Effects and Detection of Specialized Metabolites in Bumblebees

Supplementary Material

Specialized metabolites in floral resources: effects and detection in buff-tailed bumblebees

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Table S1. Purity, provider, CAS (Chemical Abstracts Service) number and naturally occurring concentrations in fresh pollen of the specialized metabolites used during the experiments. Provider, Sigma-Aldrich.

| Specialized metabolite$^{(1)}$ | CAS number | Naturally occurring concentrations in fresh pollen | Source |
|-------------------------------|------------|---------------------------------------------------|--------|
| Amygdalin ≥97%                | 29883-15-6 | 1,889 ppm                                         | London-Shafir et al. (2003) |
| Sinigrin hydrate ≥99%          | 3952-98-5  | 1,892 ppm$^{(2)}$                                 | Ares et al. (2015) |
| Scopolamine hydrochloride ≥90%| 55-16-3    | 20,014 ppm                                        | Detzel & Wink (1993) |

$^{(1)}$ Purity as well as hydrochloride and water molecules were taken into account for calculation of the substance content in pollen diets for bumblebee bioassays and in test solutions for PER experiments.

$^{(2)}$ Original data was expressed as µg/g of dry pollen mass (2.226 µg/g)
Table S2. Different score grades for the five criteria in histological examination.

| Criterion 1: Disorganization or loss of the brush-like border | Score | Description |
|-------------------------------------------------------------|-------|-------------|
|                                                             | 0     | The brush-like border was homogeneous with well-developed microvilli |
|                                                             | 1     | Focal disorganization or focal loss of the brush-like border (in maximum two cells per villus) |
|                                                             | 2     | Disorganization or loss of the brush-like border in less than 10% of villus intestinal epithelial cells |
|                                                             | 3     | Disorganization or loss of the brush-like border in 10-25% of villus intestinal epithelial cells |
|                                                             | 4     | Disorganization or loss of the brush-like border in 25-50% of villus intestinal epithelial cells |
|                                                             | 5     | Disorganization or loss of the brush-like border in more than 50% of villus intestinal epithelial cells |

| Criterion 2: Vacuolization of the epithelial cells (hydropic degeneration) | Score | Description |
|--------------------------------------------------------------------------|-------|-------------|
|                                                                         | 0     | The morphology of digestive cells appeared to be normal without cytoplasmic vacuolization |
|                                                                         | 1     | Focal cytoplasmic vacuolization (in maximum three cells per villus) |
|                                                                         | 2     | Cytoplasmic vacuolization (hydropic degeneration) in less than 10% of villus intestinal epithelial cells |
|                                                                         | 3     | Cytoplasmic vacuolization (hydropic degeneration) in 10-25% of villus intestinal epithelial cells |
|                                                                         | 4     | Cytoplasmic vacuolization (hydropic degeneration) in 25-50% of villus intestinal epithelial cells |
|                                                                         | 5     | Cytoplasmic vacuolization (hydropic degeneration) in more than 50% of villus intestinal epithelial cells |

| Criterion 3: Interstitial edema | Score | Description |
|---------------------------------|-------|-------------|
|                                 | 0     | Absence of interstitial edema in the connective tissue that forms the central axes of intestinal crypts |
|                                 | 1     | Mild interstitial edema in maximum two intestinal villi |
|                                 | 2     | Severe interstitial edema in less than 10% of intestinal villi |
|                                 | 3     | Severe interstitial edema in 10-25% of intestinal villi |
|                                 | 4     | Severe interstitial edema in 25-50% of intestinal villi |
|                                 | 5     | Severe interstitial edema in more than 50% of intestinal villi |
Table S2. Continued.

**Criterion 4: Apoptosis**

| Score | Description |
|-------|-------------|
| 0     | The morphology of digestive cells appeared to be normal without pyknotic nucleus. The nuclei had a smooth and regular appearance. |
| 1     | Apoptosis features in focal areas with pyknotic nuclei in maximum three cells per villus |
| 2     | Karyolysis, pyknosis or karyorrhexis in less than 10% of villus intestinal epithelial cells |
| 3     | Karyolysis, pyknosis or karyorrhexis in 10-25% of villus intestinal epithelial cells |
| 4     | Karyolysis, pyknosis or karyorrhexis in 25-50% of villus intestinal epithelial cells |
| 5     | Karyolysis, pyknosis or karyorrhexis in more than 50% of villus intestinal epithelial cells |

**Criterion 5: Necrosis**

| Score | Description |
|-------|-------------|
| 0     | No necrotic cells were observed both in the base and at the apex of the intestinal crypts that remained well shaped |
| 1     | Isolated necrotic cells (maximum two cells per villus) as well as cellular debris observed in the mesenteron lumen |
| 2     | Mild tissue necrosis in less than 10% of intestinal villi with necrotic areas of more than 30%, several cellular debris observed in the mesenteron lumen |
| 3     | Severe tissue necrosis in 10-25% of intestinal villi with necrotic areas of more than 30%, several cellular debris observed in the mesenteron lumen |
| 4     | Severe tissue necrosis in 25-50% of intestinal villi with necrotic areas of more than 50%, several cellular debris observed in the mesenteron lumen |
| 5     | Severe tissue necrosis in more than 50% of intestinal villi with necrotic areas of more than 50%, several cellular debris observed in the mesenteron lumen |
| 6     | Severe tissue necrosis in 100% of intestinal villi with complete desquamation of the intestinal epithelial cell layer |
Table S3. Effects of diet treatments on *B. terrestris* in micro-colonies, mean (SE) (n = 10 for each treatment). Different letters indicate significant differences (p < 0.05) (post-hoc Tukey analyses with FDR adjustment).

| Parameters                              | Amygdalin                        | Statistics<sup>(1)</sup>          |
|-----------------------------------------|-----------------------------------|-----------------------------------|
|                                        | 0% (Control) | 50% | 100% | 200% | χ² | df | p     |
| Pollen collection (g)                   | 19.52 (1.62) | 21.42 (0.96) | 20.14 (0.99) | 17.59 (1.49) | a | 6.78 | 3 | 0.079 |
| Syrup collection (g)                    | 88.77 (4.38) | 92.65 (2.24) | 89.17 (2.86) | 84.00 (3.05) | 4.82 | 3 | 0.018 |
| Pollen dilution                         | 4.66 (0.20)  | 4.37 (0.14)  | 4.47 (0.12)  | 4.98 (0.29)  | 7.55 | 3 | 0.006 |
| Mass of total eclosed offspring (g)     | 8.31 (0.62)  | 9.23 (0.64)  | 8.53 (0.43)  | 7.32 (0.60)  | 5.92 | 3 | 0.016 |
| Pollen efficiency                       | 0.43 (0.01)  | 0.43 (0.02)  | 0.43 (0.02)  | 0.42 (0.03)  | 0.10 | 3 | 0.991 |
| Larval ejection (%)                     | 22.83 (4.32) | 20.28 (3.33) | 21.80 (2.82) | 16.87 (3.25) | 3.19 | 3 | 0.363 |

| Parameters                              | Scopolamine                      | Statistics<sup>(1)</sup>          |
|                                        | 0% (Control) | 50% | 100% | 200% | χ² | df | p     |
| Pollen collection (g)                   | 10.62 (0.59) | 10.66 (0.55) | 12.30 (0.85) | 10.12 (0.82) | 5.69 | 3 | 0.128 |
| Syrup collection (g)                    | 76.33 (5.34) | 77.54 (2.61) | 81.03 (4.08) | 80.11 (3.02) | 2.12 | 3 | 0.549 |
| Pollen dilution                         | 7.22 (0.33)  | 7.40 (0.32)  | 6.71 (0.28)  | 7.65 (0.50)  | 3.77 | 3 | 0.287 |
| Mass of total eclosed offspring (g)     | 8.06 (0.66)  | 8.38 (0.82)  | 9.19 (0.51)  | 7.08 (0.86)  | 5.64 | 3 | 0.131 |
| Pollen efficiency                       | 0.76 (0.05)  | 0.78 (0.05)  | 0.76 (0.04)  | 0.74 (0.04)  | 0.74 | 3 | 0.684 |
| Larval ejection (%)                     | 25.48 (6.54) | 20.89 (5.50) | 24.98 (3.44) | 27.27 (6.39) | 0.91 | 3 | 0.684 |
Table S3. Continued.

| Parameters | Sinigrin | Statistics$^{(1)}$ |
|------------|---------|-------------------|
|             | 0% (Control) | 50% | 100% | 200% | $\chi^2$ = 1.79, df = 3, $p = 0.618$ |
| Number of eggs | 13 (6) | 18 (6) | 14 (6) | 15 (6) | $\chi^2 = 2.73$, df = 3, $p = 0.435$ |
| Number of non-isolated larvae | 31 (10) | 31 (6) | 35 (7) | 30 (10) |
| Number of pre-defecating larvae | 7 (2) | 8 (1) | 10 (2) | 7 (2) | $\chi^2 = 1.61$, df = 3, $p = 0.658$ |
| Number of post-defecating larvae | 4 (1) $^a$ | 3 (1) $^{ab}$ | 1 (1) $^b$ | 1 (1) $^b$ | $\chi^2 = 11.31$, df = 3, $p = 0.010$ |
| Number of pupae | 5 (1) | 7 (2) | 6 (1) | 6 (1) | $\chi^2 = 4.07$, df = 3, $p = 0.534$ |
| Number of non-emerged drones | 1 (1) | 1 (0) | 1 (0) | 0 (0) | $\chi^2 = 6.07$, df = 3, $p = 0.108$ |
| Number of emerged drones | 10 (1) | 8 (2) | 11 (1) | 7 (1) |

$^{(1)}\chi^2$: value of the type II Wald chi square, df: degree of freedom, $p$: p-value of the type II Wald chi square. Significant results are depicted in bold ($p < 0.05$).
Table S4. Digestive damage scores from the different diet treatments, min-max (median) (n = 3-4). Different letters indicate significant differences (p < 0.05) (post-hoc analyses). Scores significantly higher than control are depicted in bold.

| Diet treatment     | Criteria 1                          | Criteria 2                  | Criteria 3                          | Criteria 4                          | Criteria 5                          | TDS\(^{(2)}\)  |
|--------------------|-------------------------------------|----------------------------|-------------------------------------|-------------------------------------|-------------------------------------|----------------|
| Control            | 0-2 (0.5)\(^{\text{bcd}}\)         | 1-2 (1.5)\(^{\text{de}}\)  | 0-2 (1)\(^{\text{cd}}\)           | 1-3 (2)\(^{\text{bc}}\)           | 1-3 (2)\(^{\text{bc}}\)           | 4-10 (7.5)\(^{\text{bc}}\) |
| Amygdalin 50%     | 5-5 (5)\(^{\text{a}}\)             | 5-5 (5)\(^{\text{a}}\)     | 5-5 (5)\(^{\text{a}}\)           | 5-5 (5)\(^{\text{a}}\)           | 4-6 (5)\(^{\text{a}}\)           | 24-26 (25)\(^{\text{a}}\) |
| Amygdalin 100%    | 3-5 (4)\(^{\text{a}}\)             | 2-5 (3.5)\(^{\text{abc}}\) | 1-5 (4)\(^{\text{abc}}\)         | 2-5 (4.5)\(^{\text{a}}\)         | 2-6 (4)\(^{\text{ab}}\)          | 11-26 (19.5)\(^{\text{a}}\) |
| Amygdalin 200%    | 1-4 (3)\(^{\text{ab}}\)            | 3-4 (3.5)\(^{\text{abc}}\) | 1-3 (3)\(^{\text{abcd}}\)        | 3-5 (4)\(^{\text{ab}}\)          | 2-3 (3)\(^{\text{ab}}\)          | 13-18 (15.5)\(^{\text{ab}}\) |
| Scopolamine 50%   | 1-5 (5)\(^{\text{a}}\)             | 2-5 (5)\(^{\text{ab}}\)    | 1-5 (5)\(^{\text{ab}}\)          | 5-5 (5)\(^{\text{a}}\)           | 2-6 (4.5)\(^{\text{ab}}\)         | 11-26 (24.5)\(^{\text{a}}\) |
| Scopolamine 100%  | 0-1 (0.5)\(^{\text{cd}}\)          | 1-1 (1)\(^{\text{e}}\)     | 0-1 (0.5)\(^{\text{d}}\)         | 0-1 (0)\(^{\text{d}}\)           | 0-1 (0.5)\(^{\text{d}}\)         | 1-4 (2.5)\(^{\text{c}}\)       |
| Scopolamine 200%  | 0-5 (0)\(^{\text{bcd}}\)            | 1-5 (1)\(^{\text{cde}}\)   | 0-5 (1)\(^{\text{bcd}}\)         | 0-5 (1)\(^{\text{bcd}}\)         | 1-5 (1)\(^{\text{bcd}}\)          | 3-25 (3)\(^{\text{bc}}\)        |
| Sinigrin 50%      | 0-1 (0)\(^{\text{d}}\)             | 1-2 (1)\(^{\text{de}}\)    | 0-1 (0.5)\(^{\text{d}}\)         | 0-2 (1)\(^{\text{cd}}\)          | 1-1 (1)\(^{\text{cd}}\)          | 2-7 (3.5)\(^{\text{c}}\)       |
| Sinigrin 100%     | 0-5 (3)\(^{\text{abc}}\)            | 1-5 (3)\(^{\text{abcd}}\)  | 0-5 (2.5)\(^{\text{abcd}}\)      | 1-5 (4)\(^{\text{ab}}\)          | 0-6 (3)\(^{\text{ab}}\)          | 2-26 (15.5)\(^{\text{ab}}\)   |
| Sinigrin 200%     | 0-0 (0)\(^{\text{d}}\)             | 0-1 (1)\(^{\text{e}}\)     | 0-2 (0.5)\(^{\text{d}}\)         | 0-2 (1)\(^{\text{cd}}\)          | 0-1 (0.5)\(^{\text{d}}\)         | 1-5 (3)\(^{\text{c}}\)         |

Statistics\(^{(1)}\):
\[
\chi^2 = 23.42, \text{ df} = 9, \quad p = 0.005 \\
\chi^2 = 23.79, \text{ df} = 9, \quad p = 0.005 \\
\chi^2 = 17.90, \text{ df} = 9, \quad p = 0.036 \\
\chi^2 = 26.20, \text{ df} = 9, \quad p = 0.002 \\
\chi^2 = 24.04, \text{ df} = 9, \quad p = 0.004 \\
\chi^2 = 24.02, \text{ df} = 9, \quad p = 0.004
\]

\(^{(1)}\)\(\chi^2\): value of the Kruskal-Wallis tests, df: degree of freedom, p: p-value of the the Kruskal-Wallis tests. Significant results are depicted in bold (p < 0.05).

\(^{(2)}\)Total sum of damage scores.
### Table S5. Preference tests of specialized metabolites in *B. terrestris*, mean (SE) (n = 14-15 for each treatment). Different letters indicate significant differences (p < 0.05) (post-hoc Tukey analyses).

| Diet treatment            | Parameters                               |
|---------------------------|------------------------------------------|
|                           | Volume of solution consumed (mL) | Cumulative duration of feeding bouts (s) | Duration of the first contact (s) | Total duration of effective feeding (s) | Number of feeding bouts |
| Positive control (syrup)  | 28.95 (2.83)ab                       | 51.50 (7.38)ab                         | 14.69 (3.73)ab                     | 48.52 (7.45)a                   | 2 (0)b                  |
| Negative control (quinine)| 21.64 (7.24)d                        | 12.59 (5.42)c                         | 1.35 (0.50)d                       | 8.78 (4.44)c                    | 2 (0)b                  |
| Amygdalin 50%             | 18.40 (3.13)d                        | 35.94 (5.88)ab                        | 7.55 (3.98)c                       | 34.05 (5.74)ab                  | 2 (0)b                  |
| Amygdalin 100%            | 23.01 (3.67)bcd                       | 48.56 (10.33)a                        | 10.03 (7.95)c                      | 46.78 (10.25)ab                 | 1 (0)b                  |
| Amygdalin 200%            | 29.04 (4.88)abc                       | 53.19 (10.26)a                        | 33.99 (9.83)a                      | 51.13 (10.27)ab                 | 2 (0)b                  |
| Scopolamine 50%           | 37.81 (4.95)a                         | 54.94 (9.27)ab                        | 27.41 (7.78)a                      | 49.82 (8.80)ab                  | 5 (0)a                  |
| Scopolamine 100%          | 32.76 (5.09)ab                        | 42.94 (6.82)ab                        | 16.39 (3.60)ab                     | 37.49 (5.85)ab                  | 5 (0)a                  |
| Scopolamine 200%          | 34.23 (4.77)a                         | 48.13 (7.78)ab                        | 21.23 (6.37)ab                     | 37.01 (6.27)ab                  | 5 (0)a                  |
| Sinigrin 50%              | 23.01 (5.64)cd                        | 34.49 (7.75)b                         | 6.21 (2.53)cd                      | 32.61 (7.69)b                   | 2 (0)b                  |
| Sinigrin 100%             | 23.43 (5.37)bcd                       | 57.23 (10.65)a                        | 28.27 (10.90)ab                    | 50.04 (10.00)ab                 | 2 (0)b                  |
| Sinigrin 200%             | 18.14 (2.68)d                         | 57.75 (8.13)a                         | 12.87 (5.28)bc                     | 48.70 (7.34)a                   | 2 (0)b                  |

#### Statistics(1)

|     | χ² = 30.42, df = 10, p < 0.001 | χ² = 74.38, df = 10, p < 0.001 | χ² = 57.64, df = 10, p < 0.001 | χ² = 86.77, df = 10, p < 0.001 | χ² = 91.63, df = 10, p < 0.001 |

(1) χ²: value of the type II Wald chi square, df: degree of freedom, p: p-value of the type II Wald chi square. Significant results are depicted in bold (p < 0.05).