|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Population** | **Family ID** | **GOPH** | **ASCU** | **AINC** | **ASFA** | **ASYR** | **ASPEC** | **Total** |
| **Australia** | AU\_1\_2017 | 9 | 3 | 0 | 2 | 3 | 0 | 17 |
| AU\_A1\_2018 | 3 | 0 | 1 | 1 | 1 | 2 | 8 |
| AU\_A10\_2018 | 1 | 0 | 1 | 0 | 1 | 0 | 3 |
| AU\_A11\_2018 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| AU\_A12\_2018 | 2 | 2 | 1 | 1 | 2 | 2 | 10 |
| AU\_A13\_2018 | 1 | 2 | 1 | 0 | 1 | 2 | 7 |
| AU\_A14\_2018 | 1 | 0 | 1 | 1 | 0 | 1 | 4 |
| AU\_A2\_2018 | 1 | 2 | 2 | 2 | 1 | 1 | 9 |
| AU\_A5\_2018 | 2 | 1 | 1 | 0 | 2 | 0 | 6 |
| AU\_A8\_2018 | 1 | 0 | 1 | 0 | 1 | 0 | 3 |
| AU\_A9\_2018 | 1 | 1 | 0 | 1 | 1 | 0 | 4 |
|  | | **23** | **11** | **9** | **8** | **13** | **8** | **72** |
|  | |  |  |  |  |  |  |  |
| **Population** | **Family ID** | **GOPH** | **ASCU** | **AINC** | **ASFA** | **ASYR** | **ASPEC** | **Total** |
| **California** | CA\_1017.1B\_2018 | 0 | 1 | 0 | 0 | 1 | 0 | 2 |
| CA\_11\_2018 | 1 | 1 | 0 | 0 | 0 | 1 | 3 |
| CA\_13\_2017 | 1 | 0 | 0 | 1 | 0 | 0 | 2 |
| CA\_13\_2018 | 0 | 1 | 1 | 2 | 1 | 2 | 7 |
| CA\_2\_2017 | 1 | 1 | 0 | 2 | 1 | 0 | 5 |
| CA\_2\_2018 | 2 | 1 | 1 | 1 | 1 | 2 | 8 |
| CA\_23\_2018 | 0 | 1 | 1 | 1 | 1 | 1 | 5 |
| CA\_3\_2018 | 5 | 2 | 2 | 2 | 1 | 3 | 15 |
| CA\_5\_2017 | 1 | 2 | 0 | 1 | 1 | 0 | 5 |
| CA\_605.1\_2018 | 0 | 1 | 1 | 0 | 1 | 1 | 4 |
| CA\_7\_2017 | 1 | 0 | 0 | 0 | 1 | 0 | 2 |
| CA\_7\_2018 | 1 | 1 | 0 | 1 | 2 | 1 | 6 |
| CA\_8\_2017 | 1 | 1 | 0 | 0 | 2 | 0 | 4 |
| CA\_9\_2017 | 1 | 0 | 0 | 1 | 1 | 0 | 3 |
| CA\_946.1\_2018 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| CA\_X3\_2018 | 3 | 1 | 2 | 2 | 1 | 2 | 11 |
|  | | **18** | **14** | **8** | **15** | **15** | **13** | **83** |
|  | |  |  |  |  |  |  |  |
| **Population** | **Family ID** | **GOPH** | **ASCU** | **AINC** | **ASFA** | **ASYR** | **ASPEC** | **Total** |
| **Eastern North America** | ENA\_1\_2017 | 4 | 3 | 0 | 1 | 2 | 0 | 10 |
| ENA\_2\_2017 | 2 | 2 | 0 | 2 | 0 | 0 | 6 |
| ENA\_47\_2017 | 1 | 0 | 0 | 1 | 0 | 0 | 2 |
| ENA\_56\_2017 | 1 | 1 | 0 | 1 | 2 | 0 | 5 |
| ENA\_70\_2017 | 1 | 1 | 0 | 0 | 0 | 0 | 2 |
| ENA\_A\_2018 | 1 | 2 | 1 | 1 | 1 | 2 | 8 |
| ENA\_B\_2018 | 2 | 1 | 2 | 1 | 3 | 1 | 10 |
| ENA\_C\_2018 | 1 | 1 | 2 | 1 | 0 | 1 | 6 |
| ENA\_P\_2018 | 2 | 2 | 2 | 1 | 3 | 3 | 13 |
| ENA\_Q\_2018 | 3 | 1 | 2 | 1 | 2 | 2 | 11 |
| ENA\_S\_2018 | 2 | 1 | 2 | 1 | 0 | 1 | 7 |
|  | | **20** | **15** | **11** | **9** | **15** | **10** | **80** |
| **Population** | **Family ID** | **GOPH** | **ASCU** | **AINC** | **ASFA** | **ASYR** | **ASPEC** | **Total** |
| Guam | GU\_10\_2018 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| GU\_13\_2018 | 0 | 1 | 0 | 1 | 0 | 1 | 3 |
| GU\_19\_2018 | 2 | 2 | 1 | 1 | 2 | 2 | 10 |
| GU\_21\_2018 | 1 | 1 | 0 | 0 | 0 | 0 | 2 |
| GU\_25\_2018 | 2 | 1 | 1 | 1 | 1 | 1 | 7 |
| GU\_26\_2018 | 2 | 1 | 1 | 1 | 1 | 0 | 6 |
| GU\_30\_2018 | 0 | 1 | 0 | 0 | 1 | 0 | 2 |
| GU\_32\_2018 | 1 | 1 | 0 | 0 | 1 | 0 | 3 |
| GU\_38\_2018 | 2 | 1 | 1 | 1 | 1 | 2 | 8 |
| GU\_40\_2018 | 1 | 1 | 1 | 0 | 0 | 1 | 4 |
| GU\_41\_2018 | 1 | 1 | 1 | 0 | 1 | 1 | 5 |
| GU\_43\_2018 | 3 | 2 | 2 | 1 | 2 | 2 | 12 |
| GU\_901.1B\_2018 | 1 | 1 | 1 | 0 | 0 | 0 | 3 |
|  | | **16** | **15** | **9** | **6** | **10** | **10** | **66** |
|  | |  |  |  |  |  |  |  |
| **Population** | **Family ID** | **GOPH** | **ASCU** | **AINC** | **ASFA** | **ASYR** | **ASPEC** | **Total** |
| Hawaii | HI\_1\_2018 | 3 | 2 | 2 | 2 | 2 | 2 | 13 |
| HI\_10\_2017 | 2 | 3 | 0 | 1 | 3 | 0 | 9 |
| H1\_17\_2018 | 0 | 1 | 0 | 1 | 1 | 0 | 3 |
| Hi\_19\_2017 | 0 | 1 | 0 | 1 | 1 | 0 | 3 |
| HI\_19\_2018 | 2 | 1 | 1 | 1 | 0 | 1 | 6 |
| HI\_2\_2018 | 3 | 2 | 2 | 1 | 1 | 3 | 12 |
| HI\_20\_2017 | 1 | 0 | 0 | 1 | 0 | 0 | 2 |
| HI\_21\_2018 | 2 | 0 | 1 | 1 | 0 | 0 | 4 |
| HI\_22\_2017 | 6 | 3 | 0 | 1 | 2 | 0 | 12 |
| HI\_5\_2017 | 1 | 1 | 0 | 1 | 0 | 0 | 3 |
| HI\_5\_2018 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| HI\_944.2\_2018 | 1 | 0 | 2 | 0 | 0 | 1 | 4 |
|  | | **21** | **14** | **9** | **11** | **10** | **7** | **72** |
|  | |  |  |  |  |  |  |  |
| **Population** | **Family ID** | **GOPH** | **ASCU** | **AINC** | **ASFA** | **ASYR** | **ASPEC** | **Total** |
| Puerto Rico | PR\_103\_2018 | 2 | 1 | 1 | 2 | 1 | 1 | 8 |
| PR\_105\_2018 | 2 | 2 | 0 | 0 | 2 | 1 | 7 |
| PR\_107\_2018 | 1 | 2 | 2 | 0 | 2 | 2 | 9 |
| PR\_109\_2018 | 2 | 0 | 0 | 0 | 1 | 1 | 4 |
| PR\_111\_2018 | 2 | 2 | 2 | 2 | 2 | 2 | 12 |
| PR\_112\_2018 | 2 | 2 | 1 | 2 | 0 | 1 | 8 |
| PR\_113\_2018 | 0 | 1 | 2 | 0 | 1 | 0 | 4 |
| PR\_P1\_2018 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| PR\_PM2\_2018 | 1 | 1 | 0 | 0 | 0 | 0 | 2 |
| PR\_PM4\_2018 | 2 | 2 | 2 | 2 | 2 | 2 | 12 |
|  | | **14** | **14** | **10** | **8** | **11** | **10** | **67** |

**Table S1 –** Number of wing cardenolide samples for each maternal family, separated by milkweed species. Families are arranged by source population. Note that for monarchs reared in 2017, only four milkweed species (GOPH, ASCU, ASYR, ASFA) were available. In total, we analyzed cardenolides from 440 individual monarchs. Milkweed species abbreviations are as follows: GOPH = *Gomphocarpus physocarpus*, ASCU = *Asclepias curassavica*, AINC = *Asclepias incarnata*, ASFA = *Asclepias fascicularis*, ASYR *= Asclepias syriaca*, ASPEC = *Asclepias speciosa*.

|  |  |  |
| --- | --- | --- |
| **Species** | **Leaf Samples** | **Wing Samples** |
| GOPH | 54 | 112 |
| ASCU | 38 | 84 |
| AINC | 20 | 60 |
| ASFA | 20 | 59 |
| ASYR | 32 | 76 |
| ASPEC | 19 | 60 |

**Table S2 –** Number of cardenolide samples generated for leaf and wing tissue across each milkweed species.

|  |  |  |  |
| --- | --- | --- | --- |
| **Compound** | **Retention Time (Minutes)** | **Species** | **Absorbance Peak (nm)** |
| Aspecioside | 1.120 | ASYR, ASPEC | 219.13 |
| Frugoside | 5.933 | ASCU, GOPH, AINC, ASFA | 220.12 |
| Calotropin | 6.660 | ASCU, GOPH | 218.99 |
| Calactin | 7.443 | ASCU, GOPH | 218.77 |
| Digitoxin (Internal Standard) | 10.693 | All samples | 219.20 |

**Table S3** – Cardenolides present in the current study whose identities could be verified with authentic standards. Frugoside was only recorded from AINC and ASFA in trace amounts and may reflect small amounts sequestered by neonate larvae during their first ~12 hours of development on ASCU cuttings, prior to being transferred onto their focal host plants. All compounds, with the exception of digitoxin, provided by A. Agrawal and C. Duplais.

|  |  |  |  |
| --- | --- | --- | --- |
| **Species** | **Sum of Squares** | **F** | **p** |
| GOPH | 13.27 | 103.5 | <0.001 |
| ASCU | 7.72 | 57.2 | <0.001 |
| AINC | 3.93 | 15.1 | <0.001 |
| ASFA | 4.79 | 20.1 | <0.001 |
| ASYR | 7.68 | 27.0 | <0.001 |
| ASPEC | 3.19 | 9.5 | <0.001 |

**Table S4 –** MANOVA results for milkweed species level comparisons of leaf and wing cardenolide profiles. Each row corresponds to a single species-level comparison of leaf and wing cardenolides. Across all species, leaf and wing tissue contained strongly distinct cardenolide profiles, reinforcing the notion that sequestration involves active processing of leaf cardenolides.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Milkweed Species** | **Compound** | **Absolute Amount (mg/g)** | **% of Total Sequestered** | **PR Amount (mg/g)** | **Ratio (PR / Others)** |
| *Asclepias curassavica* | **Frugoside** | 4.190 | 34.4 % | 4.013 | 0.958 |
| **RT 2.150** | 1.674 | 13.8 % | 4.249 | 2.538 |
| **RT 0.830** | 1.270 | 10.4 % | 2.015 | 1.587 |
| **Calotropin** | 1.235 | 10.1 % | 0.689 | 0.558 |
| **Calactin** | 0.865 | 7.1 % | 0.968 | 1.119 |
| **RT 5.100** | 0.600 | 4.9 % | 0.598 | 0.997 |
| *Gomphocarpus physocarpus* | **Frugoside** | 1.455 | 26.9 % | 1.887 | 1.297 |
| **RT 0.830** | 0.785 | 14.5 % | 1.730 | 2.204 |
| **RT 6.383** | 0.579 | 10.7 % | 0.481 | 0.831 |
| **Calactin** | 0.564 | 10.4 % | 0.834 | 1.479 |
| **RT 1.593** | 0.544 | 10.0 % | 1.064 | 1.956 |
| **Calotropin** | 0.487 | 9.0 % | 0.335 | 0.689 |
| *Asclepias syriaca* | **Aspecioside** | 2.966 | 48.3 % | 0.128 | 0.043 |
| **RT 1.890** | 0.879 | 14.3 % | 0.098 | 0.111 |
| **RT 3.590** | 0.744 | 12.1 % | 0.357 | 0.480 |
| **RT 3.380** | 0.708 | 11.5 % | 0.320 | 0.452 |
| **RT 2.870** | 0.144 | 2.4 % | 0.031 | 0.215 |
| *Asclepias speciosa* | **Aspecioside** | 1.447 | 44.0 % | 0.096 | 0.066 |
| **RT 3.380** | 0.592 | 18.0 % | 0.104 | 0.176 |
| **RT 3.590** | 0.418 | 12.7 % | 0.037 | 0.089 |
| **RT 1.890** | 0.404 | 12.3 % | 0.363 | 0.899 |
| **RT 2.870** | 0.070 | 2.2 % | 0.000 | 0.000 |

**Table S5** – Primary sequestered cardenolide peaks across milkweed species, averaged across all monarch populations. The top six compounds are shown for *A. curassavica* and *G. physocarpus*, and the top five compounds are shown for *A. syriaca* and *A. speciosa*. For compounds whose identities are unknown, retention times are listed. Percent of total sequestered refers to within-species totals. In the second column from the right, absolute sequestered amounts are shown for the Puerto Rican population only. The rightmost column shows the ratio of sequestered cardenolides for Puerto Rican monarchs relative to species-level totals across all populations. Note that the ratio for aspecioside sequestered from A. syriaca is 0.043, corresponding to 23 times lower sequestration of this compound in Puerto Rican monarchs. For graphical depictions of chromatograms, see Figure 1A. Monarchs reared on *A. incarnata* and *A. fascicularis* contained small amounts of frugoside, RT 6.383, and RT 2.150.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Predictor** | **Sum of Squares** | **R2** | **F** | **DF** | **p** |
| Monarch population | 3.42 | 0.033 | 4.77 | 5 | <0.001 |
| Milkweed species | 51.32 | 0.494 | 119.49 | 3 | <0.001 |
| Monarch population x  milkweed species | 6.12 | 0.059 | 2.85 | 15 | <0.001 |
| Sex | 0.29 | 0.003 | 2.01 | 1 | 0.066 |
| Residual Error | 42.66 | 0.420 |  |  |  |

**Table S6 –** MANOVA results showing variation explained by milkweed species, monarch population, their interaction, and butterfly sex in the composition of sequestered cardenolides. Compared to quantitative variation in the concentration of sequestered cardenolides (see Table S7), the interaction between monarch population x milkweed species interaction term explained relatively little variation, suggesting that GxE interactions primarily involve variation in the total amount of cardenolide sequestered.

|  |  |  |  |
| --- | --- | --- | --- |
| **Predictor** | **χ2** | **DF** | **p** |
| Monarch population | 6.91 | 5 | 0.227 |
| Milkweed species | 61.55 | 3 | <0.001 |
| Monarch population x  milkweed species | 77.56 | 15 | <0.001 |
| Sex | 2.85 | 1 | 0.094 |

**Table S7 –** ANOVA results for a linear mixed model comparing total sequestered cardenolide concentrations. Here, the primary term of interest is the interaction between monarch population and milkweed species, which reflects GxE interactions for sequestration ability.

|  |  |  |  |
| --- | --- | --- | --- |
| **Model Term** | **χ2** | **DF** | **p** |
| Monarch population | 9.44 | 5 | 0.093 |
| Milkweed species | 79.41 | 3 | <0.001 |
| Sympatric / allopatric status | 0.16 | 1 | 0.687 |
| Sex | 1.34 | 1 | 0.247 |

**Table S8 –** ANOVA results for a linear mixed model directly testing for local adaptation in sequestration ability. As with Table S7, the response variable is total sequestered cardenolides in monarch wings. The primary term of interest is the sympatric/allopatric contrast, which describes the magnitude of performance difference between monarchs reared on sympatric versus allopatric host plants.

|  |  |  |  |
| --- | --- | --- | --- |
| **Species** | **Mean Cardenolide Concentration** | **Standard Deviation** | **Coefficient of Variation** |
| GOPH | 5.42 | 2.00 | 0.371 |
| ASCU | 12.17 | 4.83 | 0.397 |
| AINC | 0.45 | 0.45 | 1.002 |
| ASFA | 0.31 | 0.24 | 0.759 |
| ASYR | 6.14 | 4.18 | 0.681 |
| ASPEC | 3.29 | 3.12 | 0.949 |

**Table S9 –** Coefficient of variation in cardenolide sequestration across each milkweed species. Note that variation is lowest on GOPH and ASCU.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Monarch Population** | **Milkweed Species** | **Marginal Mean** | **SE** | **df** | **Lower CL** | **Upper CL** | **Group** |
| AU | GOPH | 5.242 | 0.795 | 115.159 | 3.667 | 6.816 | A |
| CA | GOPH | 4.819 | 0.885 | 188.960 | 3.074 | 6.564 | A |
| ENA | GOPH | 5.352 | 0.785 | 153.641 | 3.802 | 6.902 | A |
| GU | GOPH | 4.721 | 0.886 | 180.993 | 2.973 | 6.469 | A |
| HI | GOPH | 4.991 | 0.794 | 100.655 | 3.416 | 6.566 | A |
| PR | GOPH | 7.692 | 1.008 | 167.648 | 5.702 | 9.682 | A |
| AU | ASCU | 14.611 | 1.151 | 54.918 | 12.304 | 16.917 | A |
| CA | ASCU | 12.574 | 1.089 | 43.170 | 10.378 | 14.770 | AB |
| ENA | ASCU | 13.079 | 1.021 | 42.408 | 11.018 | 15.139 | A |
| **GU** | **ASCU** | **8.886** | **0.941** | **36.731** | **6.978** | **10.793** | **B** |
| HI | ASCU | 11.013 | 1.052 | 39.936 | 8.887 | 13.140 | AB |
| PR | ASCU | 14.963 | 1.026 | 41.599 | 12.892 | 17.033 | A |
| AU | ASYR | 7.164 | 0.957 | 150.575 | 5.274 | 9.054 | A |
| CA | ASYR | 5.250 | 0.905 | 107.173 | 3.456 | 7.045 | A |
| ENA | ASYR | 6.199 | 0.986 | 99.274 | 4.244 | 8.155 | A |
| GU | ASYR | 6.618 | 1.036 | 177.648 | 4.574 | 8.663 | A |
| HI | ASYR | 7.372 | 1.274 | 108.765 | 4.846 | 9.897 | A |
| **PR** | **ASYR** | **1.056** | **1.040** | **153.891** | **-0.999** | **3.111** | **B** |
| AU | ASPEC | 3.017 | 1.388 | 33.903 | 0.196 | 5.837 | A |
| CA | ASPEC | 4.656 | 1.133 | 23.151 | 2.313 | 6.999 | A |
| ENA | ASPEC | 1.910 | 1.348 | 29.558 | -0.844 | 4.665 | A |
| GU | ASPEC | 3.626 | 1.289 | 33.878 | 1.005 | 6.246 | A |
| HI | ASPEC | 4.955 | 1.481 | 63.087 | 1.994 | 7.915 | A |
| PR | ASPEC | 0.896 | 1.282 | 34.380 | -1.709 | 3.500 | A |

**Table S10** – Estimated marginal means for total sequestered cardenolide concentration for each monarch population x milkweed species. Group level differences were considered significantly different if they had non-overlapping 95% confidence intervals. Combinations of primary interest are shown in bold.

**Figure S1 –** NMDS plot of wing cardenolides from wild-caught monarchs in the Mariana Islands. Butterflies from Guam (n = 54) and Rota (n = 27) generally had indistinguishable cardenolide profiles, consistent with both populations feeding primary on the numerically dominant host *Asclepias curassavica*. Monarchs from Saipan (n = 2) included one wild-caught individual with a cardenolide fingerprint consistent with developing on A. curassavica, as well as one monarch collected on the day of its emergence on an ornamental *Calotropis gigantea* plant (point in lower right).





**Figure S2 –** Pairwise correlations among the major compounds sequestered for each of the primary milkweed species of interest. The size of the circle corresponds to the strength of the correlation, with orange dots indicating positive correlations and blue dots indicating negative correlations. Most compounds were positively correlated with each other across butterflies, with a few exceptions. Individual compounds are the same as those reported in Table S5: X5.933 is frugoside, X6.597 is calotropin, X7.403 is calactin, and X1.063 is aspecioside. Numbers in compound names correspond to retention times. Note that only the six most abundant compounds from *A. curassavica* and *G. physocarpus* are shown, and only the five most abundant compounds are shown for *A. syriaca* and *A. speciosa*. Also note that data presented here reflect retention times generated for greenhouse-reared butterflies, which were analyzed on a separate instrument from wild-caught Guam and Rota butterflies.



**Figure S3 –** Multivariate disparity in sequestered cardenolide profiles, shown separately for each milkweed species. Results shown are based on a single overall dissimilarity matrix but are faceted by milkweed species. Ellipses correspond to the 95% confidence profiles and were generated using the stat\_ellipse function. All populations appear to have generally similar overall multivariate sequestration profiles, with the potential exceptions of Puerto Rican monarchs reared on *A. speciosa* and especially on *A. syriaca*.



**Figure S4** – Example of chromatograms from two monarchs reared on A. syriaca. Australian monarchs (cyan), despite more than 150 years isolated from this ancestral North American host, still retain their ability to sequester normally. By contrast, Puerto Rican monarchs sequester poorly from A. syriaca, with average total cardenolide concentrations that are more than five times lower than all other populations. Sequestration of aspecioside in Puerto Rican populations is especially poor, with average concentrations that are nearly 25 times lower.



**Figure S5 –** Accounting for development time does not meaningfully impact inferences related to cardenolide sequestration. (A) Cardenolide sequestration efficiency, measured as cardenolide concentration divided by days from hatching to eclosion, for each *population x species* combination. Note that the results are virtually indistinguishable from those shown in Figure 4A. (B) Comparison of cardenolide concentration and cardenolide sequestration efficiency for Puerto Rican monarchs versus all other monarch populations. Puerto Rican monarchs had modestly slower development time than all other populations across all hosts (see Freedman et al. 2020a), but still had the highest sequestration efficiency on *A. curassavica* and *G. physocarpus*. Ratio is simply the value for the Puerto Rican population divided by the value for all other populations.



**Figure S6 –** There was no overall correlation between development time (measured as days from egg hatching until eclosion) and the overall quantity of cardenolide sequestered (t = 0.198, p = 0.844).



**Figure S7 –** Distribution of random intercepts for each maternal family. Error bars correspond to **±**1 SD For context, the overall intercept in the associated model is 5.54, which represents the overall average of sequestered cardenolides on GOPH, ASCU, ASYR, and ASPEC.

**Figure S8**– Estimated marginal means showing wing cardenolide concentration for only monarchs reared on *Asclepias curassavica*. Monarchs from Guam sequestered significantly lower concentrations from ASCU—their sympatric host plant—than populations from Australia, Eastern North America, and Puerto Rico. Error bars correspond to 95% confidence intervals. Results also reported in Figure 4A and Table S10.





**Figure S9** – When comparing butterfly samples whose corresponding natal plant was also analyzed (n = 154), there was little evidence for a correlation between dietary cardenolides and sequestered cardenolides. The lone species with a significantly positive correlation was A. speciosa (t = 2.822, p = 0.011).