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1 **Variation of chemical compounds in wild *Heliconiini* reveals ecological factors**
2 **involved in the evolution of chemical defences in mimetic butterflies**

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22 phylogenetic signal.

23 **Abstract**

24 Evolutionary convergence of colour pattern in mimetic species is tightly linked with the
25 evolution of chemical defences. Yet, the evolutionary forces involved in natural variations of
26 chemical defences in aposematic species are still understudied. Herein, we focus on the
27 evolution chemical defences in the butterfly tribe Heliconiini. These neo-tropical butterflies
28 contain large concentrations of cyanogenic glucosides, cyanide-releasing compounds acting
29 as predator deterrent. These compounds are either *de novo* synthesized or sequestered from
30 their *Passiflora* host-plant, so that their concentrations may depend on host-plant
31 specialization and host-plant availability. We sampled 375 wild Heliconiini butterflies across
32 Central and South America, covering 43% species of this clade, and quantify individual
33 variations in the different cyanogenic glucosides using liquid chromatography coupled with
34 tandem mass spectrometry. We detected new compounds and important variations in
35 chemical defences both within and among species. Based on the most recent and well-studied
36 phylogeny of Heliconiini, we show that ecological factors such as mimetic interactions and
37 host-plant specialization have a significant association with chemical profiles, but these effects
38 are largely explained by phylogenetic relationships. Our results therefore suggest that shared
39 ancestries largely contribute to chemical defence variation, pointing out at the interaction
40 between historical and ecological factors in the evolution of Müllerian mimicry.

41 INTRODUCTION

42 The evolution of complex phenotypes combining different traits subject to natural selection
43 raises the question of the mechanisms underlying adaptation involving multiple traits. In
44 aposematic species for instance, the defensive traits such as toxicity, and the warning
45 coloration may evolve asynchronously and can be submitted to contrasted selective
46 pressures. While the evolution of colour patterns and the selective mechanisms involved have
47 received considerable attention (Le Poul et al., 2014; Sherratt, 2008), the evolutionary origin
48 of chemical defence variations is still understudied. The effect of chemical defences on
49 predator avoidance is critical for prey survival (Ihalainen et al., 2007) and therefore central in
50 the evolution of warning colorations (Blount et al., 2009; Speed and Ruxton, 2007). By
51 sampling aposematic prey, predators learn to associate deterrent effect with a given warning
52 colour pattern and subsequently avoid any resembling prey item (Alcock, 1970a, 1970b;
53 Goodale and Sneddon, 1977). The immediate and long-term effect of defensive compounds
54 thus determines the protection gained from aposematism (Skelhorn and Rowe, 2005), and
55 therefore the evolution of colour patterns.

56 Evolutionary convergence in aposematic signal among co-occurring defended prey
57 species is frequently observed among sympatric aposematic species, because sharing a colour
58 pattern decreases individual predation risk (Müller, 1879). This results in so-called mimicry
59 rings, composed of multiple species sharing a similar warning colour pattern. Both the
60 defensive compounds and the abundance of individuals sharing a given warning colour
61 pattern determine the predation risk associated with this coloration (Sherratt, 2008).
62 Substantial quantitative variation in chemical defences is observed between mimetic species,

63 as demonstrated for instance in poison frogs (Santos and Cannatella, 2011), marine
64 gastropods opisthobranchs (Cortesi and Cheney, 2010) or insects (Arias et al., 2016;
65 Bezzerides et al., 2007; de Castro et al., 2019a). Less defended individuals may act as parasites
66 on better defended individuals by limiting predator avoidance (Rowland et al., 2010; Speed,
67 1993). The evolution of chemical defences in mimetic species is thus likely to be influenced by
68 the local abundance of the mimicry ring they belong too, as well as variations in toxin levels
69 across individuals composing the ring.

70 Nevertheless, other local ecological factors may influence the evolution of chemical
71 defences in mimetic species. In butterflies for instance, deterrent compounds, as well as
72 precursors for their synthesis, can be acquired by caterpillars during feeding on specific host-
73 plants (Jones et al., 2019; Nishida, 2002). Chemical defences may thus vary among species
74 depending on their diet (Engler and Gilbert, 2007). For instance, monarch butterflies (*Danaus*
75 *plexippus*) sequester cardenolides from milkweeds during the larval stage and are thus
76 unpalatable to birds (Brower et al., 1972). Adaptation to host-plants is thus a key evolutionary
77 factor in the origin and evolution of chemical defences in aposematic butterflies.
78 Nevertheless, because of the strength of predation on adult butterflies, the evolution of
79 chemical defences in mimetic butterflies can result from complex interactions between host-
80 plant adaptation and predation pressure. A recent survey of natural populations of two co-
81 mimetic butterfly species, the viceroy (*Limenitis archippus*) and queen (*Danaus gilippus*),
82 demonstrated that the average concentration of chemical defences increases in the viceroy
83 populations where the defended queen species is absent (Prudic et al., 2019). This effect is
84 independent from variation in defensive compounds concentrations in the host-plants (Prudic

85 et al., 2019), highlighting that the abundance of co-mimics may modulate selection exerted
86 on chemical defences in mimetic species.

87 Here we aim to disentangle the mechanisms involved in the evolution of chemical
88 defences, from neutral divergence to selective pressure of predation and host-plant
89 adaptation. We focus on the butterflies belonging to the neotropical tribe Heliconiini
90 (Nymphalidae: Heliconiinae), where colour pattern evolution and mimetic interactions have
91 been extensively documented (Joron and Iwasa, 2005; Joron and Mallet, 1998; Merrill et al.,
92 2015). Subspecies of Heliconiini are defined based on variation in colour pattern between
93 geographic locations, observed within species (Braby et al., 2012). Heliconiini butterflies
94 contain a wide diversity of defensive compounds, especially aliphatic or cyclopentenoid
95 cyanogenic glucosides (CGs) (Fig. 1) (de Castro et al., 2019a; Engler et al., 2000). CGs are
96 supposed to have a bitter and repulsive taste (Nahrstedt and Davis, 1985). Additionally, CGs
97 release toxic cyanide and chemical by-products for birds when put in contact with specific
98 degrading enzymes (Cardoso, 2019; Conn, 1980). Cyanogenic substrates and enzymes or
99 stored in different cell or tissue compartment and are mixed upon tissue disruption under a
100 predator's attack, so that Heliconiini butterflies often survive an attack after being tasted (e.g.
101 by lizard (Boyden, 1976) or avian predators (Boyden, 1976; Chai, 1996; Pinheiro and Campos,
102 2019)). Therefore, the bitter taste provided by CG and toxic metabolites may act as a chemical
103 defence because of immediate deterrent effect on predator.

104 Heliconiini caterpillars feed on *Passiflora* plants (Engler and Gilbert, 2007; Jiggins, 2016;
105 Turner, 1967), with substantial behavioural variation between species in female egg-laying
106 preferences and in larval survival on different *Passiflora* species (Benson et al., 1975; Brown,

107 1981). Around 30 different CGs have been identified in *Passiflora* (de Castro et al., 2019a;
108 Spencer and Seigler, 1987). Larvae of most Heliconiini species synthesize CGs *de novo* (Wray
109 et al., 1983), but many sequester CGs from the host-plants (Engler et al., 2000). Both synthesis
110 and sequestration of CGs is only observed in Zygaenidae (burnet moths) and Heliconiini, two
111 clades where aposematic colour patterns have evolved (Zagrobelyny et al., 2018). So far,
112 Heliconiini have been reported to sequester five cyclopentenoid CGs from *Passiflora*; the
113 diastereoisomers tetraphyllin B and epivolkenin, tetraphyllin A, gynocardin and
114 dihydrogynocardin (Fig. 1) (de Castro et al., 2019a; Engler et al., 2000). Heliconiini butterflies
115 can synthesize aliphatic CGs, linamarin and lotaustralin (Fig. 1) from the amino acids valine
116 and isoleucine, respectively (Nahrstedt and Davis, 1985). Identifying the different CGs may
117 thus allow tracking down their metabolic origins, although aliphatic linamarin and lotaustralin
118 can also be uptaken by caterpillars, as recently demonstrated in *Heliconius melpomene* (de
119 Castro et al., 2019b). The balance between sequestration from host-plants and *de novo*
120 synthesis of CGs in different species may be linked to host-plant specialization. CG
121 sequestration might be more important than synthesis in specialist species, as for instance in
122 the specialist species *Heliconius sara* and *H. sapho* containing drastically diminished CG
123 concentrations when reared on *Passiflora* species other than their specific host-plants (Engler
124 and Gilbert, 2007). Evolution of chemical defences in the Heliconiini clade can thus be
125 influenced by the adaptation to host-plants.

126 The substantial geographic variation in colour patterns and host-plants observed in the
127 Heliconiini clade (Jiggins, 2016) provides a relevant opportunity to investigate the effect of
128 selection pressure on the evolution of chemical defences in mimetic species. Based on the

129 well-studied phylogeny of Heliconiini (Kozak et al., 2015), we thus explored how phylogenetic
130 history, mimetic interactions and host-plant use can drive the evolution of chemical defence
131 in wild butterflies. We sampled butterflies throughout Heliconiini distribution, from Central to
132 South America, in order (1) to maximize the diversity of species of the Heliconiini clade (we
133 cover almost half of the tribe diversity), and (2) to assess variation in chemical defences of
134 individuals facing natural variations in host-plant availability, mimetic community abundance
135 and predator communities. Using liquid chromatography coupled to mass spectrometry (LC-
136 MS/MS), we investigate both quantitative and qualitative variation across individuals and then
137 use comparative methods to disentangle phylogenetic and ecological factors influencing the
138 evolution of chemical defences in Heliconiini.

139

140 **MATERIALS AND METHODS**

141 **Butterfly collection**

142 We sampled butterflies throughout Heliconiini distribution to collect the maximum number
143 of species. Wild butterflies were caught from 2016 to 2018 across Peru ($n = 286$), Panama
144 ($n = 45$), Ecuador ($n = 24$) and Brazil ($n = 20$), using a hand net. We used 375 individuals from
145 33 species, covering 43% of the Heliconiini tribe (Appendix. 1), and 55 subspecies (Tab. 1).
146 Individuals were killed by freezing on the day of capture (approximately -18°C). Wings were
147 cut at their attachment point to the body and preserved dried in an envelope and placed in a
148 silica gel containing box to absorb humidity. In order to preserve the integrity of CG molecules,
149 bodies were conserved in a plastic vial containing 100% methanol and kept in freezer
150 (approximately -18°C).

151

152 **Cyanogenic glucoside extraction in methanol**

153 For each butterfly specimen, the butterfly body and the methanol medium were transferred
154 in a glass tube. Methanol was evaporated at room temperature until the tissue was fully dried
155 using Savant Automatic Environmental SpeedVac System AES1010 with VaporNet. For each
156 specimen, body and wings were weighed before being crushed together into a fine powder in
157 a glass mortar and pestle using liquid nitrogen. Two mL of 100% methanol were added to the
158 powder before stirring for 1 hour at room temperature. Extracts were centrifugated for 20
159 minutes at 1600 rotations per minute, filtered using 7 mm diameter glass pipettes and cotton,
160 filtered again with a MultiScreen 0.45 µm hydrophilic, low protein binding plate, and
161 centrifuged five minutes at 3500 rotations per minute. Raw filtrates were diluted 50 times in
162 milliQ water, vortexed and stored in fridge until liquid chromatography and tandem mass
163 spectrometry (LC-MS/MS) injections.

164

165 **Liquid chromatography and tandem mass spectrometry**

166 The protocol used in this study has been previously optimized to identify and quantify CGs in
167 butterfly methanol filtrates (Briolat et al., 2019; de Castro et al., 2019a). Analytical LC-MS/MS
168 was performed using an Agilent 1100 Series LC (Agilent Technologies, Germany) coupled to a
169 High Capacity Trap-Ultra ion trap mass spectrometer (Bruker Daltonics, Germany).
170 Chromatographic separation was carried out on a Zorbax SB-C18 column (Agilent; 1.8 µM,
171 2.1x50 mm). Mobile phase A was composed by deionized water containing 0.1% (v/v) formic
172 acid. Mobile phase B was acetonitrile supplemented with 50 µM NaCl and 0.1% (v/v) formic

173 acid. The gradient was: 0 - 0.5 min, isocratic 2% B; 0.5 - 7.5 min, linear gradient 2% - 40% B;
174 7.5 - 8.5 min, linear gradient 40% - 90% B; 8.5 - 11.5 isocratic 90% B; 11.6 - 17 min, isocratic
175 2% B. Flow rate was set to 0.2 mL/min and increased to 0.3 mL/min between 11.2 to 13.5 min.
176 During the liquid chromatography step, initially neutral CGs were associated with Na⁺ cations
177 and analysed with mass spectrometer in the positive electrospray mode. The oven
178 temperature was fixed at 35°C.

179 In addition to the 375 butterfly samples, we ran blank control sample and a reference sample.
180 Blank was methanol gone through the whole protocol extraction, and the reference sample
181 was a mix of every butterfly filtrates. CGs were identified by comparison to standard solutions
182 (aliphatic were chemically synthesized at PLEN, Møller *et al.*, 2016, cyclopentenoid were
183 donated by Lawrence Gilbert and Helene Engler, Engler *et al.*, 2000). We made three
184 calibration curves based on three commercial standards: linamarin,
185 lotaustralin/epilotaustralin and amygdalin (commercial, Sigma Aldrich), from 0.1 to 20 ng/ μ L
186 each. Blanks, standards, calibration curve and reference sample were run first. The reference
187 sample was injected every ten butterfly samples.

188

189 **Chemical data analyses**

190 Mass spectra were analysed using the software Bruker Compass DataAnalysis 4.3 (x64). We
191 targeted sodium adducts [M+Na⁺] of linamarin [retention time (RT) 2.4 min at m/z 270],
192 lotaustralin [RT 5.4 min at m/z 284], epilotaustralin [RT 5.5 min at m/z 284], tetraphyllin B [RT
193 1.3 min at m/z 310], epivolkenin [RT 2.3 min at m/z 310], tetraphyllin A [RT 4.9 min at m/z
194 294], gynocardin [RT 1.4 min at m/z 326], dihydrogynocardin [RT 1.4 min at m/z 328] and

195 amygdalin [RT 6.4min at m/z 480] (Briolat et al., 2019; de Castro et al., 2019a). For every
196 targeted CG compound, the total concentration was estimated based on the Extracted Ion
197 Chromatogram (EIC) peak areas, and on a regression calculated from the standard curve (in
198 ng of CG/mL of butterfly extract). We reported the concentration of each CG in every butterfly
199 in μg of CG/mg of dried butterfly weight.

200

201 **Statistical and comparative analyses**

202 For each individual, we obtained the concentration of each of the nine studied CGs, referred
203 to as the chemical profile. By adding these nine CG concentrations, we computed the total CG
204 concentration per individual, as an estimation of the amount of chemical defences per
205 individual. All statistics were conducted in R 3.4.4 (R: The R Project for Statistical Computing,
206 2019) and RStudio 1.1.463 (RStudio, 2019). Plots were created with *ggplot2* 3.0.0 package
207 (Wickham et al., 2019).

208

209 **i) Qualitative and quantitative variation in cyanogenic glucosides**

210 We used MANOVA (Multivariate ANalysis Of Variance) to test whether the (multivariate) CG
211 profiles were different between groups (genera, species and subspecies), and we reported the
212 name of the test, Pillai's trace, degree of freedom and associated p -value. We used the Pillai's
213 test because of its robustness regarding heterogeneities in variance-covariance.

214 We used ANOVA (ANalysis Of Variance) to test whether the concentration of a specific CG was
215 different between groups. We presented statistical result of ANOVA as follow: name of the
216 test, F value (variance of the group means / mean of the within group variances), degree of

217 freedom and associated p -value. In case of a significant ANOVA (p -value < 0.050), post-hoc
218 test Tukey Honest Significant Differences (Tukey's HSD) was done to determine which group
219 was significantly different from the others. Statistical tests were run with R package *stats*
220 3.4.2. Heatmap of CG occurrence and concentration was plotted using R packages *ape* 5.1 and
221 *ggtree* 1.10.5 (Paradis, 2011; Yu et al., 2017).

222

223 **ii) Evolution of cyanogenic glucoside profiles in Heliconiini**

224 We calculated the phylogenetic signal of CG profile, *i. e.*, the extent to which trait values are
225 explained by the phylogeny, or how much closely related species resemble one another in
226 terms of CG profile (Blomberg *et al.*, 2003). We computed the K_{mult} statistic, a multivariate
227 extension of Blomberg's K test for univariate phylogenetic signal (Adams, 2014; Blomberg *et*
228 *al.*, 2003). A low phylogenetic signal (K_{mult} close to 0) indicates a low influence of the
229 phylogenetic relationships on the tested trait, whereas high value (K_{mult} close to 1) suggests
230 that the trait evolution along the phylogeny is close to Brownian motion. The multivariate
231 phylogenetic signal of quantitative CG variation across species was evaluated using K_{mult} in the
232 *geomorph* 3.0.7 R package. We calculated the phylogenetic signal in the whole Heliconiini
233 tribe, in the largest genus of the radiation: *Heliconius* and more specifically in ancient nodes
234 (pupal-mating and non-pupal-mating clades). In *Heliconius*, phenotypic races of the same
235 species often belong to different mimicry rings. Therefore, we estimated the phylogenetic
236 signal using mean CG concentrations separately at the taxonomic level of species ($n = 33$) and
237 subspecies ($n = 55$). We adapted the Heliconiini phylogenetic tree (Kozak *et al.*, 2015) by
238 pruning species not represented in our sample set. In many cases several subspecies were

239 sampled (for example: *H. hecale felix*, *H. hecale melicerta* and *H. hecale zuleika*). For the
240 subspecies-level analysis we extended the original phylogeny to include relevant subspecies
241 as follows: the terminal branch length was set equal to the decimal of the previous branch,
242 and the common branch equal to the integer part. All subspecies had same total branch
243 length. In the case of more than two subspecies, the topology was arbitrary resolved.

244

245 **iii) Phylochemospace**

246 We applied the concept of phylomorphospace, describing morphological variation across
247 species in correlation with their phylogenetic relationships (Sidlauskas, 2008). We built a
248 “phylochemospace” describing variation in concentration of multiple compounds with a
249 principal component analysis (PCA), superimposing the phylogenetic relationships among
250 subspecies. The resulting PCA visualises the variation in CGs actually occurring in the 55
251 subspecies. Packages *FactoMineR* 1.41 (Lê et al., 2008), *missMDA* 1.14 (Josse and Husson,
252 2016), and *phytools* 0.6-44 (Revell, 2012) were used.

253

254 **iv) Variation among co-mimetic subspecies and host-plant specialization**

255 We tested for differences between groups: mimicry ring, geographical range and host-plant
256 specialization. We used MANOVA and ANOVA to assess differences in CG profile and specific
257 CG concentrations respectively, both at species ($n = 33$) and subspecies ($n = 55$) level. We
258 applied Bonferroni correction as we performed several tests on the same dataset. We used
259 *stats* 3.4.2 for MANOVA and *RVAideMemoire* 0.9-72 package (Hervé, 2019) for associated

260 post-hoc test. ANOVA, associated post-hoc test and Bonferroni correction were computed
261 with *stats* 3.4.2 package as well.

262 To assess whether the observed statistically significant differences were due to shared
263 ancestry, we computed phylogenetic MANOVA and ANOVA, using *geiger* 2.0.6 (Harmon et al.,
264 2008) and *phytools* 0.6-44 packages (Revell, 2012) respectively. Phylogenetic MANOVA were
265 performed using the modified tree and mean CG concentrations per subspecies (as these
266 phylogenetical tests do not handle multiple value for one subspecies, we used mean
267 concentrations).

268 We investigated variation in total CG concentration, putatively-synthesized CG concentration
269 and putatively-sequestered CG concentration between generalist and specialist subspecies.

270 When considering the entire range of a given species across Central and South America it turns
271 out it can have a lot of host-plant species. For instance, *Agraulis vanilla* has 50 reported host-
272 plants and *Heliconius numata* 30 (Kozak, 2016). We conducted our analysis at the subspecies
273 level because locally subspecies actually use much less host-plants. In our study, generalist are
274 subspecies that feed on more than 5 host-plant species whereas specialist subspecies feed on
275 5 or less host-plant species. We adjusted this classification based on the literature.

276

277 **RESULTS**

278 **Large variations in the concentration of neo-synthesized and sequestered cyanogenic** 279 **glucosides in wild Heliconiini**

280 Across the 375 analysed Heliconiini samples, nine CGs were identified and important variation
281 in the CG profile was detected between genera and species (Tab. 2). Important variation of

282 CG profile was also detected within species, notably among different subspecies (MANOVA,
283 Pillai $\lambda_{303}^{49} = 3.513$, $p < 0.001$).

284 Regarding putatively-synthesized aliphatic CGs, linamarin was detected in all 32 out of 33
285 species, whereas lotaustralin was in all species (Fig. 2). However, the concentration of
286 linamarin was significantly different between species (ANOVA, $F_{342}^{32} = 13.77$, $p < 0.001$), and
287 individuals from the genus *Eueides* had statistically significant higher linamarin concentration
288 compared to other genera (ANOVA, $F_{368}^6 = 35.46$, $p < 0.001$; Tukey's HSD, $p < 0.001$). Similarly,
289 lotaustralin concentrations differed among species (ANOVA, $F_{342}^{32} = 4.324$, $p < 0.001$). Another
290 aliphatic CG, epilotaustralin, was detected in *Heliconius*, *Eueides*, *Dione*, *Agraulis* and *Dryas*
291 genera, with significant variation in concentration among species was (ANOVA, $F_{342}^{32} = 2.618$,
292 $p < 0.001$). These three putatively-synthesised CGs were found at the highest levels in *H.*
293 *charithonia*, which also did not contain any putatively-sequestered CGs in the two analysed
294 individuals.

295 Six putatively-sequestered CGs from *Passiflora* hostplants were measured: tetraphyllin A, a
296 diastereoisomer of tetraphyllin A, tetraphyllin B, a diastereoisomer of tetraphyllin B called
297 epivolkenin, gynocardin and dihydrogynocardin. The diastereoisomer of the tetraphyllin A
298 could be deidaclin, because this molecule is also produced by *Passiflora* species used as host-
299 plant by Heliconiini butterflies (Jaroszewski et al., 2002; Spencer et al., 1983; Tober and Conn,
300 1985). We also searched for the aromatic CGs amygdalin as it has been measured in few
301 analysed *Passiflora* species (de Castro et al., 2019a; Chassagne et al., 1996), but we did not
302 find aromatic CGs in Heliconiini butterflies, as previously reported in reared *H. melpomene* (de
303 Castro et al. 2019). The diversity of putatively-sequestered CGs and their important variations

304 between species in the wild (MANOVA, Pillai $\lambda_{342} = 1.735$, $p < 0.001$) highlight that CG
305 sequestration is widely distributed among the Heliconiini tribe, and may depend on local host-
306 plant availability and host-plant adaptation.

307

308 **Evolution of cyanogenic glucoside profiles in Heliconiini**

309 CG profiles in Heliconiini species ($n = 33$) displayed a weak but significant phylogenetic signal
310 ($K_{mult} = 0.311$, $p = 0.023$). In *Heliconius*, the largest genus in the Heliconiini radiation, the
311 phylogenetic signal was also moderate but still significant ($K_{mult} = 0.558$, $p = 0.029$). In the
312 genus *Heliconius*, many species have subspecies living in different localities, where individuals
313 display locally mimetic colour patterns. To test whether the natural selection act on the
314 evolution of defences due to the evolution of mimetic colour pattern, we then estimated the
315 phylogenetic signal in the genus *Heliconius* at the taxonomic level of subspecies ($n = 55$). We
316 observed that the phylogenetic signal of mean CG concentrations then become weaker and
317 non-significant ($K_{mult} = 0.084$, $p = 0.055$), probably because of important variation among
318 subspecies, consistent with the hypothesis of variations in the strength of selection regarding
319 defences in different mimicry rings. Intra-specific variations of defences between localities (4
320 countries, MANOVA, Pillai $\lambda_{371} = 0.546$, $p < 0.001$) could then be explained by either (1)
321 variation in the mimetic community abundance and levels of defences in co-mimetic species
322 or (2) variation in host-plant availability or host-plant specialization levels.

323

324 **Ecological factors influencing the evolution of cyanogenic glucoside profiles in Heliconiini**

325 To explore the contribution of shared ancestry on one hand, and of ecological factors
326 influencing the evolution of defences on CG variation on the other hand, we drew a
327 phylochemospace displaying average chemical profile of the different subspecies (Fig. 3). We
328 observed that subspecies belonging to distinct mimicry rings sometimes had very distinct
329 chemical profiles, e. g. *H. erato favorinus* ($n = 31$), *H. erato emma* ($n = 5$), *H. erato demophoon*
330 ($n = 3$) and *H. erato cyrbia* ($n = 1$) (MANOVA, Pillai $\lambda_{36} = 2.002$, $p < 0.001$). The distantly related
331 co-mimics *H. melpomene rosina* ($n = 4$) and *H. erato demophoon* ($n = 3$) are located closely on
332 the phylochemospace (Fig. 3), because of their similar chemical profiles (MANOVA, Pillai $\lambda_5 =$
333 0.615 , $p = 0.621$). Similarly, *H. melpomene amaryllis* ($n = 21$) and its co-mimic *H. erato*
334 *favorinus* ($n = 31$) are located closely in the phylochemospace but their CG profiles were still
335 significantly different (MANOVA, Pillai $\lambda_{50} = 0.759$, $p < 0.001$).

336 Overall, the mimicry ring was significantly associated with CG profiles, suggesting that
337 individuals from different species belonging to the same mimicry ring had similar chemical
338 defences (Tab.3). Nevertheless, this association was no longer significant when controlling for
339 shared ancestry, suggesting that the similarity in defence levels could be mainly due to
340 increased phylogenetic proximity within mimicry rings (Tab. 3).

341 The level of host-plant specialization could also influence the evolution of defence in
342 Heliconiini. Indeed, we noticed that the chemical profiles of butterflies depended on their
343 level of host-plant specialization, although this effect is mostly driven by phylogenetic
344 proximity (Tab. 3). Because there is substantial geographical variation in the level of
345 specialization, we also compared chemical defences among subspecies: individuals from host-
346 plant-specialist subspecies were generally more chemically defended (mean total [CGs] = 39.2

347 $\mu\text{g}/\text{mg DW}$) than generalist (26.5 $\mu\text{g}/\text{mg DW}$; Tab. 3, Fig. 4). Specialist subspecies sequestered
348 more CGs (19.2 $\mu\text{g}/\text{mg DW}$) than generalist subspecies (3.8 $\mu\text{g}/\text{mg DW}$; ANOVA, $F^1_{373} = 53.01$,
349 $p < 0.001$). This is pointing at the effect of host-plant specialization on chemical profiles that
350 could substantially vary among localities (note that such specialization could depend on the
351 butterfly ability to choose and survive on different plants but also on the local host-plant
352 availability).

353

354 **Geographical variation in chemical profiles**

355 In general, variation in CGs was lower within than between mimicry rings (Tab. 3). Mimicry
356 rings are composed of different species found in sympatry, they can therefore differ in local
357 abundance but also in host-plants availability. Mimetic communities exhibiting the same
358 colour pattern (*e.g.* postman colour pattern, Fig. 5) are composed of similar species, but still
359 display strikingly different chemical profiles (Fig. 5 and Fig. 6). Both colour pattern and locality
360 indeed have a significant association with chemical profiles, as well as the interaction between
361 these two factors, even when controlling for the species effect (Tab. 4). This suggests that
362 geographical variations in local abundances of mimetic patterns and/or in local host-plants
363 availability and specialisation levels may influence the defences of Heliconiini butterflies.

364

365 **DISCUSSION**

366 **Phylogenetic history partly explains the distribution of cyanogenic glucosides across**
367 **Heliconiini species**

368 We observed that mimicry rings had different levels of CG profiles and total concentrations,
369 but these differences are mostly driven by close phylogenetic relatedness among mimetic
370 species. Our results in wild-caught individuals are thus consistent with the significant
371 phylogenetic signal in CG profile observed in captive-bred Heliconiini (de Castro et al., 2019a).
372 Nevertheless, the phylogenetic signal associated with CG profile is stronger when considering
373 species rather than subspecies, suggesting that despite a strong effect of the divergence
374 between clades (ancient node), substantial variation within species are observed in our wild-
375 caught individuals, probably driven by ecological factors acting on the different mimetic
376 subspecies.

377

378 **Geographic variation in mimicry rings impacts CG profiles**

379 The important variation in CG profile observed within species is mostly explained by variations
380 between subspecies living in different geographic range. For instance, Panamanian subspecies
381 of *A. vanillae* and *H. erato* were more chemically defended than Southern subspecies of the
382 same two species. Subspecies generally differ in wing colour pattern and geographic
383 distribution, pointing at the influence of ecological factors in shaping the variation in CG
384 concentration profile in Heliconiini. Although *Heliconius* species from the pupal-mating and
385 non-pupal-mating clades are phylogenetically distant, they can be involved in the same
386 mimicry ring. This is the case for *H. erato demophoon* and *H. melpomene rosina*, which are
387 part of the postman Panama mimicry ring and presented similar CG profiles, suggesting either
388 an effect of the mimetic interactions and/or of the similarity in local host-plant chemistry. By
389 sampling wild butterflies from different countries, our study highlights that host-plant

390 interaction and geography are important ecological factors shaping variations in chemical
391 defences within species.

392

393 **How host-plant specialization shapes chemical defences**

394 Indeed, host-plant range and preference vary locally in some species (Smiley, 1978), so that
395 variation in putatively-sequestered CGs in butterflies probably reflects host-plant availability
396 and use across sampled localities. For example, *H. melpomene* has a wider range of host-plant
397 species in its eastern distribution area. In Central America it feeds on *P. menispermifolia* or *P.*
398 *oerstedii* depending on the localities but feeds preferentially on *P. platyloba* in Peru,
399 (Billington *et al.*, 1990; Jiggins, 2016). This emphasizes the plasticity in the host-plant range of
400 many Heliconiini species and the importance of local adaptation with *Passiflora* species. Local
401 patterns in host-plant use by Heliconiini is likely reflected in their CG profile.

402 The binary generalist/specialist classification used here is a rough simplification of the host-
403 plant specialization spectrum. Nevertheless, we still observed, as expected, that specialist
404 subspecies had higher concentrations of putatively-sequestered CGs (Engler and Gilbert,
405 2007; Jiggins, 2016). However, we did not detect any correlation between the level of host-
406 plant specialization and the synthesis/sequestration balance, contrary to previous studies
407 where synthesis and sequestration were shown to be negatively correlated traits, with
408 fluctuant intensity across the phylogeny (de Castro *et al.*, 2019a; Engler and Gilbert, 2007).

409 As CGs are *Passiflora* secondary metabolites, their production may vary in space, time
410 and across tissues depending on abiotic and biotic conditions exert on plant. Thus, reported
411 putatively-sequestered CGs in our study on wild butterflies are potentially a subset of the CGs

412 contained in locally-available *Passiflora* host-plants. The evolution of Heliconiini chemical
413 defence profile would thus be shaped by both host-plant specialization of the different
414 butterfly species and available *Passiflora* host-plants variations across the geographical areas.

415

416 **Variability of CG profiles within mimicry rings and Müllerian mimicry**

417 Variation in CG concentrations between mimicry rings observed here had already been
418 reported in a study based on colorimetric assays (to investigate total CG concentration per
419 individual regardless of each CG identity) (Arias et al., 2016). This effect of mimicry on the
420 individuals belonging to different co-occurring mimicry rings are thus not necessarily equally
421 defended, and potentially perceived with different degrees of aversion by predators. Recently,
422 an experiment using domestic chicks shows that beyond a certain CG concentration, birds
423 learned to avoid the prey at a similar speed (Chouteau et al., 2019). Variations in the level of
424 CGs observed within and among mimicry rings might thus not directly translate into variation
425 in learning behaviour by predators, so that the evolution of high chemical defence in some
426 Heliconiini would not necessarily be promoted by natural selection exerted by predators in
427 mimetic prey. Furthermore, it is currently unknown whether predator rejection behaviour
428 depends on the total concentration of CG or is mostly shaped by the presence of key CGs with
429 a particularly repellent taste. Chemical defences are also a complex cocktail (Speed et al.,
430 2012) with components acting through synergetic or antagonist effects.

431 Predator communities and strength in predation pressure acting on aposematic prey vary in
432 space and time, as demonstrated in the field using artificial poison frogs and caterpillars
433 (Chouteau and Angers, 2011; Mappes et al., 2014). Predator sensibility to detect bitterness of

434 CGs and to endure unpleasant taste vary (Li and Zhang, 2014), as well as their tolerance
435 towards cyanide (Cardoso, 2019). Indeed, based on how hungry they are, avian predators may
436 decide to feed on unpalatable butterflies (Chai, 1986; Marshall, 1908). The geographic
437 variation in chemical profile detected here might therefore be influenced by both host-plant
438 availability and composition of predator communities. But the strong phylogenetic signal
439 detected on CG profiles, and the high sensitivity of predator to CG suggests that the evolution
440 of elevated levels of chemical defence is not directly related to colour pattern evolution.

441

442 **Conclusions**

443 Our study sheds light on the evolution of CGs in Heliconiini butterflies, and highlights the
444 strong effect of evolutionary history in the variation of CG profile observed between species.
445 Variation in CG profiles between mimicry rings seems to be mostly driven by phylogenetic
446 relatedness between mimetic species. Nevertheless, the strong variation observed between
447 individuals belonging to different mimicry rings within species suggests that other ecological
448 factors might be at play. Some species seem to rely on *de novo* synthesis only, whereas other
449 species mostly perform CG sequestration from *Passiflora* host-plants. Many species rely on a
450 combination of these two pathways for CG acquisition, which contributes to substantial
451 variation of chemical profiles both between species and among species. Geographic variation
452 in host-plants, but also abundance of mimicry rings could also influence the CG profile: the
453 individual predation risk is indeed lower in abundant mimicry rings as compared to rare ones
454 (Chouteau et al., 2016), so that selection for higher distastefulness might be higher in localities
455 where a given mimicry ring is at low density. Ecological studies estimating local host-plant and

456 predator community variations, as well as local abundances of mimetic communities would
457 now be required to better understand the selective pressures shaping chemical defence
458 evolution in mimetic species.

459

460 **AUTHORSHIP CONTRIBUTIONS**

461 The study was conceived by V.L, B.N, O.S and M.E. Specimens were collected by O.S, K.M.K
462 and V.L. S.B welcomed O.S at the Department of Plant and Environmental Sciences, University
463 of Copenhagen, Denmark, so she could performed the chemical analyses with help from E.C..
464 Statistical analyses were done by O.S. O.S wrote the manuscript with contributions from all
465 authors. All authors participated in constructive discussions and approved manuscript final
466 version.

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491 **DATA ACCESSIBILITY**

492 Raw data file describing each compound and concentration per individual is available on
493 Dryad following the link: <https://doi.org/10.5061/dryad.ghx3ffbjt>

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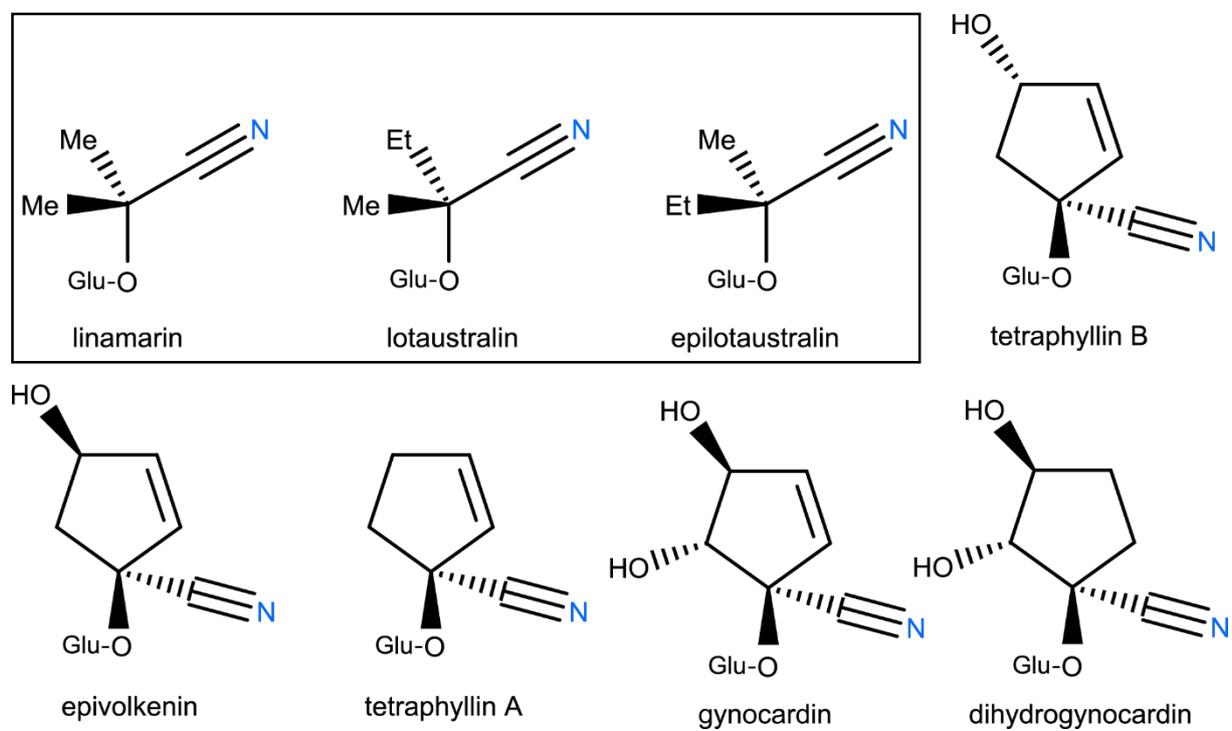
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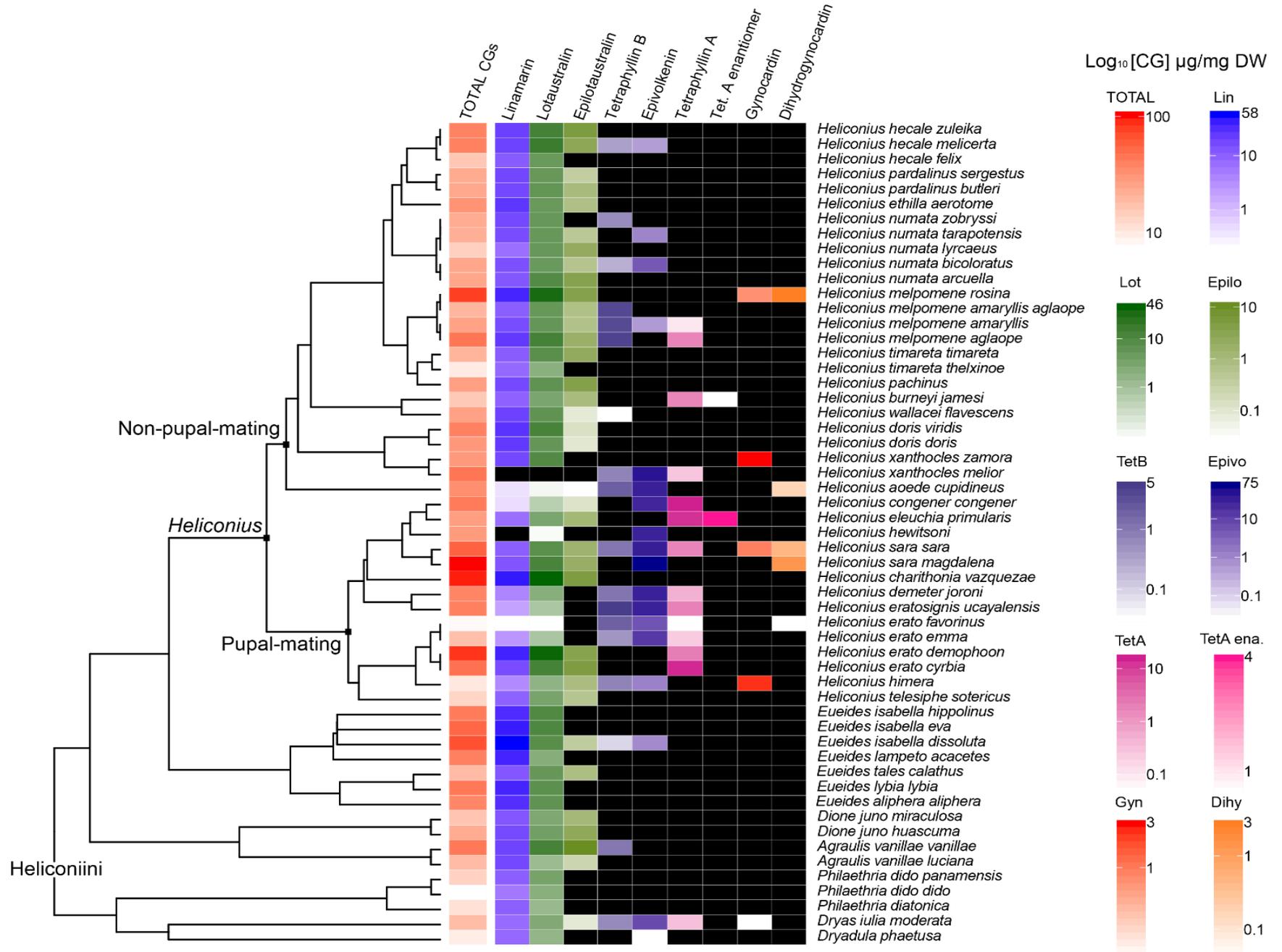
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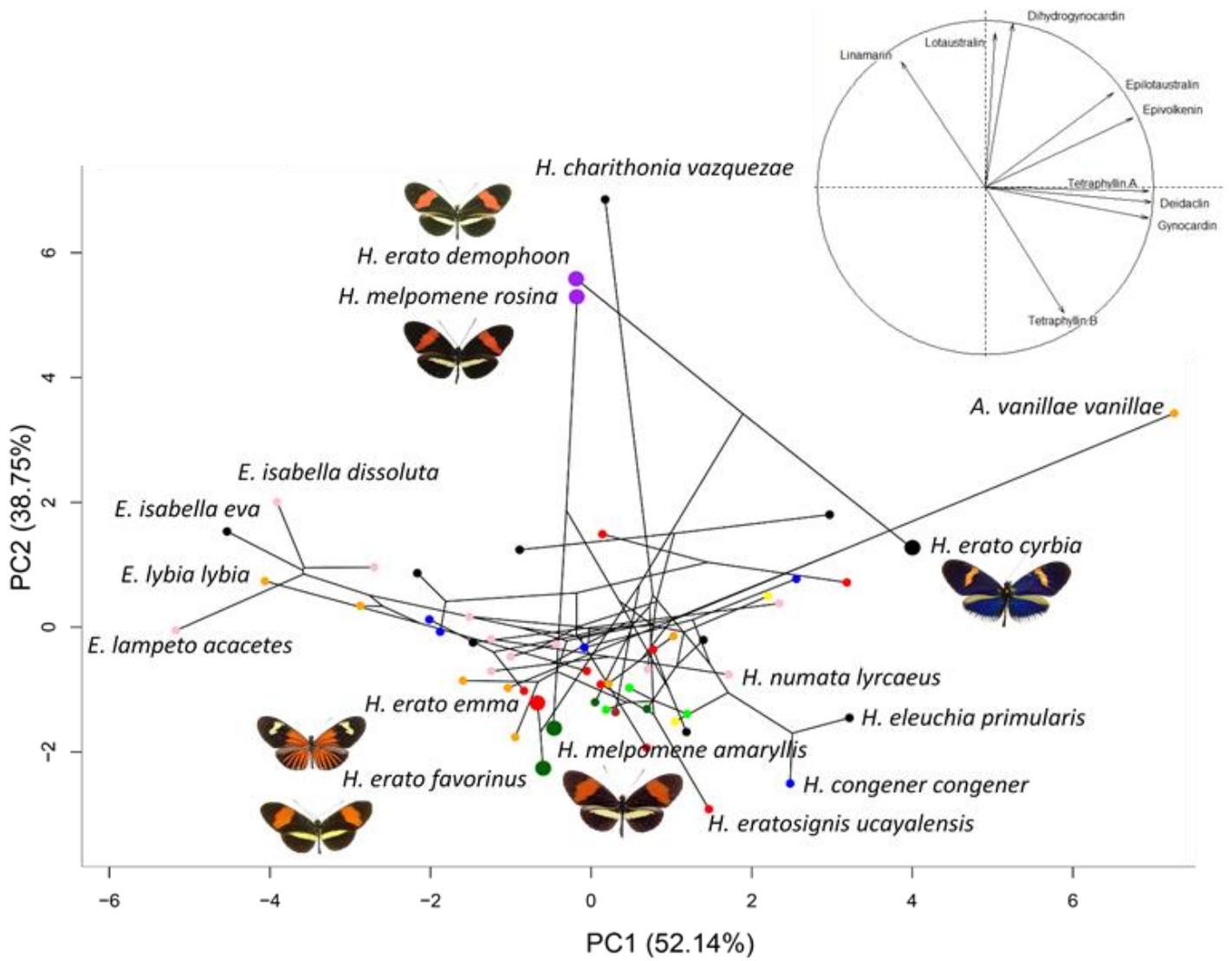


647

648 Figure 1. Cyanogenic glucosides identified in Heliconiini. Framed molecules are aliphatic CGs
 649 synthesized by Heliconiini, followed by cyclopentenoid CGs sequestered from *Passiflora*
 650 plants. Glucose group is symbolized by "Glu". For the first time in Heliconiini, we report
 651 epilotaustralin and a stereoisomer of tetraphyllin A (putatively the deidacline, which is not
 652 represented here because it was not firmly identified during this study).



654 Figure 2. Qualitative and quantitative variations for the nine studied cyanogenic glucosides
655 across Heliconiini subspecies. Phylogenetic tree is adapted from (Kozak et al., 2015). The left
656 column represents the total CG mean concentration ($n = 375$ individuals in 55 subspecies).
657 Following column presents the average of each CG concentration. Concentrations are in μg of
658 CG per mg of dried weigh (body + wings) in a logarithmic scale. A black box signifies either the
659 absence of the CG or insufficient data for measurement. A coloured filled box indicates that
660 the corresponding CG has been reported in at least one individual of the species. Colour
661 gradient is from white corresponding to the minimum reported concentration to the darkest
662 colour corresponding to the maximal reported concentration.

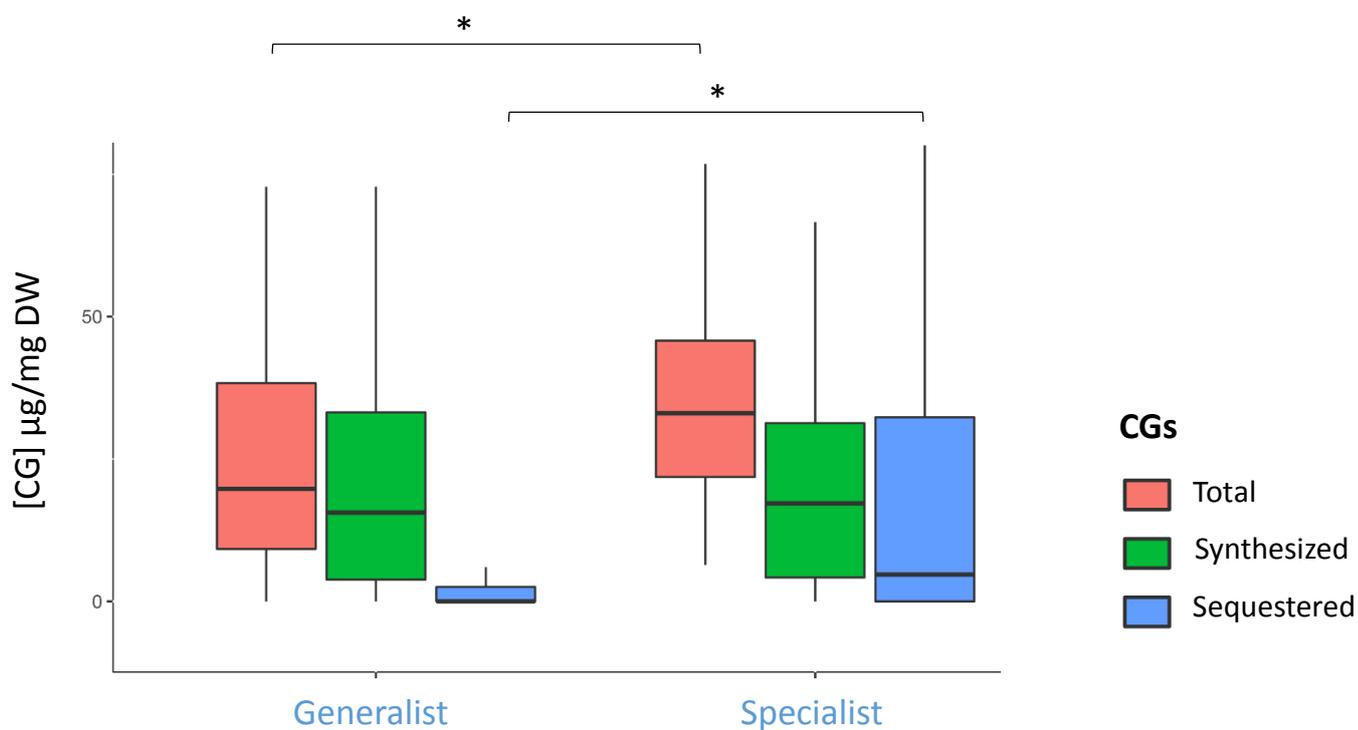


Mimicy ring

- Blue ● Green ● Postman Panama ● Postman Reverse ● Tiger
- Dennis ray ● Orange ● Postman Ecuador/Peru ● Rayed yellow ● Other

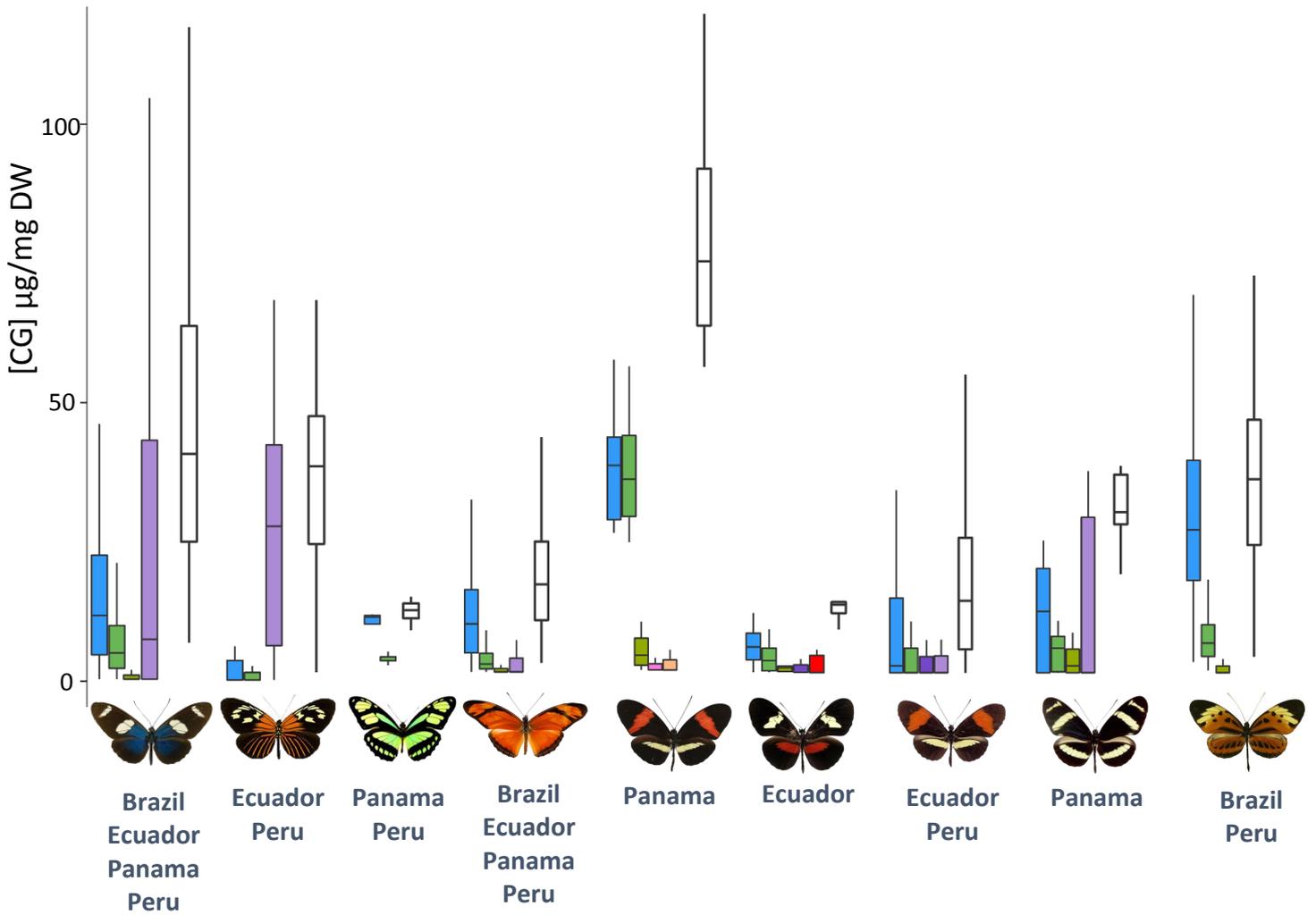
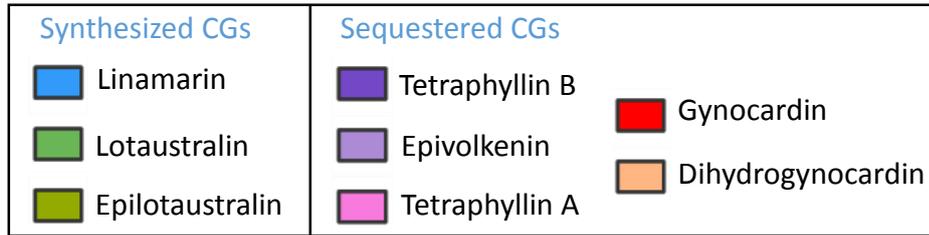
663

664 Figure 3. Phylochemospace depicting the relationships between phylogenetic history and the
665 mean CG concentration in Heliconiini subspecies. Visualization in 2 dimensions of the
666 distribution of the variation in CG profiles. Dark line represents the phylogenetic tree modified
667 from Kozak *et al.*, (2015) to plot subspecies used in our analyses ($n = 55$ subspecies). Dots are
668 mean imputed CG profile per subspecies. Colour indicates the mimicry ring subspecies belong
669 to (Supp. 1). *Heliconius erato* subspecies from distinct mimicry rings also differ in their mean
670 chemical profiles (*H. e. cyrbia* in the “Other” mimicry ring from Ecuador, *H. e. emma* from
671 Dennis-ray ring from Peru, *H. e. favorinus* from Postman ring from Peru and *H. e. demophoon*
672 from Postman ring from Panama). *H. erato* and *H. melpomene* subspecies have increased size
673 dot and are illustrated by a photo.



674

675 Figure 4. Amount of chemical defences according to host-plant specialization. CG
 676 concentrations are given in $\mu\text{g}/\text{mg}$ of dried body mass. We pooled generalist subspecies ($n =$
 677 210 individuals distributed in 32 subspecies) on the left and specialist subspecies ($n = 165$
 678 individuals distributed in 23 subspecies) on the right. We represented the total amount of CG
 679 (red boxplot) that sums synthesized (green boxplot) and sequestered (blue boxplot) CG
 680 concentrations. Asterix shows significant statistical difference.



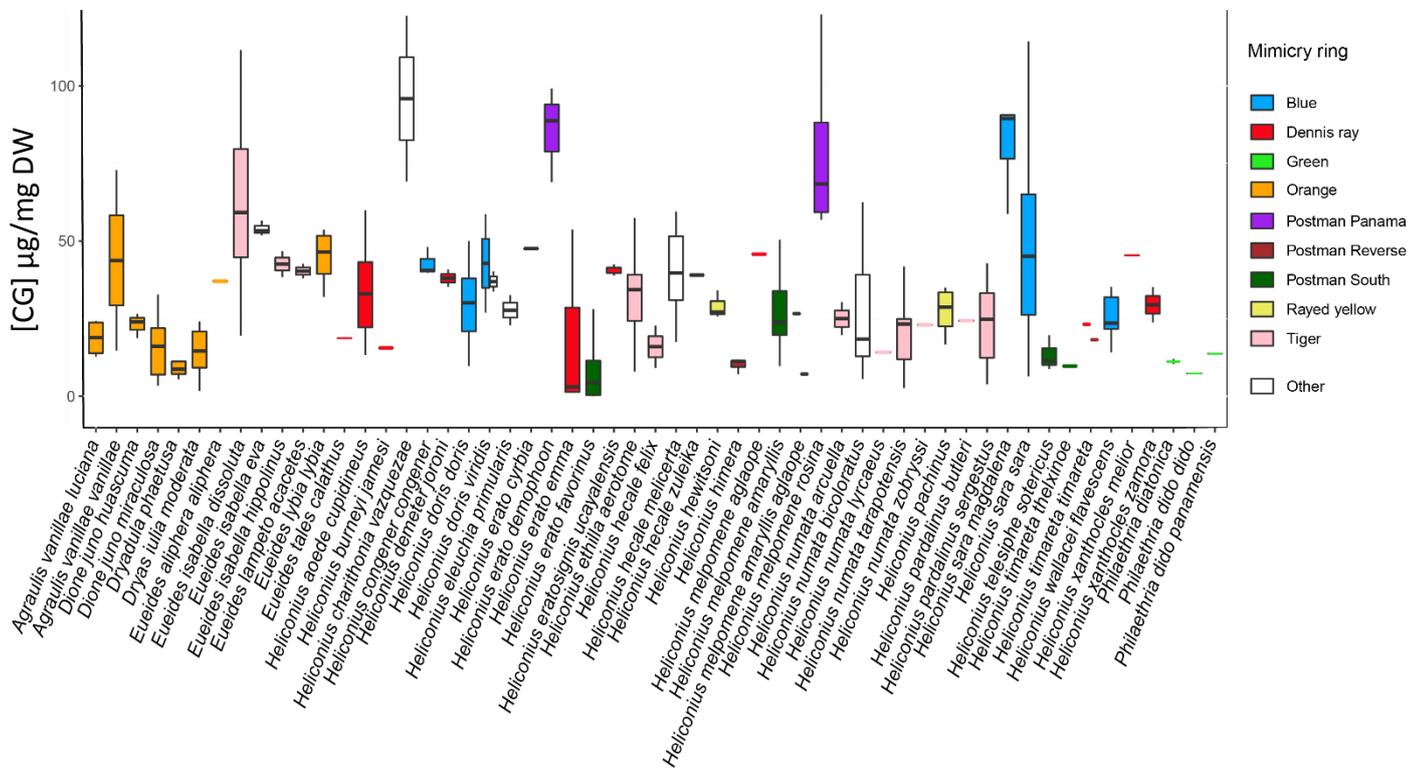
681

682 Figure 5. Variation in chemical profiles of individuals from the nine studied mimicry rings,

683 located in different regions of Central and South America. CG concentrations are given in

684 $\mu\text{g}/\text{mg DW}$. Mimicry rings from left to right, with illustrations of the colour pattern: blue (6

685 subspecies, $n = 66$ individuals), Dennis ray (10 subspecies, $n = 39$), green (3 subspecies, $n = 4$),
686 orange (8 subspecies, $n = 73$), postman Panama (2 subspecies, $n = 7$), postman reverse (2
687 subspecies, $n = 6$), postman from Ecuador and Peru (5 subspecies, $n = 57$), rayed yellow (2
688 subspecies, $n = 7$), tiger (11 subspecies, $n = 78$). White boxplots are mean total CG
689 concentration.



690 Figure 6. Total CG concentration per subspecies. Concentrations are given in $\mu\text{g}/\text{mg DW}$.

691 Boxplot colours correspond to the associated mimicry ring with legend on the right.

692 Subspecies are listed in alphabetical order from left to right ($n = 55$ subspecies).

Mimicry ring	Subspecies
<p>Blue</p> 	<p><i>Heliconius congener congener</i> <i>Heliconius doris doris</i> <i>Heliconius doris viridis</i> (blue morph) <i>Heliconius sara magdalena</i> <i>Heliconius sara sara</i> <i>Heliconius wallacei flavescens</i></p>
<p>Dennis ray</p> 	<p><i>Eueides tales calathus</i> <i>Heliconius aoede cupidineus</i> <i>Heliconius burneyi jamesi</i> <i>Heliconius demeter joroni</i> <i>Heliconius erato emma</i> <i>Heliconius eratosignis ucayalensis</i> <i>Heliconius melpomene aglaope</i> <i>Heliconius timareta timareta</i> <i>Heliconius xanthocles melior</i> <i>Heliconius xanthocles zamora</i></p>
<p>Green</p> 	<p><i>Philaethria diatonica</i> <i>Philaethria dido dido</i> <i>Philaethria dido panamensis</i></p>
<p>Orange</p> 	<p><i>Agraulis vanillae luciana</i> <i>Agraulis vanillae vanillae</i> <i>Dione junno huascuma</i> <i>Dione junno miraculosa</i> <i>Dryadula phaetusa</i> <i>Dryas iulia moderata</i> <i>Eueides aliphera aliphera</i> <i>Eueides lybia lybia</i></p>
<p>Postman Panama</p> 	<p><i>Heliconius erato demopoon</i> <i>Heliconius melpomene rosina</i></p>
<p>Postman Ecuador/Peru</p> 	<p><i>Heliconius erato favorinus</i> <i>Heliconius melpomene amaryllis X aglaope</i> <i>Heliconius telesiphe sotericus</i> <i>Heliconius timareta thelxinoe</i></p>

Mimicry ring	Subspecies (continued)
Postman reverse 	<i>Heliconius himera</i> <i>Heliconius timareta timareta</i>
Rayed yellow 	<i>Heliconius hewitsoni</i> <i>Heliconius pachinus</i>
Tiger 	<i>Eueides isabella dissoluta</i> <i>Eueides isabella hippolinus</i> <i>Eueides lampeto acacetes</i> <i>Heliconius ethilla aerotome</i> <i>Heliconius hecale felix</i> <i>Heliconius numata arcuella</i> <i>Heliconius numata lyrcaeus</i> <i>Heliconius numata tarapotensis</i> <i>Heliconius numata zobryssi</i> <i>Heliconius pardalinus butleri</i> <i>Heliconius pardalinus sergestus</i>
Other	<i>Heliconius melpomene amaryllis X aglaope</i> <i>Eueides isabella eva</i> <i>Heliconius charithonia vazquezae</i> <i>Heliconius doris viridis</i> (red morph) <i>Heliconius eleuchia primularis</i> <i>Heliconius erato cyrbia</i> <i>Heliconius hecale melicerta</i> <i>Heliconius hecale zuleika</i> <i>Heliconius numata bicoloratus</i>

696

697 Table 1. Subspecies are divided in nine mimicry rings. Geographically isolated, phenotypically
698 unique and hybrid individuals were assigned to “Other”. Subspecies belonging to the same
699 mimicry ring share a given colour pattern within the same locality. Mimicry rings and
700 subspecies within are listed in alphabetical order.

Species	Linamarin	Lotaustralin	Epilotaustralin	Tetraphyllin B	Epivolkenin	Tetraphyllin A	Tetraphyllin A stereoisomer	Gynocardin	Dihydrogynocardin
<i>Agraulis vanillae</i>	17.91±8.43	5.10±8.80	3.74±7.58	0.34±0.84	0.00	0.00	0.00	0.00	0.00
<i>Agraulis vanillae luciana</i>	17.28±5.17	1.22±0.91	0.24±0.48	0.00	0.00	0.00	0.00	0.00	0.00
<i>Agraulis vanillae vanillae</i>	19.16±16.43	12.85±14.28	10.74±11.81	1.02±1.45	0.00	0.00	0.00	0.00	0.00
<i>Dione juno</i>	13.28±7.08	2.98±3.62	1.40±1.38	0.00	0.00	0.00	0.00	0.00	0.00
<i>Dione juno huascuma</i>	16.65±2.41	3.88±2.37	2.57±3.13	0.00	0.00	0.00	0.00	0.00	0.00
<i>Dione juno miraculosa</i>	12.50±7.63	2.77±3.90	1.13±0.59	0.00	0.00	0.00	0.00	0.00	0.00
<i>Dryadula phaetusa</i>	7.96±3.32	1.57±1.19	0.00	0.00	0.03±0.07	0.00	0.00	0.00	0.00
<i>Dryas iulia moderata</i>	7.47±10.32	2.51±4.58	0.09±0.28	0.63±1.71	7.50±10.56	0.31±1.37	0.00	0.13±0.61	0.00
<i>Eueides aliphera aliphera</i>	30.66	6.40	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Eueides isabella</i>	54.18±31.07	8.39±5.53	0.30±0.75	0.07±0.36	0.73±3.60	0.00	0.00	0.00	0.00
<i>Eueides isabella dissoluta</i>	58.11±33.81	7.93±6.11	0.38±0.83	0.09±0.40	0.93±4.04	0.00	0.00	0.00	0.00
<i>Eueides isabella eva</i>	43.38±4.58	10.55±2.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Eueides isabella hippolinus</i>	33.02±6.55	9.58±0.68	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Eueides lampeto acacetes</i>	38.15±1.47	2.20±1.83	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Eueides lybia lybia</i>	37.51±8.33	7.15±2.71	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Eueides tales calathus</i>	12.45	5.48	0.81	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius aoede cupidineus</i>	0.40±1.15	0.11±0.28	0.03±0.12	2.02±9.49	31.04±14.70	0.00	0.00	0.00	0.17±0.39
<i>Heliconius burneyi jamesi</i>	9.23	2.98	0.95	0.00	0.00	1.67	0.77	0.00	0.00
<i>Heliconius charithonia vazquezae</i>	45.18±13.08	45.78±24.24	4.91±0.45	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius congener congener</i>	0.45±0.77	0.55±0.95	0.12±0.20	0.00	25.96±22.48	15.74±26.85	0.00	0.00	0.00
<i>Heliconius demeter joroni</i>	3.93±1.94	2.08±0.16	0.00	1.08±1.52	30.45±3.79	0.48±0.68	0.00	0.00	0.00
<i>Heliconius doris</i>	25.44±7.88	7.73±8.58	0.11±0.25	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius doris doris</i>	24.37±8.28	5.50±7.36	0.09±0.27	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius doris viridis</i>	27.56±7.64	12.20±10.18	0.13±0.27	0.00	0.00	0.00	0.00	0.00	0.00

<i>Heliconius eleuchia primularis</i>	6.84±9.67	3.07±4.35	1.14±1.61	0.00	0.00	12.34±6.92	4.34±1.86	0.00	0.00
<i>Heliconius erato</i>	3.77±10.68	3.58±11.12	0.39±1.49	1.58±3.91	6.07±10.02	0.58±2.35	0.00	0.00	0.03±0.21
<i>Heliconius erato cyrbia</i>	15.42	12.89	4.97	0.00	0.00	14.30	0.00	0.00	0.00
<i>Heliconius erato demopoon</i>	38.82±9.22	41.21±5.29	3.50±3.94	0.00	0.00	2.11±2.04	0.00	0.00	0.00
<i>Heliconius erato emma</i>	2.57±3.57	0.81±1.60	0.00	0.47±0.66	13.49±18.06	0.22±0.50	0.00	0.00	0.00
<i>Heliconius erato favorinus</i>	0.19±0.41	0.08±0.45	0.00	1.97±4.38	5.65±8.53	0.05±0.27	0.00	0.00	0.04±0.23
<i>Heliconius eratosignis ucayalensis</i>	1.87±1.66	0.79±0.56	0.00	5.51±4.41	30.54±8.74	1.89±2.61	0.00	0.00	0.00
<i>Heliconius ethilla aerotome</i>	26.30±10.38	5.02±2.66	0.74±0.73	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius hecale</i>	17.88±7.53	13.66±8.23	2.45±2.98	0.23±0.70	0.33±0.99	0.00	0.00	0.00	0.00
<i>Heliconius hecale felix</i>	10.94±5.66	5.02±3.93	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius hecale melicerta</i>	19.74±7.71	16.62±8.05	2.79±3.17	0.35±0.86	0.50±1.22	0.00	0.00	0.00	0.00
<i>Heliconius hecale zuleika</i>	20.59	13.16	5.29	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius hewitsoni</i>	0.00	0.09±0.16	0.00	0.00	28.91±4.55	0.00	0.00	0.00	0.00
<i>Heliconius himera</i>	3.44±2.77	1.66±1.95	0.91±1.28	0.77±1.06	1.24±2.77	0.00	0.00	2.22±3.28	0.00
<i>Heliconius melpomene</i>	18.51±11.97	9.79±11.69	1.13±1.87	3.31±4.66	0.39±1.87	0.13±0.49	0.00	0.10±0.54	0.38±1.48
<i>Heliconius melpomene aglaope</i>	24.60	13.09	1.75	4.57	0.00	1.75	0.00	0.00	0.00
<i>Heliconius melpomene amaryllis</i>	15.82±8.52	5.56±3.31	0.67±0.96	3.78±5.10	0.55±2.20	0.10±0.44	0.00	0.00	0.00
<i>Heliconius melpomene amaryllis aglaope (hybrid)</i>	10.15±5.99	5.25±2.47	0.71±0.65	4.03±4.27	0.00	0.00	0.00	0.00	0.00
<i>Heliconius melpomene rosina</i>	37.34±15.00	34.61±15.06	3.74±3.93	0.00	0.00	0.00	0.00	0.72±1.45	2.74±3.44
<i>Heliconius numata</i>	14.52±8.33	5.29±3.75	0.74±1.10	0.15±0.65	2.96±7.20	0.00	0.00	0.00	0.00
<i>Heliconius numata arcuella</i>	12.26±4.15	9.16±2.68	3.58±0.73	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius numata bicoloratus</i>	14.41±9.00	4.92±3.42	0.58±0.86	0.24±0.88	4.76±9.02	0.00	0.00	0.00	0.00
<i>Heliconius numata lyrcaeus</i>	6.72	5.27	2.19	0.00	0.00	0.00	0.00	0.00	0.00

<i>Heliconius numata tarapotensis</i>	15.5±8.49	5.23±4.55	0.47±0.81	0.00	1.09±3.79	0.00	0.00	0.00	0.00
<i>Heliconius numata zobryssi</i>	17.13	5.27	0.00	0.61	0.00	0.00	0.00	0.00	0.00
<i>Heliconius pachus</i>	17.01±4.95	6.31±1.97	3.98±2.72	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius pardalinus</i>	17.80±8.89	5.38±3.18	0.42±0.63	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius pardalinus butleri</i>	17.56±0.92	5.84±0.28	0.97±1.37	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius pardalinus sergestus</i>	17.83±9.55	5.32±3.41	0.34±0.51	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius sara</i>	10.59±10.80	8.07±8.41	1.49±3.00	0.94±6.19	38.17±40.18	1.55±9.95	0.00	0.81±2.71	0.56±1.33
<i>Heliconius sara magdalena</i>	12.98±14.65	11.63±8.28	1.88±0.89	0.00	75.92±40.87	0.00	0.00	0.00	1.3±1.8
<i>Heliconius sara sara</i>	10.28±10.41	7.61±8.42	1.43±3.17	1.07±6.58	33.20±37.86	1.76±10.58	0.00	0.92±2.87	0.46±1.25
<i>Heliconius telesiphe sotericus</i>	9.42±3.59	3.26±2.26	0.57±0.53	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius timareta</i>	9.76±1.85	5.76±3.36	1.53±1.82	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius timareta thelxinoe</i>	7.82	1.92	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius timareta timareta</i>	10.73±1.11	7.69±0.68	2.30±1.75	0.00	0.00	0.00	0.00	0.00	0.00
<i>Heliconius wallacei flavescens</i>	20.09±8.87	6.37±2.44	0.08±0.26	0.03±0.10	0.00	0.00	0.00	0.00	0.00
<i>Heliconius xanthocles</i>	11.70±10.41	6.15±6.45	0.00	0.25±0.43	14.80±25.64	0.09±0.16	0.00	1.80±1.60	0.00
<i>Heliconius xanthocles melior</i>	0.00	0.00	0.00	0.75	44.41	0.27	0.00	0.00	0.00
<i>Heliconius xanthocles zamora</i>	17.55±3.41	9.23±5.12	0.00	0.00	0.00	0.00	0.00	2.70±0.52	0.00
<i>Philaethria diatonica</i>	9.85±0.34	1.34±0.79	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Philaethria dido</i>	7.87±3.54	2.68±0.93	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Philaethria dido dido</i>	5.37	2.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Philaethria dido panamensis</i>	10.38	3.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00

701

702 Table 2. Mean concentration and associated standard deviation for each compound detected.
703 We present data for both species and subspecies. CG concentrations are given in $\mu\text{g}/\text{mg}$ of
704 dried body mass.

MANOVA on mean per subspecies ($n = 55$)	Regular	Phylogenetic
	Mimicry ring *	
	Pillai $^9_{36} = 2.736, p < 0.001$	Pillai $^9_{36} = 2.736, p = 0.582$
	Host-plant specialization *	
MANOVA on inter-individual variation ($n = 375$)	Regular	
	Mimicry ring *	
	Pillai $^{10}_{364} = 1.209, p < 0.001$	
	Host-plant specialization *	
	Pillai $^1_{373} = 0.165, p < 0.001$	

705

706 Table 3. Comparisons of CG profile (MANOVA) between and among mimicry rings and host-
707 plant specialization levels. To compare the effect of mimicry rings and host-plant
708 specialization on CG profiles with phylogenetic effect, we performed a MANOVA using the
709 mean concentration per subspecies ($n = 55$ subspecies). Then MANOVA were performed on
710 CG profiles using the whole dataset to test for inter-individual variation ($n = 375$ individuals),
711 without testing the effect of phylogeny.

712 * Note that each factor was tested using an independent MANOVA.

Regular MANOVA on inter-individual variation ($n = 375$)						
	Degree of Freedom	Pillai	F-statistic	Degrees of freedom of the numerator	Degrees of freedom of the denominator	p -value associated with the F statistic
Colour pattern	9	1.455	$F^9_{325} = 6.965$	81	2925	$p < 0.001$
Locality	3	1.167	$F^3_{325} = 22.544$	27	957	$p < 0.001$
Colour pattern + Locality	29	0.540	$F^8_{325} = 2.607$	72	2592	$p < 0.001$
Species	8	2.371	$F^{28}_{325} = 4.153$	252	2925	$p < 0.001$
Specialization	1	0.247	$F^1_{325} = 11.546$	9	317	$p < 0.001$

713

714 Table 4. Variation of CG chemical profile between individuals ($n = 375$). MANOVA tests if there

715 is difference for the CG chemical profiles between groups (listed in left column). Residuals =

716 325.

Genre	Species	subspecies	Female	Male	TOTAL	Country	Specialization
<i>Agraulis</i>	<i>vanillae</i>	<i>luciana</i>	1	3	4	Peru	Generalist
<i>Agraulis</i>	<i>vanillae</i>	<i>vanillae</i>	1	1	2	Panama	Generalist
<i>Dione</i>	<i>juno</i>	<i>huascuma</i>	1	2	3	Panama	Generalist
<i>Dione</i>	<i>juno</i>	<i>miraculosa</i>	5	8	13	Peru	Generalist
<i>Dryadula</i>	<i>phaetusa</i>	NA	2	6	8	Peru/Ecuador	Generalist
<i>Dryas</i>	<i>iulia</i>	<i>moderata</i>	14	24	38	Peru/Panama/Brazil	Generalist
<i>Eueides</i>	<i>isabella</i>	<i>dissoluta</i>	8	11	19	Peru	Generalist
<i>Eueides</i>	<i>isabella</i>	<i>eva</i>	0	3	3	Panama	Generalist
<i>Eueides</i>	<i>isabella</i>	<i>hippelinus</i>	0	2	2	Peru	Generalist
<i>Eueides</i>	<i>lampeto</i>	<i>acacetes</i>	1	1	2	Peru	Generalist
<i>Eueides</i>	<i>aliphera</i>	<i>aliphera</i>	1	0	1	Brazil	Generalist
<i>Eueides</i>	<i>lybia</i>	<i>lybia</i>	0	4	4	Brazil	Generalist
<i>Eueides</i>	<i>tales</i>	<i>calathus</i>	0	1	1	Ecuador	Generalist
<i>Heliconius</i>	<i>aoede</i>	<i>cupidineus</i>	9	13	22	Peru	Specialist
<i>Heliconius</i>	<i>burneyi</i>	<i>jamesi</i>	0	1	1	Peru	Specialist
<i>Heliconius</i>	<i>charithonia</i>	<i>vazquezae</i>	0	2	2	Panama	Generalist
<i>Heliconius</i>	<i>congener</i>	<i>congener</i>	0	3	3	Ecuador	Specialist
<i>Heliconius</i>	<i>demeter</i>	<i>joroni</i>	2	0	2	Peru	Specialist
<i>Heliconius</i>	<i>doris</i>	<i>doris</i>	3	5	8	Peru	Specialist
<i>Heliconius</i>	<i>doris</i>	<i>viridis</i>	2	2	4	Panama	Specialist
<i>Heliconius</i>	<i>eleuchia</i>	<i>primularis</i>	0	2	2	Ecuador	Specialist
<i>Heliconius</i>	<i>erato</i>	<i>cyrbia</i>	0	1	1	Ecuador	Generalist
<i>Heliconius</i>	<i>erato</i>	<i>demophoon</i>	2	1	3	Panama	Generalist
<i>Heliconius</i>	<i>erato</i>	<i>emma</i>	1	4	5	Peru	Generalist
<i>Heliconius</i>	<i>erato</i>	<i>favorinus</i>	11	20	31	Peru	Generalist
<i>Heliconius</i>	<i>eratosignis</i>	<i>ucayalensis</i>	0	3	3	Peru	Specialist
<i>Heliconius</i>	<i>ethilla</i>	<i>aerotome</i>	5	16	21	Peru	Specialist
<i>Heliconius</i>	<i>hecale</i>	<i>felix</i>	0	2	2	Peru	Generalist
<i>Heliconius</i>	<i>hecale</i>	<i>melicerta</i>	2	4	6	Panama	Generalist
<i>Heliconius</i>	<i>hecale</i>	<i>zuleika</i>	0	1	1	Panama	Generalist
<i>Heliconius</i>	<i>hewitsoni</i>	NA	0	3	3	Panama	Specialist
<i>Heliconius</i>	<i>himera</i>	NA	2	3	5	Ecuador	Specialist
<i>Heliconius</i>	<i>melpomene</i>	<i>aglaope</i>	1	0	1	Peru	Specialist
<i>Heliconius</i>	<i>melpomene</i>	<i>amaryllis</i>	5	16	21	Peru	Specialist
<i>Heliconius</i>	<i>melpomene</i>	<i>amaryllis*aglaope</i>	1	2	3	Peru	Specialist
<i>Heliconius</i>	<i>melpomene</i>	<i>rosina</i>	1	3	4	Panama	Specialist

Genre	Species	subspecies	Female	Male	TOTAL	Country	Specialization
<i>Heliconius</i>	<i>numata</i>	<i>arcuella</i>	2	0	2	Peru	Generalist
<i>Heliconius</i>	<i>numata</i>	<i>bicoloratus</i>	4	15	19	Peru	Generalist
<i>Heliconius</i>	<i>numata</i>	<i>lyrcaeus</i>	1	0	1	Peru	Generalist
<i>Heliconius</i>	<i>numata</i>	<i>tarapotensis</i>	2	10	12	Peru	Generalist
<i>Heliconius</i>	<i>numata</i>	<i>zobryssi</i>	0	1	1	Brazil	Generalist
<i>Heliconius</i>	<i>pachinus</i>	NA	2	2	4	Panama	Generalist
<i>Heliconius</i>	<i>pardalinus</i>	<i>butleri</i>	1	1	2	Peru	Generalist
<i>Heliconius</i>	<i>pardalinus</i>	<i>sergestus</i>	3	11	14	Peru	Generalist
<i>Heliconius</i>	<i>sara</i>	<i>magdalena</i>	2	3	5	Panama	Specialist
<i>Heliconius</i>	<i>sara</i>	<i>sara</i>	16	22	38	Peru/Ecuador/Brazil	Specialist
<i>Heliconius</i>	<i>telesiphe</i>	<i>sotericus</i>	0	3	3	Ecuador	Specialist
<i>Heliconius</i>	<i>timareta</i>	<i>thelxinoe</i>	0	1	1	Peru	Specialist
<i>Heliconius</i>	<i>timareta</i>	<i>timareta</i>	0	2	2	Ecuador	Specialist
<i>Heliconius</i>	<i>wallacei</i>	<i>flavescens</i>	2	8	10	Peru/Brazil	Specialist
<i>Heliconius</i>	<i>xanthocles</i>	<i>melior</i>	0	1	1	Peru	Specialist
<i>Heliconius</i>	<i>xanthocles</i>	<i>zamora</i>	2	0	2	Ecuador	Specialist
<i>Philaethria</i>	<i>diatonica</i>	NA	0	2	2	Peru	Generalist
<i>Philaethria</i>	<i>dido</i>	<i>dido</i>	0	1	1	Peru	Generalist
<i>Philaethria</i>	<i>dido</i>	<i>panamensis</i>	1	0	1	Panama	Generalist

719

720 Appendix 1. detailed list of sampled butterfly subspecies ($n = 375$ individuals), with number of
721 females ($n = 119$) and males ($n = 256$) as well as provenance country (Brazil, Ecuador, Panama
722 or Peru). Some species do not have subspecies name so it was “NA” assigned. Right column
723 “Specialization” indicates whether subspecies are generalists (feed on wide panel of *Passiflora*
724 plants) or specialists (feed on a restricted range of *Passiflora* plants).