Australian native flower colours: does nectar

reward drive bee pollinator flower preferences?

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Abstract

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24   Colour is an important signal that flowering plants use to attract insect pollinators

25   like bees. Previous research in Germany has shown that nectar volume is higher for flower

26   colours that are innately preferred by European bees, suggesting an important link between

27   colour signals, bee preferences and floral rewards. In Australia, flower colour signals have

28   evolved in parallel to the Northern hemisphere to enable easy discrimination and detection

29   by the phylogenetically ancient trichromatic visual system of bees, and native Australian

30   bees also possess similar innate colour preferences to European bees. We measured 59

31   spectral signatures from flowers present at two preserved native habitats in South Eastern

32   Australia and tested whether there were any significant differences in the frequency of

33   flowers presenting higher nectar rewards depending upon the colour category of the flower

34   signals, as perceived by bees. We also tested if there was a significant correlation between

35   chromatic contrast and the frequency of flowers presenting higher nectar rewards. For the

36   entire sample, and for subsets excluding species in the Asteraceae and Orchidaceae, we

37   found no significant difference among colour categories in the frequency of high nectar

38   reward. This suggests that whilst such relationships between flower colour signals and

39   nectar volume rewards have been observed at a field site in Germany, the effect is likely to

40   be specific at a community level rather than a broad general principle that has resulted in

41   the common signalling of bee flower colours around the world.

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Introduction

Many floral traits play a role in the reproduction of animal-pollinated angiosperms [1–5]. Colour is one of the most important signals used by flowering plants to communicate to their pollinators [6–10]. Flowers typically present nutritional rewards like nectar to entice floral visitors [6,11–15], and nectar is a reward that can promote learning and neural changes in a bees visual system [16]. What relationship between floral colour signals and nectar as a reward that promotes motivation in insect pollinators, should evolve in plants? Whilst nectar has been well studied in flowering plants [15,17–20], the question of a potential relationship has been rarely considered because many animals have very different colour vision to humans [21]. Thus, colour is not an unambiguous trait, and to test colour as a factor in a biologically meaningful way it is necessary to map how relevant pollinators like bees perceive and use colour information.

Both male and female fitness of plants should often benefit from pollinator visits, especially given widespread pollen-limitation of seed set [22]. Such fitness benefits for plants will be especially strong with flower visitors like bees that tend to be 'flower constant,' that is, to use colour signals to repeatedly visit flowers of the same plant species [23]. In turn, the ability of pollinators to assess which floral colour signals are more reliable predictors of nutritional rewards will affect the foraging success of individuals [24] and thus the subsequent success of bee colonies [14,25–27]. This leads to an interesting hypothesis that bees may have evolved colour preferences because visiting flowers with higher rewards improves foraging performance while flowers gain increased pollination services by signaling higher rewards in a reciprocal selection loop that promotes the evolution of certain flower colours [28].

71   Colour vision requires multiple photoreceptors with different sensitivities (Jacobs

72   2018). The spectral sensitivities of photoreceptors in many bee species have been

73   empirically determined, showing that the trichromatic colour vision of bees is highly

74   conserved and predates the evolution of flowers [29]. To yield colour information,

75   photoreceptor signals have to be antagonistically processed in a brain [21]. Such colour

76   opponent mechanisms in bees have been empirically recorded [30–32]. Knowing this

77   information enables the construction of a colour space that accurately represents colour

78   information, and the colour Hexagon is an opponent colour space that represents the visual

79   capabilities of trichromatic bees [33]. In the current manuscript we use capitals (e.g.

80   BLUE) to convey a region in bee colour space (e.g. see Fig. 1a), and 'blue' to refer to how

81   humans typically describe colour stimuli, following the convention proposed by [34]).

82   Interestingly, both honeybees and bumblebees show similar distinct preferences for

83   short wavelength 'blue' stimuli that frequently have loci in the UV-BLUE, BLUE and/or

84   BLUE-GREEN sectors of bee colour space [28,35–40]. It has also been recently shown

85   that native bees in Australia show a significant colour preference for stimuli in the BLUE

86   and BLUE-GREEN regions of bee colour space [41,42]. These potentially common bee

87   colour preferences provide a plausible explanation for why bee pollinated flowers around

88   the world frequently share similar distributions in colour space [43].

89   Floral colour distributions in natural plant communities have recently been

90   documented from a geographically wide array of sites [3,44–52] including several recent

91   studies that use sophisticated modeling of pollinator colour vision [43,52–54]. Where bees

92   are present in both the Northern [3,46,55] and Southern [56–58] Hemispheres, flowers tend

93   to have evolved colour signals that are efficiently processed by bee trichromatic vision. In

94   Australia it has been observed at both a continental level [56,58] and a local community

95   level [43,54] that flower colours share a very similar distribution in colour space to flower

96 colour distributions in the Northern Hemisphere where bees with a blue preference are the

97 dominant pollinator. Evolutionary change in floral colour appears not to be strongly

98 limited by phylogenetic constraints [43,46,47,53,58], and it is of high value to understand

99 what does promote very consistent flower colouration around the world when considering

100 the visual capabilities of bees.

101 In a study at a field site near Berlin, Germany, flowers were most frequently found

102 to be in the UV-BLUE region of colour space and these flowers also contained higher

103 nectar rewards [28]. In the current study, we seek to understand if floral colours at a

104 community level in Australia also show a significant association with nectar rewards that is

105 consistent with the pattern found in Germany [28], and thus whether floral nectar reward

106 might be an important driver linking bee preferences and flower colours.

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109 # Materials and Methods

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111 ## Sites and data collection

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113 We collected data from two natural communities in central Victoria, Australia:

114 Baluk Willam Flora Reserve (37°55′32″S, 145°20′45″E), 40 km south east of Melbourne,

115 and Boomers Reserve (37°37′39″S, 145°15′21″E), approximately 35 km north east of

116 Melbourne. Both sites are *Eucalyptus* woodland with well-developed shrub and herb layers

117 containing a high diversity of orchid species. These communities have been protected to

118 maintain native vegetation. Flowers were sampled from March 2010 to May 2011. Species

119 were identified with the aid of several local floras [59–66]. A list of species included in

120 this study is given in online Appendix A.

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Nectar measurement

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124   We used the floral nectar data of 59 bee pollinated flowering plant species in our

125 analysis. Nectar collection and measurement methods are detailed in [54]. Briefly, newly

126 opened flowers were place in pollinator exclusion nets for 24 hours to allow nectar

127 accumulation. Flowers were then excised, and soluble sugars were extracted by immersing

128 whole flowers in known volume of distilled water followed by an acid treatment to reduce

129 all sugars into hexoses. Subsequently, we measured the concentration of soluble sugars

130 using standard spectrophotometric methods and back calculated the total sugar content of

131 each flower [67]. Quantity of sucrose present in each flowering plant species is listed in

132 online supplementary [54] and Appendix A.

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Colour measurement

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136   Reflectance spectra from 300 to 700 nm wavelength were measured on a minimum

137 of three flowers for each species using an Ocean Optics spectrophotometer (Dunedin,

138 Florida, USA) with a PX-2 pulsed xenon light source. A UV-reflecting white standard was

139 used to calibrate the spectrophotometer. Spectra from multiple flowers were averaged

140 within each species. For flowers with multiple colours, such as areas with and without a

141 UV component, we obtained reflectance spectra of the two colours covering the largest

142 surface area of the flower.

Colour space representation

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145   Floral reflectance spectra were converted to positions in a hexagonal colour space,

146   a two-dimensional representation of the excitation levels of the three different classes of

147   photoreceptors in a hymenopteran insect's visual system [33]. This model is widely

148   accepted as a representation of bee trichromatic vision in comparative studies of flower

149   evolution [3,28,34,39,41,43,50,53,55,56,68,69]. The exact photoreceptor sensitivities of

150   native Australian bees are currently unknown, but relying on the phylogenetic conservation

151   of spectral sensitivity peaks of hymenopteran photoreceptors [29], we use a general

152   hymenopteran visual model based on a vitamin A1 template for photopigments [70] with

153   sensitivity peaks at 350 nm (ULTRAVIOLET: UV), 440 nm (BLUE: B) and 540 nm

154   (GREEN: G) (cf. [56]). We calculated the relative probability of photon capture (*P*) by

155   each of the UV, B, and G photoreceptors by numerically integrating the product of the

156   spectral sensitivity function of each one of the (*i* = 3) photoreceptors $S_i(\lambda)$, the diffuse

157   spectral reflectance of each flower $I(\lambda)$ and the spectral distribution of the ambient

158   illumination $D(\lambda)$ expressed as photon flux [71]. All spectral functions were expressed

159   from 300 to 650 nm at 10 nm steps:

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161    $P_i = R_i \int_{300}^{650} S_i(\lambda)I(\lambda)D(\lambda)d\lambda$.   (1)

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163   The coefficient R_i in equation 1 represents von Kries adaptation and is used to normalize

164   each of the photoreceptors to the illumination reflected from the background [33]:

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166    $R_i = 1 / \int_{300}^{650} S_i(\lambda)I_B(\lambda)D(\lambda)d\lambda$,   (2)

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168   where $I_B(\lambda)$ is the spectral reflectance of the background. We used the average reflectance

169   from 20 species of *Eucalyptus* (Average Green Leaf) as background reflectance ($I_B(\lambda)$) for

170   our calculations. We used a open sky, daylight ambient illumination equivalent to CIE

171   6,500 K [72], that represent typical daylight conditions for foraging insects [73]. The

172   transduction of photoreceptor captures (P) into receptor excitations (E) is given by

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174    $E = P/P+1.$ (3)

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176   The receptor excitations (E_{UV}, E_B *and* E_G) are plotted on orthogonal axes, each of

177   unit length, and the colour locus of a flower is the vector sum of the individual excitations.

178   A colour locus can be represented by Cartesian coordinates in a hexagonal space using

179   equations 4 [33]:

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181    $x = \sin(60°)(E_G - E_{UV})$ (4a)

182    $y = E_B - 0.5(E_G - E_{UV}).$ (4b)

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184   Colour contrast in Hexagon space was calculated as the Euclidean distance from the centre

185   of the colour space, representing the adapting background, to the locus of a flower [74].

186   Hexagon coordinates for all flower species are given in online Appendix A.

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188   Data analysis

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190   Colour categories

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192   For each species, we calculated the polar coordinates (angle and magnitude) of the

193   floral colour locus in the hexagonal colour model (Fig 1a). The angle is a measurement of

194   'hue' in the hexagon space [33]. Samples were subsequently classified into one of the six

195   colour categories proposed by [34] based on their respective hue value: BLUE (B), BLUE-

196   GREEN (BG). GREEN (G), UV-GREEN (UG), UV (U), and UV-BLUE (UB).

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Does sugar content vary among colour categories?

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200   Flowers were subsequently classified as either having a 'high' or 'low' soluble sugar

201   content relative to the median soluble sugar amount for the entire flower sample, following

202   the method used in [28,75,76]. We used a set of contingency tables to test for significant

203   differences in proportion of high and low soluble sugar content per color category, against

204   a null hypothesis of equality of proportion per color group. Contingency tables excluded

205   the single sample present in the UV color group (see results section for details). To avoid

206   problems associated with using the χ^2 distribution with small sample sizes in some colour

207   categories, probability values from the chi-square test were obtained using 100,000 Monte

208   Carlo simulations.

209   A second contingency test was conducted after excluding three species from the

210   family Asteraceae because the soluble sugar content for these species was measured from a

211   compound 'head' rather than individual flowers. Median soluble sugar amount was thus

212   recalculated from the remaining species and the response variable was reformulated using

213   the updated soluble sugar threshold value.

214   Finally, we constructed a third table excluding plant species in both Asteraceae and

215   orchidaceae, given the prevalence of potential food deception in many orchids [77,78]. As

216   before, the median soluble sugar content was updated, and the remaining flower species

217   were subsequently reclassified as being high or low.

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219   Correlation between sugar content and chromatic contrast

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221   In addition to hue, chromatic contrast with a background (see *Colour space*

222   *representation* above) is an element of colour that may be relevant to pollinators. We

223   tested for a potential correlation between chromatic contrast of flower loci to the leaf green

224   background in hexagon colour space and soluble sugar content. The analysis was done first

225   on the entire data set, and subsequently for the two data subsets following the same

226   rationale used for the contingency tests. All correlation tests were performed using

227   Kendall's tau (τ) statistic as the test makes no assumptions on the underlying distribution

228   of the data [79]. All analyses were performed using R base package version 3.6.1 (05-07-

229   2019).

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231

232   **Fig 1: Flower colour and nectar in Australian native plant flowers. a.** Distribution of

233   59 flowering plant species in hexagon colour space: non-orchids (●) and orchids (∗). b.

234   Frequency of sampled species classified on each of the six Hexagon categories along with

235   the corresponding global pattern of distribution (red line) of plant species taken from the

236   surveys of plant communities in Germany, Australia, and Nepal [34,43,46,56]. c. Plant

237   flower soluble sugars by colour category; thick lines represent medians, boxes represent

238   the 25% and 75% interquartile ranges, and thin vertical bars represent 2.5 and 97.5 %

239   quantiles of the data distribution. Names of the different Hexagon sectors are abbreviated:

240   BLUE (B), BLUE-GREEN (BG). GREEN (G), UV-GREEN (UG), UV (U), and UV-

241   BLUE (UB) as described by [34].

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Results

The distribution of species among hexagon sectors appeared uneven (Fig 1a, b), as did the distribution of soluble floral sugar per sector (Fig 1c). Only one sample was classified into the UV hexagon sector: *Hypericum pygmae* (Clusiaceae) and this hexagon sector was thus excluded from all subsequent analyses. The scarcity of flowers in UV sector is consistent with previous studies [34].

Median soluble sugar content per flower ± median absolute deviation (MAD) for the sample excluding *Hypericum pygmae* was 392 ± 377 μg. Following categorization of species based on this threshold value (Fig 2a), we found no significant difference among hexagon sectors in the proportion of species with a high sugar content ($\chi^2 = 3.97$, $P = 0.466$).

Results from the second contingency table (median sugar soluble sugar = 383 ± 359 μg), which excluded species from the family Asteraceae also failed to reject the null hypothesis of equality in the proportion of species with a high amount of soluble sugar ($\chi^2 = 2.15$, $P = 0.765$, Fig 2b).

Fig 2: Proportion of species with a 'high' amount of soluble sugar for each one of the five categories including (panel a) and excluding species from the family Asteraceae (panel b).

268   Finally, the threshold soluble sugar value for non-orchid, non-aster species

269   contingency table was 367 ± 406 µg. For the third model we also found no evidence

270   rejecting the null hypothesis of equality in the proportion of high-reward species among

271   the different colour categories ($\chi^2 = 2.97$, $P = 0.693$). This result thus suggests that the low

272   amount of soluble sugars present in orchid species at our field site was not a factor

273   affecting a potentially the outcome of the initial model.

274   Chromatic contrast revealed no significant relation to floral sugar content at our

275   field site. Tests using Kendall's tau (τ) statistic failed to reject the null hypothesis of

276   independence between soluble nectar content and chromatic contrast for all the subsets

277   considered (Table 1).

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281   **Table 1**: Results of correlation test testing for a potential relation between soluble sugar
282   content and chromatic contrast considering various sample subsets: Complete data set
283   including all flowers, data set excluding the only species allocated to the UV hexagon
284   sector *Hypericum pygmae*, subset also excluding family Asteraceae, and all non-orchid
285   species. A non-parametric (Kendall tau (τ)) correlation coefficient was calculated in all
286   cases.

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Data set	τ	P
Complete data set	-0.016	0.86
Excluding flower in UV sector	-0.019	0.83
Excluding Asteraceae	-0.059	0.528
Excluding Orchidaceae	0.003	0.999

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Discussion

It was hypothesised by Darwin [80] that insects may evolve innate preferences to aid the efficient location of profitable flowers, and bees do have both innate spatial [5,81] and colour [28,35–40] preferences. In Germany, where important bee species like honeybees and bumbles have innate preferences for short wavelength blue flowers, it was found that flowers in the UV-BLUE category contained significantly higher volumes of nectar than those in other hue categories in bee colour space [28]. Several studies suggest that nectar volume can influence the behaviour of foraging bees [11,12,19,20,82]. Indeed, it has also been shown that introduced species with flowers that contain higher volume rewards can out-compete resident flowers by attracting more bee pollinators [83].

Nonetheless, flower colours in different parts of the world have very similar distributions in bee colour space [56], which is also consistent with evidence that bees have phylogenetically conserved colour visual systems including preferences for short wavelength stimuli [29,43]. Thus, understanding whether flowers in different communities have colours that predict higher reward levels in a consistent fashion is of value for understanding what traits promote bee choices, and the potential major drivers that influence flower signal evolution.

In the current study we considered flower colour signaling from two communities in south-eastern Australia that had similar flower colours in bee colour space, and were also similar to bee pollinated flower colours found elsewhere across Australia and around the world [3,43,46,55,56]. We found no evidence that a particular hue predicted a higher reward level within flowers. These results from native plant communities suggest that a simple, direct link between flower rewards and the preferred bee hue of 'blue' is not an explanation of flower colour preferences in Australia, and may not be in other locations

318   around the world. Reciprocal selection between plants and their pollinators can reach

319   different evolutionary equilibria among local populations, leading to a geographic mosaic

320   of trait values of the interacting parties [84]. The previously observed higher rewards for

321   bee preferred blue flowers in Germany [28] may thus be a local equilibrium, and it will be

322   of value to map more communities to understand if and how flower colour predicts nectar

323   rewards around the world.

324   Given the evidence that bee colour preferences may influence how flowers evolve

325   similar spectral signals at several different locations around the world [34,43,46,56,57,85],

326   it is interesting to consider what traits other than nectar rewards might promote bee

327   preferences. Plausible alternative lines of investigation could include how the spectral

328   overlap of photoreceptors when combined with opponent processes at a neural level

329   enhance both colour discrimination [55,86–88] and colour detection [89] in a way that is

330   most efficient for finding flowers [90]. This in turn could enhance neural mechanisms to

331   promote innate colour preferences. By itself, this mechanism of spectral tuning cannot be

332   the sole explanation for a stronger blue preference, since there is also spectral overlap and

333   enhanced signal processing at longer wavelengths [55,85]. However, many common

334   background stimuli reflect at longer wavelengths [91], so having innate brain preferences

335   for shorter wavelength 'blue' stimuli might enable bees to efficiently detect stimuli that

336   have a very high probability of being a rewarding flower given that very few natural

337   colours are blue. Interestingly, UV absorbing flowers that appear 'white' to humans are

338   very common within this short wavelength range of preferred colours in bee colour space,

339   and additionally have the advantage of having strong modulation of the long wavelength

340   bee receptor that is implicated in enhancing signal detection at a distance [68,92–95].

341   To better understand the complexities of these multiple complex factors it is

342   important to collect more flower data at a plant community level around the world, as well

343   as continue to map the sensory capabilities of different pollinators [16,87].

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356   **Author contributions**: MS, AGD and MB designed the study. MS conducted the

357   field work, data collections, plant identification. MS and MB did the data curations and lab

358   experiment. MS, and AGD mapped the floral reflectance spectra to the bee-vision colour

359   space model. MS, JEG, MB, AGD performed the statistical analyses. All authors

360   interpreted the results and wrote the manuscript. The authors declare that they have no

361   conflict of interest involving the work reported here.

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631

Supplementary information:

633

634 **Appendix** A: provides the data used in current analysis. The '.csv' file includes hexagon

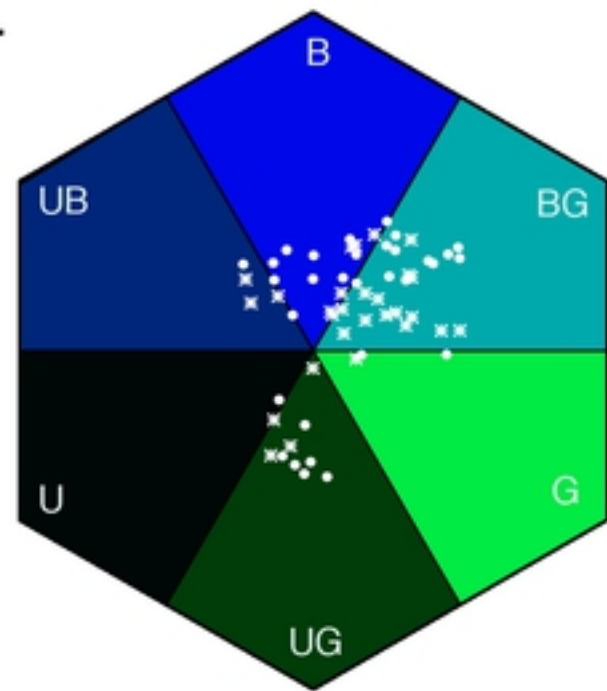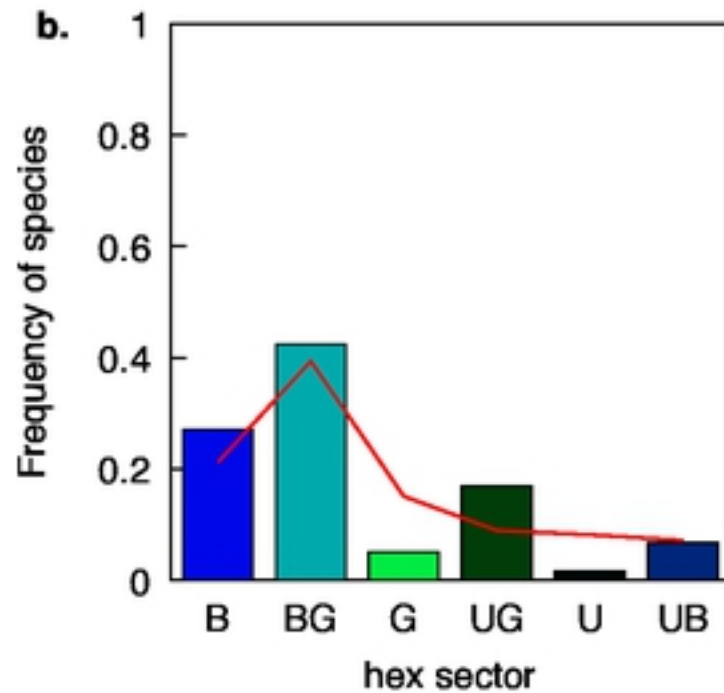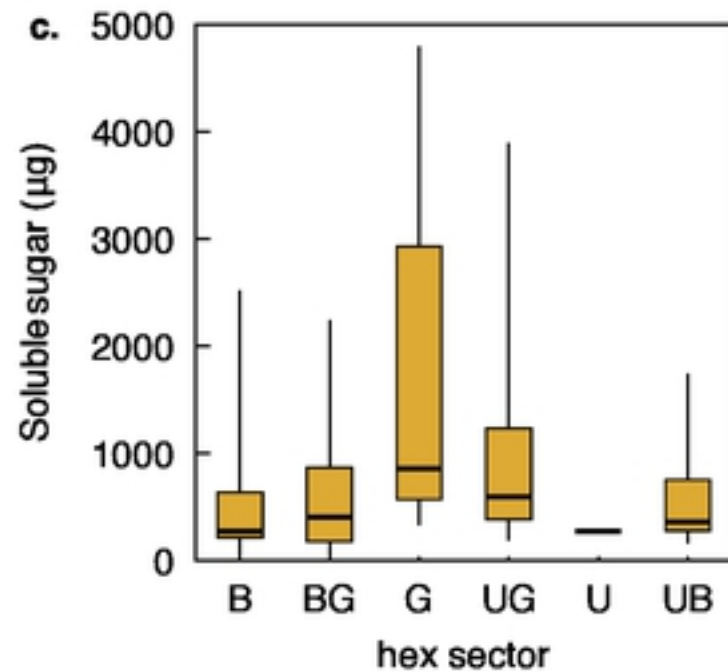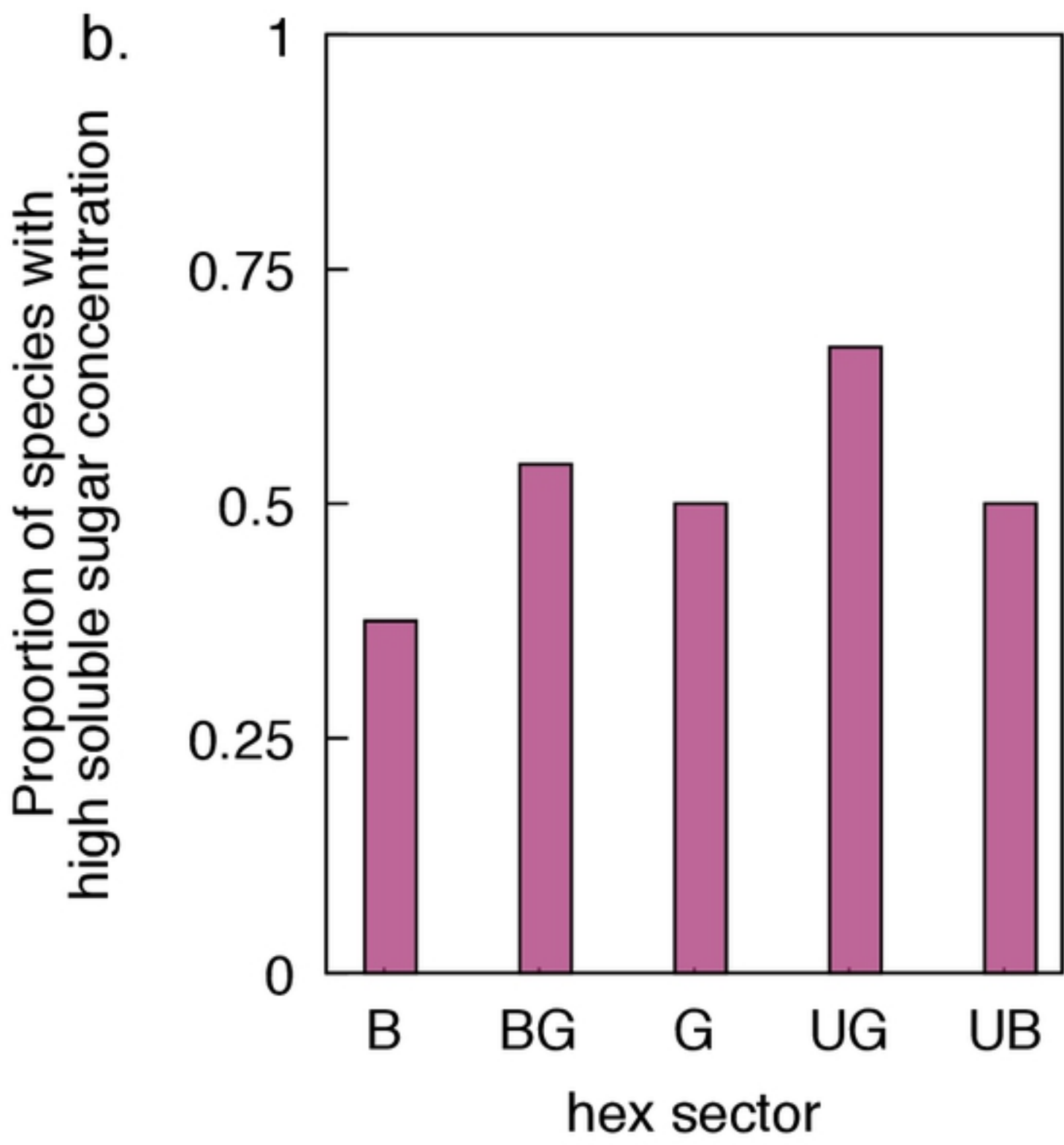635 x and y unit, sucrose amount (microgram) and pollination categorization.

Figure 1

Figure 2