', *Molecules*, 28(18), pp. 1–45. doi:
3465 10.3390/molecules28186526.
- 3466 Falk, S. J. and Lewington, R. (2015) *Field guide to the bees of Great Britain and Ireland*. British
3467 Wildlife Publishing.
- 3468 Feldlaufer, M. F. *et al.* (1986) 'Biosynthesis of makisterone A and 20-hydroxyecdysone from
3469 labeled sterols by the honey bee, *Apis mellifera*', *Archives of Insect Biochemistry and Physiology*,
3470 3(5), pp. 415–421. doi: 10.1002/arch.940030502.
- 3471 Feldlaufer, M. F. *et al.* (1993) 'Neutral sterols and ecdysteroids of the solitary cactus bee *Diadasia*
3472 *rinconis* cockerell (hymenoptera: Anthophoridae)', *Archives of Insect Biochemistry and*
3473 *Physiology*, 23(2), pp. 91–98. doi: 10.1002/arch.940230205.
- 3474 Feldlaufer, M. F. and Harrison, D. J. (2020) 'Neutral sterols in honey bee (*Apis mellifera*) feces',
3475 *Journal of Apicultural Research*, 59(5), pp. 1033–1036. doi: 10.1080/00218839.2020.1753917.
- 3476 Feldlaufer, M. F., Svoboda, J. A. and E.W. Herbert, J. (1986) 'Makisterone A and 24-
3477 methylenecholesterol from the ovaries of the honey bee, *Apis mellifera* L.', *Experientia*, 42, pp.
3478 200–201. doi: 10.31857/s0475145021060021.
- 3479 Ferrer, A. *et al.* (2017) 'Emerging roles for conjugated sterols in plants', *Progress in Lipid*
3480 *Research*, 67, pp. 27–37. doi: 10.1016/j.plipres.2017.06.002.
- 3481 Fitzpatrick, Ú. *et al.* (2007) 'Rarity and decline in bumblebees - A test of causes and correlates in
3482 the Irish fauna', *Biological Conservation*, 136(2), pp. 185–194. doi: 10.1016/j.biocon.2006.11.012.
- 3483 Fowler, A. E. *et al.* (2020) 'Sunflower pollen reduces a gut pathogen in worker and queen but not

- 3484 male bumble bees', *Ecological Entomology*, 45(6), pp. 1318–1326. doi: 10.1111/een.12915.
- 3485 Franchi, G. G. *et al.* (1996) 'Types of carbohydrate reserves in pollen: Localization, systematic
3486 distribution and ecophysiological significance', *Flora*, 191(2), pp. 143–159. doi: 10.1016/S0367-
3487 2530(17)30706-5.
- 3488 Furse, S., Koch, H., *et al.* (2023) 'Sterol and lipid metabolism in bees', *Metabolomics*, 19(78), pp.
3489 1–19. doi: 10.1007/s11306-023-02039-1.
- 3490 Furse, S., Martel, C., *et al.* (2023) 'Sterol composition in plants is specific to pollen, leaf,
3491 pollination and pollinator', *Phytochemistry*, 214, pp. 1–10. doi: 10.1016/j.phytochem.2023.113800.
- 3492 Gallet, A. *et al.* (2003) 'Cholesterol modification of Hedgehog is required for trafficking and
3493 movement, revealing an asymmetric cellular response to Hedgehog', *Developmental Cell*, 4(2), pp.
3494 191–204. doi: 10.1016/S1534-5807(03)00031-5.
- 3495 Goulson, D. *et al.* (2005) 'Causes of rarity in bumblebees', *Biological Conservation*, 122(1), pp. 1–
3496 8. doi: 10.1016/j.biocon.2004.06.017.
- 3497 Goulson, D. *et al.* (2015) 'Bee declines driven by combined stress from parasites, pesticides, and
3498 lack of flowers', *Science*, 347(6229). doi: 10.1126/science.1255957.
- 3499 Goulson, D. and Darvill, B. (2004) 'Niche overlap and diet breadth in bumblebees; are rare species
3500 more specialized in their choice of flowers?', *Apidologie*, 35, pp. 55–63. doi: 10.1051/apido.
- 3501 Goulson, D., Lye, G. C. and Darvill, B. (2008) 'Decline and conservation of bumble bees', *Annual
3502 Review of Entomology*, 53, pp. 191–208. doi: 10.1146/annurev.ento.53.103106.093454.
- 3503 Hagedorn, H. H. and Burger, M. (1968) 'Effect of the Age of Pollen Used in Pollen Supplements
3504 on Their Nutritive Value for the Honeybee. II. Effect of Vitamin Content of Pollens', *Journal of
3505 Apicultural Research*, 7(2), pp. 97–101. doi: 10.1080/00218839.1968.11100196.
- 3506 Hanley, M. E. *et al.* (2008) 'Breeding system, pollinator choice and variation in pollen quality in
3507 British herbaceous plants', *Functional*, 22(4), pp. 592–598. doi: 10.1111/j.1365-
3508 2435.2008.01415.x.
- 3509 Henríquez-Piskulich, P., Hugall, A. F. and Stuart-Fox, D. (2024) 'A supermatrix phylogeny of the
3510 world's bees (Hymenoptera: Anthophila)', *Molecular Phylogenetics and Evolution*, 190. doi:
3511 10.1016/j.ympev.2023.107963.
- 3512 Herbert, E. W. *et al.* (1980) 'Sterol utilization in honey bees fed a synthetic diet: Effects on brood
3513 rearing', *Journal of Insect Physiology*, 26(5), pp. 287–289. doi: 10.1016/0022-1910(80)90135-3.
- 3514 Hicks, D. M. *et al.* (2016) 'Food for pollinators: Quantifying the nectar and pollen resources of
3515 urban flower meadows', *PLoS ONE*, 11(6), pp. 1–37. doi: 10.1371/journal.pone.0158117.
- 3516 Hines, H. M. (2008) 'Historical biogeography, divergence times, and diversification patterns of
3517 bumble bees (Hymenoptera: Apidae: Bombus)', *Systematic Biology*, 57(1), pp. 58–75. doi:
3518 10.1080/10635150801898912.
- 3519 Howard, D. C. *et al.* (2003) 'Estimating the extent and change in Broad Habitats in Great Britain',

- 3520 *Journal of Environmental Management*, 67(3), pp. 219–227. doi: 10.1016/S0301-4797(02)00175-
3521 5.
- 3522 Hutchinson, L. A. *et al.* (2021) ‘Using ecological and field survey data to establish a national list of
3523 the wild bee pollinators of crops’, *Agriculture, Ecosystems and Environment*, 315, pp. 1–9. doi:
3524 10.1016/j.agee.2021.107447.
- 3525 Ischebeck, T. (2016) ‘Lipids in pollen — They are different’, *Biochimica et Biophysica Acta -*
3526 *Molecular and Cell Biology of Lipids*, 1861(9), pp. 1315–1328. doi: 10.1016/j.bbalip.2016.03.023.
- 3527 Janson, E. M. *et al.* (2009) ‘Same host-plant, different sterols: Variation in sterol metabolism in an
3528 insect herbivore community’, *Journal of Chemical Ecology*, 35(11), pp. 1309–1319. doi:
3529 10.1007/s10886-009-9713-6.
- 3530 Jing, X. and Behmer, S. T. (2020) ‘Insect sterol nutrition: Physiological mechanisms, ecology, and
3531 applications’, *Annual Review of Entomology*, 65, pp. 251–271. doi: 10.1146/annurev-ento-011019-
3532 025017.
- 3533 Kamilar, J. M. and Cooper, N. (2013) ‘Phylogenetic signal in primate behaviour, ecology and life
3534 history’, *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368(1618). doi:
3535 10.1098/rstb.2012.0341.
- 3536 Keck, F. *et al.* (2016) ‘phylosignal: an R package to measure, test, and explore the phylogenetic
3537 signal’, *Ecology and Evolution*, 6(9), pp. 2774–2780. doi: 10.1002/ece3.2051.
- 3538 Kleijn, D. and Raemakers, I. (2008) ‘A retrospective analysis of pollen host plant use by stable and
3539 declining bumble bee species’, *Ecology*, 89(7), pp. 1811–1823. doi: 10.1890/07-1275.1.
- 3540 van Klink, R. *et al.* (2020) ‘Meta-analysis reveals declines in terrestrial but increases in freshwater
3541 insect abundances’, *Science*, 368(6489), pp. 417–420. doi: 10.1126/SCIENCE.AAX9931.
- 3542 Knittelfelder, O. *et al.* (2020) ‘Sterols as dietary markers for *Drosophila melanogaster*’, *Biochimica*
3543 *et Biophysica Acta - Molecular and Cell Biology of Lipids*, 1865(7), pp. 1–9. doi:
3544 10.1016/j.bbalip.2020.158683.
- 3545 Kriesell, L., Hilpert, A. and Leonhardt, S. D. (2017) ‘Different but the same: bumblebee species
3546 collect pollen of different plant sources but similar amino acid profiles’, *Apidologie*, 48(1), pp.
3547 102–116. doi: 10.1007/s13592-016-0454-6.
- 3548 Lau, P. *et al.* (2022) ‘Assessing pollen nutrient content: a unifying approach for the study of bee
3549 nutritional ecology’, *Phil. Trans. R. Soc. B*, 377(1853).
- 3550 Mandel, J. R. *et al.* (2019) ‘A fully resolved backbone phylogeny reveals numerous dispersals and
3551 explosive diversifications throughout the history of Asteraceae’, *Proceedings of the National*
3552 *Academy of Sciences of the United States of America*, 116(28), pp. 14083–14088. doi:
3553 10.1073/pnas.1903871116.
- 3554 Martínez-Bauer, A. E. *et al.* (2021) ‘Novel pollen analogue technique shows bumblebees display
3555 low floral constancy and prefer sites with high floral diversity’, *Landscape Ecology*, 36, pp. 3231–
3556 3247. doi: 10.1007/s10980-021-01304-9.

- 3557 Mascarenhas, J. P. (1993) ‘Molecular mechanisms of pollen tube growth and differentiation’, *The*
3558 *Plant Cell*, 5(10), pp. 1303–1314. doi: 10.2307/3869783.
- 3559 Mellema, S. *et al.* (2002) ‘The ethanolic fermentation pathway supports respiration and lipid
3560 biosynthesis in tobacco pollen’, *The Plant Journal*, 30(3), pp. 329–336. doi: 10.1046/j.1365-
3561 313X.2002.01293.x.
- 3562 Michez, D. *et al.* (2008) ‘Phylogeny and host-plant evolution in Melittidae s.l. (Hymenoptera :
3563 Apoidea)*’, *Apidologie*, 39, pp. 146–162.
- 3564 Moerman, R. *et al.* (2017) ‘Pollen nutrients better explain bumblebee colony development than
3565 pollen diversity’, *Insect Conservation and Diversity*, 10(2), pp. 171–179. doi: 10.1111/icad.12213.
- 3566 Mondy, N. and Corio-Costet, M. F. (2000) ‘The response of the grape berry moth (*Lobesia*
3567 *botrana*) to a dietary phytopathogenic fungus (*Botrytis cinerea*): The significance of fungus
3568 sterols’, *Journal of Insect Physiology*, 46(12), pp. 1557–1564. doi: 10.1016/S0022-1910(00)00085-
3569 8.
- 3570 Müller, A. and Kuhlmann, M. (2008) ‘Pollen hosts of western palaeartic bees of the genus
3571 *Colletes* (Hymenoptera: Colletidae): The Asteraceae paradox’, *Biological Journal of the Linnean*
3572 *Society*, 95(4), pp. 719–733. doi: 10.1111/j.1095-8312.2008.01113.x.
- 3573 Narayanan, S. *et al.* (2016) ‘Wheat leaf lipids during heat stress: I. High day and night
3574 temperatures result in major lipid alterations’, *Plant Cell and Environment*, 39(4), pp. 787–803.
3575 doi: 10.1111/pce.12649.
- 3576 National Biodiversity Data Centre (2022) *Protecting rare pollinators: Great Yellow Bumblebee,*
3577 *All-Ireland Pollinator Plan How-to Guide 6*. 19.
- 3578 Natural England (2013) *NCA Profile: 04 Cheviots (NE457)*. Available at:
3579 <https://publications.naturalengland.org.uk/publication/4820746454958080?category=587130>.
- 3580 Natural England (2015) *NCA Profile: 132 Salisbury Plain and West Wiltshire Downs (NE479)*.
3581 Available at:
3582 <https://publications.naturalengland.org.uk/publication/5001829523914752?category=587130>.
- 3583 Nowakowski, M. and Pywell, R. (2016) *Habitat Creation and Management for Pollinators*. Centre
3584 for Ecology & Hydrology, Wallingford, UK. doi: 10.1080/00016470052943982.
- 3585 Ogle, D. H. *et al.* (2023) ‘FSA: Simple Fisheries Stock Assessment Methods’. Available at:
3586 <https://cran.r-project.org/package=FSA>.
- 3587 Oksanen, J. *et al.* (2022) ‘vegan: Community Ecology Package’. Available at: [https://cran.r-](https://cran.r-project.org/package=vegan)
3588 [project.org/package=vegan](https://cran.r-project.org/package=vegan).
- 3589 OpenTreeOfLife *et al.* (2019) ‘Open Tree of Life Synthetic Tree Version 3.2’. Zenodo. doi:
3590 10.5281/zenodo.3937742.
- 3591 Pacini, E. and Hesse, M. (2005) ‘Pollenkitt - Its composition, forms and functions’, *Flora:*
3592 *Morphology, Distribution, Functional Ecology of Plants*, 200(5), pp. 399–415. doi:
3593 10.1016/j.flora.2005.02.006.

- 3594 Paludo, C. R. *et al.* (2018) ‘Stingless Bee Larvae Require Fungal Steroid to Pupate’, *Scientific*
3595 *Reports*, 8(1), pp. 1–10. doi: 10.1038/s41598-018-19583-9.
- 3596 Paradis, E. and Schliep, K. (2019) ‘ape 5.0: an environment for modern phylogenetics and
3597 evolutionary analyses in R’, *Bioinformatics*, 35, pp. 526–528. doi: 10.1093/bioinformatics/bty633.
- 3598 Pardee, G. L. *et al.* (2022) ‘Life-history traits predict responses of wild bees to climate variation’,
3599 *Proceedings of the royal society B*, 289.
- 3600 Pelletier, L. and McNeil, J. N. (2003) ‘The effect of food supplementation on reproductive success
3601 in bumblebee field colonies’, *Oikos*, 103(3), pp. 688–694. doi: 10.1034/j.1600-0706.2003.12592.x.
- 3602 Pennell, M. W. *et al.* (2014) ‘geiger v2.0: an expanded suite of methods for fitting
3603 macroevolutionary models to phylogenetic trees’, *Bioinformatics*, 30, pp. 2216–2218. doi:
3604 10.1093/bioinformatics/btu181.
- 3605 Pernal, S. F. and Currie, R. W. (2000) ‘Pollen quality of fresh and 1-year-old single pollen diets for
3606 worker honey bees (*Apis mellifera* L.)’, *Apidologie*, 31(3), pp. 387–409. doi:
3607 10.1051/apido:2000130.
- 3608 Pinheiro, J., Bates, D. and R Core Team (2023) ‘nlme: Linear and Nonlinear Mixed Effects
3609 Models’. Available at: <https://cran.r-project.org/package=nlme>.
- 3610 Posit team (2023) ‘RStudio: Integrated Development Environment for R’. Boston, MA. Available
3611 at: <http://www.posit.co/>.
- 3612 Potts, S. G. *et al.* (2003) ‘Linking bees and flowers: How do floral communities structure
3613 pollinator communities?’, *Ecology*, 84(10), pp. 2628–2642. doi: 10.1890/02-0136.
- 3614 Potts, S. G. *et al.* (2010) ‘Global pollinator declines: Trends, impacts and drivers’, *Trends in*
3615 *Ecology and Evolution*, 25(6), pp. 345–353. doi: 10.1016/j.tree.2010.01.007.
- 3616 Praz, C. J., Müller, A. and Dorn, S. (2008) ‘Specialized bees fail to develop on non-host pollen: do
3617 plants chemically protect their pollen’, *Ecology*, 89(3), pp. 795–804.
- 3618 R Core Team (2023) ‘R: A Language and Environment for Statistical Computing’. Vienna,
3619 Austria. Available at: <https://www.r-project.org/>.
- 3620 R Hackathon *et al.* (2020) ‘phylobase: Base Package for Phylogenetic Structures and Comparative
3621 Data’. Available at: <https://cran.r-project.org/package=phylobase>.
- 3622 Raine, N. E. and Rundlöf, M. (2024) ‘Pesticide Exposure and Effects on Non-*Apis* Bees’, *Annual*
3623 *Review of Entomology*, 69, pp. 551–576. doi: 10.1146/annurev-ento-040323-020625.
- 3624 Requier, F. *et al.* (2017) ‘The carry-over effects of pollen shortage decrease the survival of
3625 honeybee colonies in farmlands’, *Journal of Applied Ecology*, 54(4), pp. 1161–1170. doi:
3626 10.1111/1365-2664.12836.
- 3627 Roberts, D. W. (2023) ‘labdsv: Ordination and Multivariate Analysis for Ecology’. Available at:
3628 <https://cran.r-project.org/package=labdsv>.
- 3629 Rotheray, E. L., Osborne, J. L. and Goulson, D. (2017) ‘Quantifying the food requirements and

- 3630 effects of food stress on bumble bee colony development’, *Journal of Apicultural Research*, 56(3),
3631 pp. 288–299. doi: 10.1080/00218839.2017.1307712.
- 3632 Roulston, T. H. and Cane, J. H. (2002) ‘The effect of pollen protein concentration on body size in
3633 the sweat bee *Lasioglossum zephyrum* (Hymenoptera: Apiformes)’, *Evolutionary Ecology*, 16(1),
3634 pp. 49–65. doi: 10.1023/A:1016048526475.
- 3635 Roulston, T. H., Cane, J. H. and Buchmann, S. L. (2000) ‘What governs protein content of pollen:
3636 Pollinator preferences, pollen-pistil interactions, or phylogeny?’, *Ecological Monographs*, 70(4),
3637 pp. 617–643. doi: 10.1890/0012-9615(2000)070[0617:WGPCOP]2.0.CO;2.
- 3638 Roulston, T. H. I. and Cane, J. H. (2000) ‘Pollen nutritional content and digestibility for animals’,
3639 *Plant Systematics and Evolution*, 222, pp. 187–209.
- 3640 Rundlöf, M. *et al.* (2014) ‘Late-season mass-flowering red clover increases bumble bee queen and
3641 male densities’, *Biological Conservation*, 172, pp. 138–145. doi: 10.1016/j.biocon.2014.02.027.
- 3642 Russo, L. *et al.* (2020) ‘Warming increases pollen lipid concentration in an invasive thistle, with
3643 minor effects on the associated floral-visitor community’, *Insects*, 11(1), pp. 1–10. doi:
3644 10.3390/insects11010020.
- 3645 Santerre, R. (2023) *The nutritional constraint of sterols in the origin and evolution of bees.*
3646 University of Mons.
- 3647 Scheper, J. *et al.* (2014) ‘Museum specimens reveal loss of pollen host plants as key factor driving
3648 wild bee decline in the Netherlands’, *Proceedings of the National Academy of Sciences of the*
3649 *United States of America*, 111(49), pp. 17552–17557. doi: 10.1073/pnas.1412973111.
- 3650 Schwery, O. and O’Meara, B. C. (2016) ‘MonoPhy: a simple R package to find and visualize
3651 monophyly issues’, *PeerJ Computer Science*, 2, p. e56. doi: 10.7717/peerj-cs.56.
- 3652 Slowikowski, K. (2023) ‘ggrepel: Automatically Position Non-Overlapping Text Labels with
3653 “ggplot2”’. Available at: <https://cran.r-project.org/package=ggrepel>.
- 3654 Somme, L. *et al.* (2015) ‘Pollen and nectar quality drive the major and minor floral choices of
3655 bumble bees’, *Apidologie*, 46(1), pp. 92–106. doi: 10.1007/s13592-014-0307-0.
- 3656 Steffan, S. A. *et al.* (2019) ‘Omnivory in bees: Elevated trophic positions among all major bee
3657 families’, *American Naturalist*, 194(3), pp. 414–421. doi: 10.1086/704281.
- 3658 Svoboda, J. A. *et al.* (1980) ‘Sterol utilization in honey bees fed a synthetic diet: Analysis of
3659 prepupal sterols’, *Journal of Insect Physiology*, 26(5), pp. 291–294. doi: 10.1016/0022-
3660 1910(80)90135-3.
- 3661 Svoboda, J. A. *et al.* (1983) ‘Comparison of sterols of pollens, honeybee workers, and prepupae
3662 from field sites’, *Archives of Insect Biochemistry and Physiology*, 1(1), pp. 25–31. doi:
3663 10.1002/arch.940010104.
- 3664 Svoboda, J. A. *et al.* (1986) ‘Selective sterol transfer in the honey bee: Its significance and
3665 relationship to other hymenoptera’, *Lipids*, 21(1), pp. 97–101.

- 3666 Svoboda, J. A. and Feldlaufer, M. F. (1991) 'Neutral sterol metabolism in insects', *Lipids*, 26, pp.
3667 614–618.
- 3668 Svoboda, J. A., Herbert, E. W. and Thompson, M. J. (1983) 'Definitive evidence for lack of
3669 phytosterol dealkylation in honey bees', *Experientia*, 39(10), pp. 1120–1121. doi:
3670 10.1007/BF01943139.
- 3671 Svoboda, J. A., Herbert, E. W. and Thompson, M. J. (1986) 'Sterols of organs involved in brood
3672 food production and of royal jelly in honey bees', *Insect Biochemistry*, 16(3), pp. 479–482. doi:
3673 10.1016/0020-1790(86)90024-7.
- 3674 Svoboda, J. A. and Lusby, W. R. (1986) 'Sterols of phytophagous and omnivorous species of
3675 Hymenoptera', *Archives of Insect Biochemistry and Physiology*, 3(1), pp. 13–18. doi:
3676 10.1002/arch.940030103.
- 3677 Svoboda, J. A. and Thompson, M. J. (1987) 'Variability in Steroid Metabolism Among
3678 Phytophagous Insects', in *Ecology and Metabolism of Plant Lipids*. American Chemical Society,
3679 pp. 176–186. doi: 10.1021/bk-1987-0325.ch011.
- 3680 Timberlake, T. P., Vaughan, I. P. and Memmott, J. (2019) 'Phenology of farmland floral resources
3681 reveals seasonal gaps in nectar availability for bumblebees', *Journal of Applied Ecology*, 56(7), pp.
3682 1585–1596. doi: 10.1111/1365-2664.13403.
- 3683 Ugine, T. A. *et al.* (2019) 'Prey nutrient content creates omnivores out of predators', *Ecology*
3684 *Letters*, 22(2), pp. 275–283. doi: 10.1111/ele.13186.
- 3685 Valitova, J. N., Sulkarnayeva, A. G. and Minibayeva, F. V. (2016) 'Plant sterols: Diversity,
3686 biosynthesis, and physiological functions', *Biochemistry (Moscow)*, 81(8), pp. 819–834. doi:
3687 10.1134/S0006297916080046.
- 3688 Vanderplanck, M. *et al.* (2014) 'How does pollen chemistry impact development and feeding
3689 behaviour of polylectic bees?', *PLoS ONE*, 9(1), pp. 1–9. doi: 10.1371/journal.pone.0086209.
- 3690 Vanderplanck, M. *et al.* (2017) 'The importance of pollen chemistry in evolutionary host shifts of
3691 bees', *Scientific Reports*, 7, pp. 1–10. doi: 10.1038/srep43058.
- 3692 Vanderplanck, M. *et al.* (2018) 'Is non-host pollen suitable for generalist bumblebees?', *Insect*
3693 *Science*, 25(2), pp. 259–272. doi: 10.1111/1744-7917.12410.
- 3694 Vanderplanck, M., Gilles, H., *et al.* (2020) 'Asteraceae Paradox : Chemical and Mechanical
3695 Protection of Taraxacum Pollen', *Insects*, 11(5). doi: <https://doi.org/10.3390/insects11050304>.
- 3696 Vanderplanck, M., Zerck, P.-L., *et al.* (2020a) 'Generalized host-plant feeding can hide sterol-
3697 specialized foraging behaviors in bee–plant interactions', *Ecology and Evolution*, 10(1), pp. 150–
3698 162. doi: 10.1002/ece3.5868.
- 3699 Vanderplanck, M., Zerck, P.-L., *et al.* (2020b) 'Sterol addition during pollen collection by bees:
3700 another possible strategy to balance nutrient deficiencies?', *Apidologie*, 51(5), pp. 826–843. doi:
3701 10.1007/s13592-020-00764-3.
- 3702 Vaudo, A. D., Dyer, L. A. and Leonard, A. S. (2023) 'Pollen nutrition structures bee and plant

- 3703 community interactions’, 121(3), pp. 1–11. doi: 10.1073/pnas.
- 3704 Villette, C. *et al.* (2015) ‘Plant Sterol Diversity in Pollen from Angiosperms’, *Lipids*, 50(8), pp.
3705 749–760. doi: 10.1007/s11745-015-4008-x.
- 3706 Weete, J. D. (1973) ‘Sterols of the fungi: Distribution and biosynthesis’, *Phytochemistry*, 12(8), pp.
3707 1843–1864. doi: 10.1016/S0031-9422(00)91502-4.
- 3708 Wickham, H. (2016) *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.
3709 Available at: <https://ggplot2.tidyverse.org>.
- 3710 Wickham, H. *et al.* (2019) ‘Welcome to the Tidyverse’, *Journal of Open Source Software*, 4(43), p.
3711 1686. doi: 10.21105/joss.01686.
- 3712 Williams, N. M. and Lonsdorf, E. V. (2018) ‘Selecting cost-effective plant mixes to support
3713 pollinators’, *Biological Conservation*, 217, pp. 195–202. doi: 10.1016/j.biocon.2017.10.032.
- 3714 Williams, P. H. *et al.* (2008) ‘A simplified subgeneric classification of the bumblebees (genus
3715 *Bombus*)’*, *Apidologie*, 39, pp. 46–74.
- 3716 Wood, T. J. *et al.* (2019) ‘Narrow pollen diets are associated with declining Midwestern bumble
3717 bee species’, *Ecology*, 100(6). doi: 10.1002/ecy.2697.
- 3718 Wood, T. J. *et al.* (2021) ‘Global patterns in bumble bee pollen collection show phylogenetic
3719 conservation of diet’, *Journal of Animal Ecology*, 90(10), pp. 2421–2430. doi: 10.1111/1365-
3720 2656.13553.
- 3721 Yourstone, J., Varadarajan, V. and Olsson, O. (2023) ‘Bumblebee flower constancy and pollen
3722 diversity over time’, *Behavioral Ecology*, 34(4), pp. 602–612. doi: 10.1093/beheco/acad028.
- 3723 Yu, G. *et al.* (2017) ‘ggtree: an R package for visualization and annotation of phylogenetic trees
3724 with their covariates and other associated data.’, *Methods in Ecology and Evolution*, 8(1), pp. 28–
3725 36. doi: 10.1111/2041-210X.12628.
- 3726 Yu, G. *et al.* (2018) ‘Two methods for mapping and visualizing associated data on phylogeny using
3727 ggtree.’, *Molecular Biology and Evolution*, 35(2), pp. 3041–3043. doi: 10.1093/molbev/msy194.
- 3728 Yu, G. (2020) ‘Using ggtree to Visualize Data on Tree-Like Structures’, *Current Protocols in
3729 Bioinformatics*, 69(1), p. e96. doi: 10.1002/cpbi.96.
- 3730 Yu, G. (2023) ‘aplot: Decorate a “ggplot” with Associated Information’. Available at:
3731 <https://cran.r-project.org/package=aplot>.
- 3732 Zanne, A. E. *et al.* (2014) ‘Three keys to the radiation of angiosperms into freezing environments’,
3733 *Nature*, 506, pp. 89–92. doi: 10.1038/nature12872.
- 3734 Zu, P. *et al.* (2021) ‘Pollen sterols are associated with phylogeny and environment but not with
3735 pollinator guilds’, *New Phytologist*, 230(3), pp. 1169–1184. doi: 10.1111/nph.17227.
- 3736