TITLE PAGE

Title: Evolutionary and ecological processes influencing chemical defense variation in an aposematic and mimetic *Heliconius* butterfly

Short title: Chemical defense variation in a butterfly

Authors: Anniina L. K. Mattila[1,6*]; Chris D. Jiggins[2]; Øystein H. Opedal[1,3]; Gabriela Montejo-Kovacevich[2]; Érika de Castro[2]; William O. McMillan[4]; Caroline Bacquet[5]; Marjo Saastamoinen[1,6]

Author affiliations:

1. Research Centre for Ecological Change, Organismal and Evolutionary Biology Research Programme, University of Helsinki, Helsinki, Finland
2. Department of Zoology, University of Cambridge, Cambridge, UK
3. Department of Biology, Lund University, Lund, Sweden
4. Smithsonian Tropical Research Institute, Gamboa, Panama
5. Universidad Regional Amazónica de Ikiam, Tena, Ecuador
6. Helsinki Life Science Institute, University of Helsinki, Helsinki, Finland

Corresponding author: Anniina L. K. Mattila: anniina.mattila@helsinki.fi, *current affiliation: Finnish Museum of Natural History (LUOMUS), University of Helsinki, Helsinki, Finland

ABSTRACT

Chemical defences against predators underlie the evolution of aposematic coloration and mimicry, which are classic examples of adaptive evolution. Surprisingly little is known about the roles of ecological and evolutionary processes maintaining defence variation, and how they may feedback to shape the evolutionary dynamics of species. Cyanogenic *Heliconius* butterflies exhibit diverse warning color patterns and mimicry, thus providing a useful framework for investigating these questions. We studied intraspecific variation in *de novo* biosynthesized cyanogenic toxicity and its potential ecological and evolutionary sources in wild populations of *Heliconius erato* along environmental gradients, in common-garden broods and with feeding treatments. Our results demonstrate substantial intraspecific variation, including detectable variation among broods reared in a common garden. The latter estimate suggests considerable evolutionary potential in this trait, although predicting the response to selection is likely complicated due to the observed skewed distribution of toxicity values and the signatures of maternal contributions to the inheritance of toxicity. Larval diet contributed little to toxicity variation. Furthermore, toxicity profiles were similar along steep rainfall and altitudinal gradients, providing little evidence for these factors explaining variation in biosynthesized toxicity in natural populations. In contrast, there were striking differences in the chemical profiles of *H. erato* from geographically distant populations, implying potential local adaptation in the acquisition mechanisms and levels of defensive compounds. The results highlight the extensive variation and potential for adaptive evolution in defense traits for aposematic and mimetic species, which may contribute to the high diversity often found in these systems.

KEYWORDS: chemical defenses, aposematism, mimicry, evolvability, *Heliconius*

MAIN TEXT

INTRODUCTION

Chemical defenses are a common means for animals to gain protection against predators. Defensive chemicals can be acquired from the larval or adult diet (sequestered) and/or biosynthesized *de novo* by the organism (Bowers, 1992; de Castro et al., 2019; Opitz & Müller, 2009). Chemical defenses are often coupled with bright color patterns, which local predators learn to associate with toxicity or unpalatability of the prey, a phenomenon known as aposematism (Ruxton et al., 2018). This can also lead to mimicry among co-occurring prey species, one of the best studied examples of Darwin's theory of evolution by natural selection and the subject of one of evolution's oldest mathematical models (Müller, 1879). Because palatability can vary along a continuum (Arias et al., 2016; Prudic et al., 2019; Speed, 1999; Speed et al., 2012; Turner, 1984), mimicry can take several forms; Batesian, where a palatable mimic exploits an unpalatable model, Müllerian, in which unpalatable species copy one another for mutual benefit, or Quasi-Batesian, where a less well protected species acts in a parasitic manner, diluting the protection of the better defended species (Mallet, 1999; Speed, 1993, 1999; Turner, 1987).

These different mimicry relationships all imply predictable differences in toxicity among mimetic species and individuals (Speed, 1999; Turner, 1987), and yield different predictions about the origins of diversity in warning coloration and mimicry, which is often greater than predicted by classic aposematic theory (Briolat et al., 2019; Joron & Mallet, 1998; Speed, 1999). For example, alternation between different mimicry types could influence patterns of convergent vs. advergent selection (where selective pressures cause phenotypic convergence of one species on another, but not vice versa) (Mallet, 1999; Mallet & Joron, 1999; Speed, 1999). However, there are many open questions related to the roles of ecological and evolutionary processes shaping chemical defense variation in aposematic and mimetic species (Mallet & Joron, 1999; Speed et al., 2012; Ruxton et al., 2018; Briolat et al., 2019). One outstanding current question is "what drives variation in defensive toxins within and among prey populations?"

Extensive variation in defensive toxins within species and across mimicry rings has been demonstrated repeatedly (Speed et al., 2012). Indeed, unpalatability levels of mimic species can range from equal to very uneven (Arias et al., 2016; Bowers, 1992; Ritland & Brower, 1991). Potential sources of this

variation include host-plant chemistry, which often varies with the environment and is influenced by resource availability (Massad et al., 2011). Host-plant chemistry can be a major source of intraspecific variation particularly in sequestered defensive compounds (Bowers, 1992; Opitz & Müller, 2009), as seen in many butterfly species (Camara, 1997; Gardner & Stermitz, 1988; Hay-Roe & Nation, 2007). Detoxification of host plant defensive chemicals can also negatively affect an individual's resource budget, leading to variation in investment in defenses (Lindstedt et al., 2010; Reudler et al., 2015). Similarly, species that have evolved the ability to biosynthesize defensive compounds often vary extensively in their defensive chemicals. In this case, the origin of both inter- and intraspecific variation is less clear (Ruxton et al., 2018; Burdfield-Steel et al., 2019; Zvereva & Kozlov, 2016). Sources of variation may include biotic factors, such as the energy and resources available for toxin synthesis, interactions with life-history traits including age and reproductive stage, and general condition as assayed by e.g. immunological status (Bowers, 1992; Smilanich et al., 2009; Zvereva & Kozlov, 2016). This variation could simply be maintained by drift if toxicity profiles are selectively neutral, for example if acquiring and maintaining defenses does not incur costs for the individual, and variation in defense level does not affect predator behavior (Speed et al., 2012; Briolat et al., 2019).

In those species that sequester and those that synthesize defensive compounds, there are also several potential adaptive explanations for the persistence of variation in nature (Speed et al., 2012). The selection pressures acting on chemical defense traits are expected to be complex, as chemically defended herbivores are often involved in close coevolutionary arms-races with their host plants, predators and parasitoids (Turner, 1984; Ruxton et al., 2018). The costs of producing and maintaining toxicity may also result in frequency- or density-dependent selection for resource optimization (Speed et al., 2012). Such costs could lead to energetic trade-offs (Bowers, 1992; Fordyce & Nice, 2008; Lindstedt et al., 2020) and automimicry (the occurrence of palatable "cheaters" in a chemically defended population; Brower et al., 1970; Speed et al., 2012). Evolutionary explanations for chemical defense variation are only plausible if such variation is genetically determined, and sufficient genetic variation is available for selection to act on. Therefore, knowledge about the heritability and evolvability of chemical defense traits is fundamental in evaluating the roles of evolutionary *vs.* ecological processes in the origins of the extensive variation observed in these traits. Overall, empirical data unraveling the patterns and origins of chemical defense variation are

needed to help build a more complete understanding of the evolution and diversification of aposematic and mimetic systems.

Here, we investigate aspects of chemical defense variation in neotropical *Heliconius* butterflies. With their incredible diversity in wing coloration, they are a well-known example of aposematism and mimicry (Jiggins, 2017; Merrill et al., 2015), and provide a powerful study system in which to assess the role of ecology and evolution in chemical defense variation within species and populations. *Heliconius* larvae feed exclusively on passion vines (*Passiflora*), with which they have radiated and speciated through a coevolutionary arms race (Benson et al., 1975; de Castro et al., 2018). Almost 30 different cyanogenic glucoside structures have been reported in the *Passiflora* genus (de Castro et al., 2019) and only the most simple of these compounds can be sequestered by *Heliconius* larvae (de Castro et al., 2019; Engler et al., 2000). *Heliconius* larvae and adult butterflies can additionally biosynthesize the cyanogens linamarin and lotaustralin *de novo* from aliphatic amino acids (Nahrstedt & Davis, 1983). Although biosynthesis is not uncommon in chemically defended animals (Bowers, 1992; Ruxton et al., 2018), it is uncommon among chemically defended Lepidoptera (Nishida, 2002). There is substantial variation in the amounts and types of cyanogens among different *Heliconius* species (Arias et al., 2016; Cardoso & Gilbert, 2013; de Castro et al., 2019; Engler-Chaouat & Gilbert, 2007; Sculfort et al., 2020). Some of this is explained by the level of diet specialization, which is a major determinant of the relative roles of cyanogen sequestration from plants and *de novo* biosynthesis (de Castro et al., 2019; Engler-Chaouat & Gilbert, 2007). The level of sequestered defenses is also known to be affected by variation in the chemical content of the host plants (Engler-Chaouat & Gilbert, 2007; Hay-Roe & Nation, 2007), whereas the sources of variation in biosynthesis are less well understood.

We studied ecological and evolutionary aspects of intraspecific variation in biosynthesized cyanogenic toxicity in *Heliconius erato* using a combination of extensive field collections and common-garden rearing experiments. *Heliconius erato* provides an interesting study subject in terms of variation in chemical defenses, warning coloration patterns and mimicry relationships, as it has a diverse array of color forms associated with local color pattern mimicry rings, and is found in ecologically diverse habitats across a large geographic range. Our field collections exploit two strong environmental gradients: a rainfall gradient with a five-fold difference in rainfall, and a steep altitudinal gradient in the Andes. We use these data to

129 tackle three questions: 1) What is the extent of natural variation in toxicity within and among populations of
130 *H. erato*? 2) Can some of this variation be explained by environmental gradients or highly divergent habitat
131 types across a wider geographic scale? 3) How much of the variation in biosynthesized chemical defenses is
132 heritable and evolvable, and how much is governed by environmental (particularly dietary) variance? By
133 examining our results in the context of the well-studied mimicry system of *Heliconius* butterflies, we discuss
134 the potential wider implications of our findings for aposematic and mimetic systems.
135

136 **METHODS**

137 **Study sites and field collection of butterflies and host plants**

138 *Heliconius erato demophoon* and its typical host plant *P. biflora* were collected in Panama along a steep
139 rainfall gradient (the Smithsonian Tropical Research Institute Physical Monitoring Program;
140 https://biogeodb.stri.si.edu/physical_monitoring/) from three populations (Wet, Intermediate and Dry study
141 sites; Fig 1A, Supplementary File 4), and *H. erato lativitta* from High and Low altitude sites on the Eastern
142 slope of the Andes in Ecuador (Fig. 1A, Supplementary File 4). Butterflies were caught using a hand net and
143 transported live to the laboratory. All individuals caught from the Panamanian study areas (n = 92, of which
144 34, 28 and 30 individuals were caught from Dry, Intermediate and Wet sites, respectively) were sexed,
145 weighed, and preserved in 1 ml 100% MeOH (body excluding wings and one half of the thorax) on the day
146 of capture (males) or after being allowed to oviposit in an insectary (females, see Supplementary File 4). At
147 the Ecuadorian sites, fertilized females were brought to a common garden environment, allowed to oviposit
148 and raised separately according to the altitude where they were collected. The thorax of 21 F1 individuals
149 from each altitude (total n=42) were sampled in 1 ml 100% MeOH.

150 *Passiflora biflora* leaf samples (one to four leaves pooled per plant individual, see
151 Supplementary File 4) were collected from the Panama Dry (n = 20), Intermediate (n = 11) and Wet (n = 20)
152 study areas (Fig. 1A), and preserved fresh in 1ml 100% MeOH. In addition, 50 cm cuttings, all from
153 different plant individuals, were sampled from the Dry (n = 12) and Wet (n = 12) study areas for greenhouse
154 cultivation.
155

156 **Host plant greenhouse cultivation and treatments**

157 *Passiflora biflora* were cultivated in greenhouses (75% relative humidity; 8-20 hrs: 25°C, light; 20-8 hrs:
158 20°C, dark) in a soil mixture (50% compost, 20% coir, 15% perlite, 15% sand or gravel) (see Supplementary
159 File 4 for details on cultivation methods and watering treatments). Standard (std)-treated *P. biflora* were used
160 as oviposition plants for the *H. erato* parental generation and larval diet for the F1 families not included in
161 feeding treatments (see below). The quality of host plants in *H. erato* feeding treatments was manipulated
162 with watering treatments, motivated by the importance of water availability for many cyanogenic plants
163 (Gleadow & Woodrow, 2002; Hayden & Parker, 2002; Liang, 2003; Vandegeer et al., 2013). The *P. biflora*
164 cuttings collected from the Dry and Wet study areas in Panama were cultivated in a full-factorial
165 combination of origins and water treatments, resulting in four host plant types. Plants in the dry treatment
166 were kept at 75% relative humidity and watered two to three times weekly with about 1/3 of the water
167 volume compared to the wet treatment (constant water availability, relative humidity up to 100%), such that
168 the surface soil was allowed to dry and the leaves slightly droop between watering events. The dry treatment
169 thus aimed to mimic conditions of drought stress.
170
171 **Butterfly rearing, breeding design and feeding treatments**
172 Laboratory *H. erato* populations descendent of the wild Dry population were established in greenhouse
173 conditions (see Supplementary File 4 for details on rearing methods). Larvae were reared on std-type *P.*
174 *biflora*. All butterflies were individually marked, and were allowed to fly, mate, and oviposit freely in 2×2×2
175 m mesh cages with potted std-type *P. biflora* and a standardized diet of sugar solution supplemented with
176 amino acids provided *ad libitum*. The butterfly cages were observed throughout the day for mating pairs, and
177 mated females were moved to individual 2×2×2 m mesh cages and allowed to oviposit on std-type *P. biflora*.
178 Eggs of 14 females (eight mated with a known father, average no. of full-sibs/family = 8, see Supplementary
179 File 2) were collected, and hatched larvae were reared in full-sib groups fed on std-type *P. biflora* until
180 pupation.
181 The effect of larval dietary cyanogen content and quality on adult butterfly biosynthesized
182 toxicity was studied with feeding treatments. We expected larval dietary quality to affect butterfly toxicity
183 due to potential variation in the acquisition of nitrogenous resources for cyanogen biosynthesis (Cardoso and
184 Gilbert 2013), possible detoxification costs (Lindstedt et al., 2010; Reudler et al., 2015) and the general

importance of larval diet for fitness traits in holometabolous insects (e.g. Boggs & Freeman, 2005). The offspring of six mated pairs (both parental identities known, average no. of full-sibs/family = 29, Supplementary File 2) were included in the feeding treatments, where eggs from each mother were divided equally into four groups and transferred onto each of the four *P. biflora* treatment types. Individuals of the F1 generation were collected within three days following emergence (unmated and unfed), weighed, and preserved in 100% MeOH (body excluding wings and one-half of thorax).

Butterfly toxicity analyses with ^{1}H-NMR

The concentrations of the two cyanogenic compounds biosynthesized by *Heliconius* larvae and adults, linamarin and lotaustralin, were analyzed from the butterfly samples using nuclear magnetic resonance (^{1}H-NMR) with Bruker Avance III HD NMR spectrometer (Bruker BioSpin, Germany) (see Supplementary File 4 for details on sample extraction and ^{1}H-NMR methods). The data were processed and analyzed using Bruker TopSpin software. The peaks of cyanogen compounds were identified based on comparison of sample ^{1}H spectra with the ^{1}H spectra of authentic reference samples of linamarin and lotaustralin. Note that we analyzed only the biosynthesized cyanogenic compounds (not sequestered cyanogenic compounds nor other chemicals potentially influencing unpalatability in *Heliconius*; see Jiggins, 2017), and *Heliconius* "cyanogenic toxicity" or "toxicity" refer here to biosynthesized cyanogenic toxicity. Our study species has been considered generalist or oligophagous on *Passiflora*, largely acquiring its cyanogenic toxicity through *de novo* biosynthesis of cyanogens (Engler-Chaouat & Gilbert, 2007; Merrill et al., 2013), and the cyanogen profile of its main host plant *P. biflora*, also used in our experimental setup, consists of complex compounds which *Heliconius* larvae are unable to sequester (de Castro et al., 2019; Engler-Chaouat & Gilbert, 2007).

All statistical analyses of the study were undertaken using R 3.4.4 (R Core Team, 2018). The values of cyanogen concentrations in the wild-collected samples were normalized with a square-root-transformation before applying ANOVA to test for population differences. Distributions of cyanogen traits were also explored by inspection of truncated weighted density distributions (accounting for the skewness of data towards near-zero values) using the "sm" R package (Bowman & Azzalini, 2014). For the analyses using common-garden data, the data were normally distributed and untransformed values were used.

213 **Host plant toxicity and quality analyses**

214 The cyanogenic content of the *Passiflora biflora* samples was analyzed using liquid chromatography-mass
215 spectrometry (LC-MS/MS) with Agilent 1100 Series LC (Agilent Technologies, Germany) hyphenated to a
216 Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, Germany) following the procedure of de
217 Castro et al. (2019)(see Supplementary File 4 for details on sample extraction, spectrometry and analyses).
218 Mass spectral data were analyzed with the native data analysis software (Compass DataAnalyses, Bruker
219 Daltonics). The cyanogen compounds of *P. biflora* were detected and quantified following de Castro et al.
220 (2019).

221 Plant quality traits assumed to be proxies of nutritional value, including nitrogen/carbon ratio
222 (a proxy of protein/carbohydrate-ratio), were measured from the greenhouse-cultivated *P. biflora* treatment
223 types (average n = 63) with Dualex® Scientific+ leaf clip meter (Cerovic et al. 2012; Supplementary File 4).
224 We tested for differences in cyanogen levels and plant quality traits between the treatment groups in an
225 ANOVA including plant origin, treatment and their interaction.

226

227 **Estimation of variance components, heritability, evolvability and maternal effects in *Heliconius***
228 **biosynthesized cyanogenic toxicity**

229 The concentration of the two tested biosynthesized cyanogens, linamarin and lotaustralin were strongly
230 correlated with each other (r = 0.897). Cyanogen concentration was not correlated with adult body mass in
231 either sex ($F_{1,193}$ = 0.52, P = 0.470 for total cyanogen concentration; mass data available for n = 196
232 individuals). Adult mass also did not differ between the feeding treatments ($F_{4,186}$=0.88, P= 0.480; model
233 including feeding treatment, sex and their interaction), and was not included as a variable in further analyses.

234 Variance components of *Heliconius erato* biosynthesized cyanogenic toxicity were analyzed
235 based on the two-generation full-sib/half-sib breeding design (Lynch & Walsh, 1998b) by fitting linear
236 mixed models applying the "animal model" framework (Kruuk, 2004; Wilson et al., 2010), in which the full
237 pedigree (relatedness matrix) is incorporated as an individual-level random effect. The models were fitted
238 using ASReml-R Version 4 (Butler et al., 2017), an R package that fits linear mixed models using Residual
239 Maximum Likelihood (REML) estimation. The two-generation pedigree consisted of 20 broods and their
240 parents, and included mother-offspring, father-offspring, full-sibling and paternal half-sibling relationships

241 (n = 322 individuals, Supplementary File 2). Of the 20 broods, six were included in the feeding treatments
242 receiving four different *P. biflora* types as larval diet (Supplementary File 2). Individuals of the parental
243 generation were assumed to be unrelated. With the materials available, we maximized our power to detect
244 genetic variation by generating as many family groups as possible. Because the number of families was
245 limited, we also analyzed a large number of offspring per family, which allowed precise estimates of family
246 means. Because our breeding design resulted mostly in full-sib families, we have limited power to
247 distinguish additive-genetic from other genetic variance components (dominance, epistasis, maternal/paternal
248 effects). We thus consider our genetic-variance estimate as a 'broad-sense' genetic variance, although the
249 estimate approaches the narrow-sense estimate more than a simple full-sib design due to the parent-offspring
250 and half-sib relations in the breeding design (Lynch & Walsh, 1998b).

251 Animal models were fitted separately for the three biosynthesized cyanogenic toxicity traits
252 (concentration of linamarin, lotaustralin and total cyanogens). The primary models (referred to as "Animal
253 Model1") included the individual breeding value as a random effect, and the fixed effects of sex and feeding
254 treatment (variance explained by fixed effects computed using the method of Villemereuil et al. (2018); see
255 also Supplementary File 4). "Animal Model2" included additionally the random effect of maternal identity.
256 We computed broad-sense heritabilities (H^2) as the proportion of the total phenotypic variance (V_P)
257 explained by the (broad-sense) genetic variance component (V_G). When calculating heritabilities we included
258 the variance due to sex in the total phenotypic variance, but we did not include the variance due to the
259 treatment because this component of the variance is unlikely to represent natural variation (Villemereuil et
260 al., 2018). Heritabilities are useful measures of the resemblance between offspring and parents, but are poor
261 standardized measures of evolutionary potential (Hansen et al., 2011). As a measure of evolutionary
262 potential, we therefore computed mean-scaled evolvabilities (e_μ) as $e_\mu = V_G/\mu^2$, where μ is the trait mean (of
263 common garden brood data trait values), which gives the expected percent change in the trait mean per
264 generation under a unit strength of selection (Hansen & Houle, 2008; Hansen et al., 2011).

265 Given that our design included mostly full-sib groups, we obtained an additional estimate of
266 the component of toxicity variance explained by brood by estimating the intra-class correlation coefficient
267 (ICC), i.e. repeatability, with a linear mixed model approach. For this, we implemented the "rptR" package

268 (Stoffel et al., 2017), with brood identity as the grouping factor, a Gaussian distribution and 1000 parametric
269 bootstraps to quantify uncertainty.

270 To further explore the role of maternal contributions to the inheritance of cyanogenic toxicity,
271 we estimated maternal-effect coefficients following Falconer´s dilution model, which assumes that offspring
272 phenotype is a direct, linear function of maternal phenotype, beyond the contribution of maternally
273 transmitted genes (Lande & Price, 1989; Lynch & Walsh, 1998a; Walsh & Lynch, 2018b). Thus, the
274 interpretation of this 'maternal effect' is related to specific measured traits (here, cyanogenic toxicity), and is
275 different from the typical formulation of 'maternal environmental effects' (influence of the maternal
276 environment on offspring traits). We estimated maternal effect coefficients (m) as the difference in slope
277 between a mother-offspring (mo) and a father-offspring (fo) regression, i.e. $m = \beta_{mo} - \beta_{fo}$ (Lande & Price,
278 1989; Lynch & Walsh, 1998a). We fitted the parent-offspring regressions as linear mixed models with the
279 *lme4* R package (Bates et al., 2014), including family as a random factor and maternal or paternal toxicity
280 phenotype as explanatory variables of offspring toxicity phenotype.

281

282 **RESULTS**

283 First, we investigated the ecological origins of variation in defensive traits by studying toxicity of butterflies
284 along steep gradients in rainfall and altitude (Fig. 1A). Toxicity levels were similar between populations
285 within the gradients, and variation in butterfly toxicity was not detectably associated with toxicity variation
286 of the major host plant species along the rainfall gradient (Fig. 1B). A comparison of geographically distant
287 natural populations of *H. erato* from Panama and Ecuador showed that populations of the same species can
288 have substantially different levels of biosynthesized toxicity (Fig. 1B). Furthermore, within natural *H. erato*
289 populations sampled along the rainfall gradient in Panama, the distribution of biosynthesized cyanogenic
290 toxicity was consistently skewed, so that a large proportion of individuals had a noticeably low level of
291 toxicity compared with a smaller proportion of highly toxic individuals within all sampled populations (Fig.
292 1C). Finally, we partitioned the variation in toxicity into genetic and environmental components, with
293 particular focus on the variance originating from diet. Common-garden pedigree data comprising 20 broods
294 and more than 300 *Heliconius erato* individuals revealed a moderate but detectable component of variance
295 explained by brood identity (around 12% of the total variance; Fig. 1D). However, further analyses

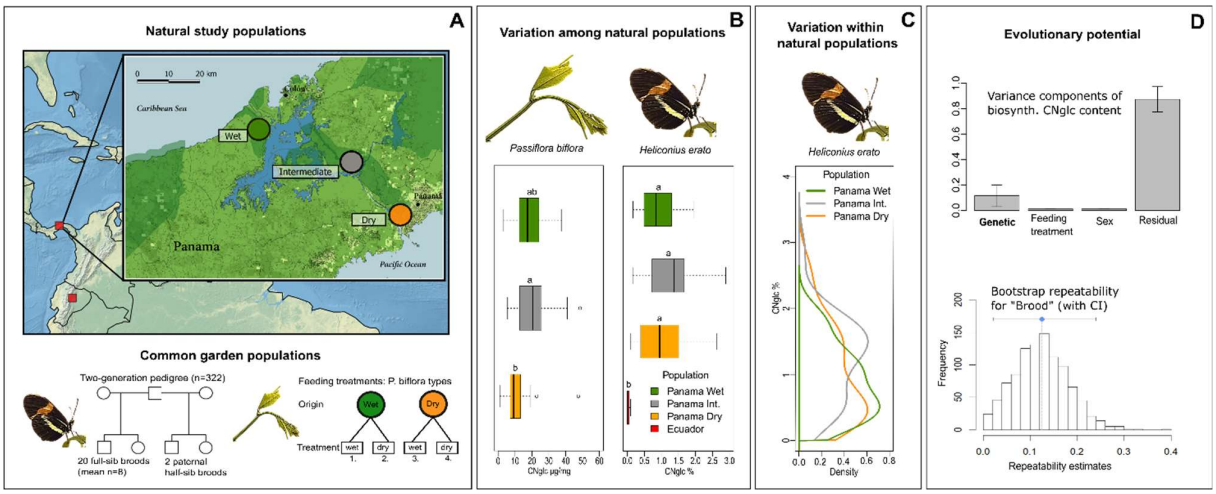

Figure 1. Natural variation and evolutionary potential of biosynthesized cyanogenic toxicity in *Heliconius erato*. **A)** Top panel: Locations of the natural study populations in Central and South America (Panama and Ecuador, respectively), with a more detailed map of study areas along the rainfall gradient in Panama. Bottom panel: Experimental setups for common garden populations of *H. erato* and host plant *Passiflora biflora*. **B)** Variation among natural populations in total- and total biosynthesized cyanogenic (CNglc) toxin concentrations in *P. biflora* and *H. erato*, respectively. Small-case letters indicate statistically significant differences between populations (Tukey HSD $P<0.05$). **C)** Zero-truncated kernel density distributions of total biosynthesized cyanogenic toxicity in natural populations of *H. erato* in Panama. **D)** Top panel: Variance components of *H. erato* biosynthesized cyanogenic toxicity (with S.E.) based on pedigree data (see panel A and Table 2). Bottom panel: Repeatability estimates with brood identity as the grouping factor and 1000 parametric bootstraps to quantify uncertainty.

suggested that the among-brood variance may not primarily represent additive genetic variance but rather be driven by maternal environmental or genetic influences on offspring toxicity phenotype. In contrast, nutritional quality indicators and cyanogenic toxicity of the host plant *Passiflora biflora*, which varied in response to water availability treatments, had no effect on the biosynthesized toxicity level of the adult butterflies that had fed on these plants as larvae. Together, these results demonstrate high levels of intraspecific variation that is not detectably driven by the ecological factors studied here. They also indicate complex modes of inheritance in this chemical defense trait of an aposematic and mimetic *Heliconius* butterfly.

Wide natural variation in toxicity within populations and between regions, but not along environmental gradients

The distribution of biosynthesized cyanogen levels, as estimated from the concentrations of biosynthesized compounds linamarin and lotaustralin, was similar across natural populations of *H. erato* sampled along an

environmental rainfall gradient in Panama (Fig. 1C). A large proportion of individuals had low toxicity values (e.g. 40% of individuals had total cyanogen concentrations falling within the lower 25% of toxicity values), while a small proportion of individuals had a considerably higher toxicity level (e.g. 5% of individuals had total cyanogen concentrations within the highest 25% of toxicity values). Total cyanogen concentration and concentration of linamarin did not differ among the three Panamanian populations (Fig. 1B, Table 1, Supplementary file 1). The concentration of lotaustralin differed among populations ($F_{2, 89}$ = 7.97, $P < 0.001$), with the Intermediate population having higher concentrations than the two populations at the opposite extremes along the rainfall gradient (Supplementary file 1).

		***H. erato* total cyanogens %**							***P. biflora* total cyanogens (µg/mg)**				
Country	**Population**	Mean	SD	*Dry*	*Int*	*Wet*	*Low*	*High*	Mean	SD	*Dry*	*Int*	*Wet*
Panama	*Dry*	1.064	0.709						11.850	10.073			
Panama	*Intermediate*	1.271	0.631	0.432					21.544	13.586	**0.038**		
Panama	*Wet*	0.907	0.521	0.905	0.098				18.005	7.953	0.146	0.627	
Ecuador	*Lowland*	0.011	0.028	**<0.001**	**<0.001**	**<0.001**							
Ecuador	*Highland*	0.026	0.033	**<0.001**	**<0.001**	**<0.001**	0.936						

Table 1. Total cyanogen concentrations (mean, standard deviation (SD) and *P*-values of Tukey HSD pairwise comparisons) in natural populations of *Heliconius erato* and *Passiflora biflora* sampled along environmental gradients in Panama and Ecuador. Statistically significant pairwise comparisons are marked in bold. See Supplementary file 1 for full results table with different cyanogen compounds shown separately.

The total cyanogen levels of the host plant *Passiflora biflora* differed among the natural populations along the rainfall gradient in Panama ($F_{2, 48}$ = 3.65, P = 0.034; Fig. 1B, Table 1). The Dry population had substantially lower cyanogen levels compared with both the Intermediate and Wet populations (Fig 1B, Table 1). In contrast, levels of cyanogen did not vary substantially across populations of *H. erato* (Fig. 1B, Table 1, Supplementary file 1).

The populations along the altitudinal gradient in Ecuador did not differ in biosynthesized cyanogenic toxicity from each other, but differed markedly from their Panamanian conspecifics (Fig. 1B, Table 1). Most Ecuadorian samples lacked both linamarin and lotaustralin (Supplementary file 1). Linamarin was detected in only 29% of samples, most of which originated from the high-altitude site (75% of those with any signal), and the average biosynthesized cyanogen level in these samples was very low (0.063%; Fig. 1B, Table 1, Supplementary file 1), which is below the minimum level observed in the Panamanian populations.

Biosynthesized toxicity does not vary with diet but varies among broods

The level of biosynthesized cyanogenic toxicity varied two-fold among the 20 common-garden reared broods (range of brood averages = 0.48% - 0.88% of dry mass, Fig. 2A). Phenotypic variance components of cyanogenic toxicity estimated with an animal model including the full pedigree as a random effect and fixed effects of sex and feeding treatment (Model1) revealed a statistically significant genetic variance component (V_G) for total biosynthesized cyanogen concentration level (Fig. 1D, Table 2, Supplementary File 1), corresponding to a broad-sense heritability (H^2) of 0.115 (H^2(linamarin) = 0.139, H^2(lotaustralin) = 0.067; Table 2, Supplementary File 1). We confirmed the presence of detectable among-brood variance in a simpler brood-level analysis, in which brood identity explained 12.5% of the variation in biosynthesized cyanogen concentration across common-garden reared broods (repeatability R = 0.125, SE = 0.059, CI = [0.015, 0.245], P = 6.02e-05). Among-brood variance could originate from several sources including 1) additive genetic variance, 2) maternal environmental or genetic effects, and 3) shared environment, and below we explore these options further.

First, we consider the estimated broad-sense genetic variance component and estimate evolutionary potential of toxicity, assuming the genetic component consists of largely additive genetic variance. The broad-sense evolvability of total cyanogen concentration was e_μ=1.55%, SE ± 0.89 (e_μ(linamarin) = 1.89%, e_μ(lotaustralin)= 1.04%; Table 2, Supplementary File 1), meaning that the trait mean is expected to change by 1.55% per generation per unit strength of selection (Hansen & Houle, 2008; Hansen et al., 2011).

					Genetic variance component, heritability and evolvability				Other variance components (V/V_P)				Total phenotypic variance
Model type	Cyanogen trait	Trait mean (μ)	No. Obs.	No. broods	V_G	P V_G	**H^2** (V_G/V_P)	**e_μ** (V_G/μ^2); %	V_{mat}/V_P	V_{trm}/V_P	V_{sex}/V_P	V_R/V_P	V_P
1	Total CNglc %	0.772	322	20	0.0093	0.037	0.115	1.555		0.011	0.011	0.874	0.080
1	Linamarin %	0.577	322	20	0.0063	0.020	0.139	1.887		0.001	0.013	0.848	0.045
1	Lotaustralin %	0.195	322	20	0.0004	0.130	0.067	1.039		0.008	0.004	0.929	0.006
2	Total CNglc %	0.772	322	20	1.0e-7	1.000	1.4e-6	1.7e-5	0.102	0.043	0.002	0.895	0.073

Table 2. Summary of variance components of *Heliconius* biosynthesized cyanogenic (CNglc) toxicity estimated with REML animal models. V_{mat}, V_{trm}, V_{sex} and V_R are the variance components for maternal effects, host plant feeding treatment, sex, and residual variance, respectively. See Supplementary File 1 for full model results.

Our baseline estimates of repeatability, as well as broad-sense heritability and evolvability (Model 1) thus showed that there is moderate but detectable variation among broods in biosynthesized

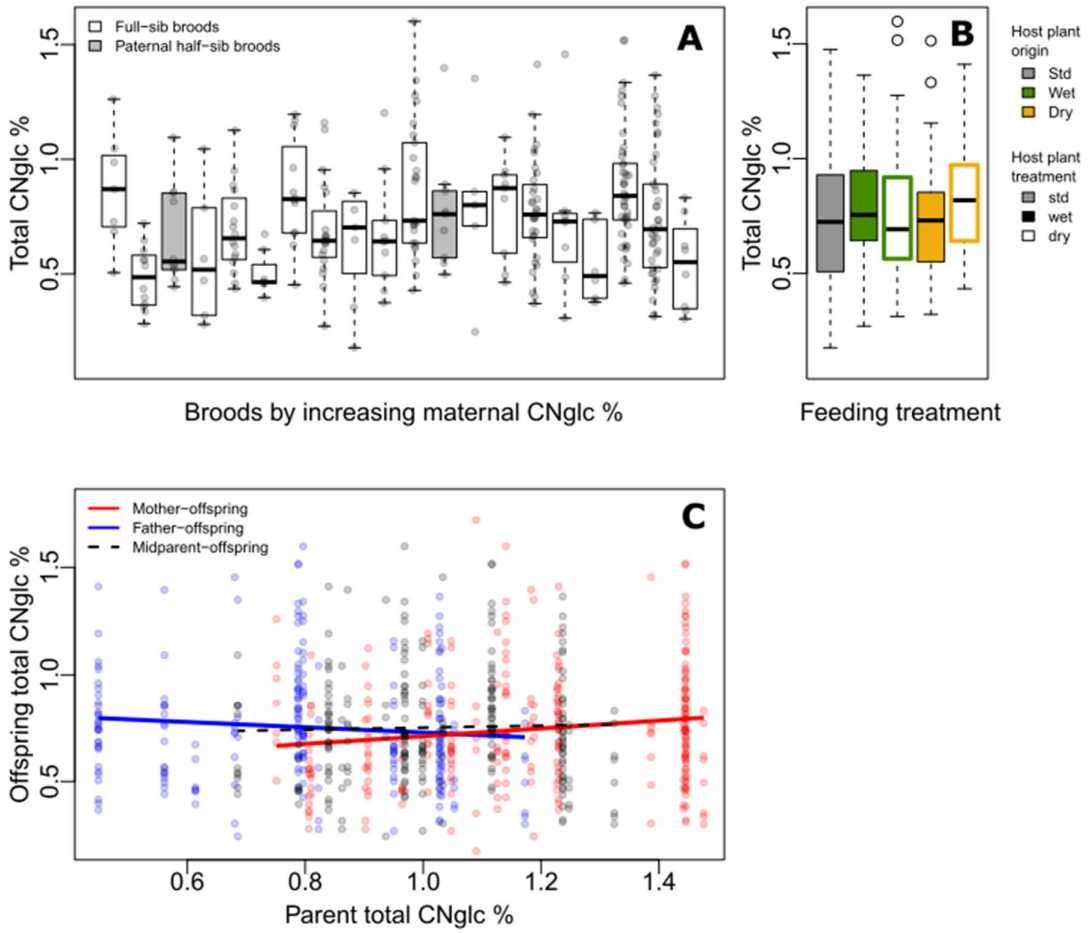

Figure 2. A) Variation in total biosynthesized cyanogen (CNglc) concentration (% dry mass) among full-sibling broods of *Heliconius erato* by increasing maternal CNglc % (one pair of full-sib broods are paternal half-siblings, in grey) **B)** Total biosynthesized CNglcs concentration (% dry mass) in newly-emerged adult individuals fed with different *Passiflora biflora* host plant treatment types (see Fig. 1A) as larvae. **C)** Parent-offspring regressions of total biosynthesized CNglcs concentration.

toxicity. We then estimated maternal and paternal contributions to this variation. When including maternal identity as a second random effect in the animal model (Model2), this maternal variance component explained most of the variance explained by the genetic variance component in Model1, whereas V_G became negligible, illustrating that the two random terms explain essentially the same variance component (i.e. the among-brood variance; Table 2, Supplementary File 1). Parent-offspring regressions revealed that offspring cyanogen concentration was positively associated with the cyanogen concentration of the mother (slope = 0.181 ± 0.071, t = 2.539, P = 0.012 for total cyanogen concentration; Fig. 2C), while the association between fathers and offspring showed a negative, but statistically non-significant trend (slope = -0.122 ± 0.083, t = -

359 1.469, $P = 0.143$ for total cyanogen concentration). In turn, the midparent-offspring regression was nearly
360 flat (slope = 0.046 ± 0.107, $t = 0.435$, $P = 0.664$ for total cyanogen concentration; Fig. 2C). These regression
361 slopes translate into a maternal-effect coefficient for total cyanogen concentration of $m = 0.261$, $CI = [-$
362 $0.099, 0.614]$ (m(linamarin)= 0.288, CI = [-0.094, 0.687]; m (lotaustralin)= 0.193, $CI = [-0.135, 0.520]$),
363 which implies the existence of positive maternal effects in biosynthesized cyanogenic toxicity. However,
364 uncertainty in these maternal effects remains notable, as illustrated by the wide confidence intervals.
365 Finally, we investigated other sources, particularly environmental origins of variance. Larvae
366 reared on different host plant types (Fig. 1A) did not differ in biosynthesized cyanogenic toxicity as adults
367 (Fig. 2B), and the host plant type explained a negligible proportion of variance in butterfly toxicity level
368 ($V_{trm}/V_P = 0.011$; Fig. 1D, Table 2, Supplementary File 1). This is despite the strong effect of the watering
369 treatments on the cyanogenic toxicity and quality indicators of the host plants (e.g. nitrogen-carbon balance;
370 see Supplementary File 3), and also indicates that among-brood variance is unlikely to be explained by
371 common environment effects related to dietary quality. Sex was also a negligible source of variation in
372 toxicity ($V_{sex}/V_P = 0.011$; Fig. 1D, Table 2, Supplementary File 1). Despite the common-garden conditions
373 (outside of dietary treatments) that all individuals experienced throughout their life cycle, the residual
374 (unexplained) variance component of cyanogen concentration remained large ($V_{res}/V_P = 0.874$ for total
375 cyanogens, Fig. 1D, Table 2, Supplementary File 1). This large amount of unexplained variance is also
376 demonstrated by a difference in toxicity level between the parental and offspring generation ($F_{1, 320} = 27.42$,
377 $P < 0.001$, effect size (Hedges' g) = 0.962 for total cyanogens), despite the two generations sharing common
378 garden conditions.
379
380 **DISCUSSION**
381 We have documented extensive variation in chemical defenses, both within and across, natural populations
382 of *H. erato*. Wide variation in defensive toxins has often been found in chemically defended prey, yet the
383 sources of this variation remains largely unclear (Speed et al., 2012). Our data shows that intraspecific
384 variation in *de novo* synthesized chemicals was not strongly associated with environmental conditions and
385 levels of biosynthesized toxicity were similar in butterflies collected across steep environmental gradients.
386 Similarly, our experimental work consistently showed that variation in host plant biochemical profiles and

387 nutritional quality do not contribute to variation in *H. erato* biosynthesized toxicity. Instead, the variation has
388 a moderate but detectable broad-sense genetic component (12.5% of variation explained by brood), which
389 could, given that it was largely composed of additive genetic effects, imply substantial evolutionary potential
390 in biosynthesized chemical defenses of *H. erato*. Our broad-sense heritability estimate of biosynthesized
391 cyanogenic toxicity (0.115 ± 0.086) is close to the average heritability value of physiological traits (0.12 ±
392 0.05), based on a meta-analysis of a wide range of traits and taxa (Hansen et al., 2011). This estimate differs
393 markedly, though, from that obtained in a recent study on toxicity in warningly colored wood tiger moths
394 (*Arctia plantaginis*), in which no evidence for a genetic component to variation in the secretion amounts of
395 *de novo* synthesized chemical defenses could be detected (Burdfield-Steel et al., 2018; Lindstedt et al.,
396 2020). A few other studies have estimated genetic components of insect chemical defense variation, with
397 some detecting a genetic component (Eggenberger & Rowell-Rahier, 1992; Holloway et al., 1993; Yezerski
398 et al., 2004) and others not (Müller et al., 2003). Our study adds on these previous work by investigating
399 chemical defense variation across environments and mimicry rings.

400 Our estimated value for mean-scaled evolvability of cyanogenic toxicity, which gives a better
401 standardized measure of evolutionary potential (Hansen & Houle, 2008; Hansen et al., 2011) is high (1.55 ±
402 0.89%), especially when compared to the average value of physiological traits (0.49 ± 0.14%), as well as the
403 median evolvability of all studied traits across taxa (0.26 ± 0.03%; see Hansen et al., 2011). The evolvability
404 of e_{μ}=1.55% means that the mean cyanogenic toxicity can be expected to change by 1.55% per unit strength
405 of selection per generation. This high estimate of evolvability would imply that the trait has the potential to
406 evolve rapidly, especially in conditions of strong selection. The effect would be magnified given the short
407 generation time (usually one to two months) of these tropical butterflies. For example, assuming *Heliconius*
408 has six generations per year, and increased cyanogenic toxicity is selected for with selection as strong as
409 selection on fitness as a trait (β_{μ}= 100%, see Hereford et al., 2004), the trait mean would have the potential to
410 increase by 9.7% in just one year.

411 However, our data from experimental crosses indicate that the inheritance of biosynthesized
412 cyanogenic toxicity has a more complex basis, beyond pure additive genetic effects. Particularly, there is
413 evidence for a central role for maternal effects. This is supported by our observations that 1) the maternal
414 effect component fitted in our animal model seemed to explain the same component of variance as the broad-

415 sense genetic component, as well as that 2) midparent-offspring and father-offspring regressions were non-
416 significant while mother and offspring toxicity values were positively correlated. Given that mothers were
417 raised in common garden conditions, and environmental variance is thus expected to be low, these effects
418 likely have a genetic or epigenetic basis. At this point, it is unclear if the maternal effects that we document
419 here are the result of maternally transmitted genes, epigenetic effects, direct transfer of toxins from mother to
420 offspring in eggs (Nahrstedt & Davis, 1983), or other types of maternal influence on the offspring phenotype
421 (Lande & Price, 1989; Lynch & Walsh, 1998a; Walsh & Lynch, 2018b). Females are the heterogametic sex
422 in Lepidoptera, but gene content of the highly heterochromatic *W* chromosome is poorly explored.
423 *Heliconius* eggs have very high concentrations of cyanogenic toxins (de Castro et al., 2020; Nahrstedt &
424 Davis, 1983) and the relationship between the provisioning of compounds in eggs by mothers and the level
425 of these compounds in adults could be explored in future studies. We did not detect any paternal effects,
426 even though *Heliconius* males are known to transfer a nuptial gift containing biosynthesized cyanogenic
427 compounds to females at mating (Cardoso & Gilbert, 2007).
428 Independent of what is driving the maternal effects, the occurrence of a maternal effect of the
429 type estimated here, defined simply as an effect of the mothers phenotype on the offspring's phenotype (as in
430 Falconer´s dilution model; Lande & Price, 1989; Lynch & Walsh, 1998a), could considerably strengthen the
431 response of the trait to selection. Although there is much uncertainty in how maternal effects will affect the
432 response to selection (Walsh & Lynch, 2018a), the predicted cumulative selection response with maternal
433 effects under Falconer´s model for a trait with a maternal effect coefficient of $m = 0.25$, which is comparable
434 to the $m = 0.261$ estimated here for cyanogenic toxicity, would over time exceed that of a trait with $m = 0$ by
435 as much as 50% (assuming constant heritability) (Walsh & Lynch, 2018a). This effect, however, is expected
436 to dilute over generations. The maternal coefficient should also be interpreted with caution because of
437 remaining uncertainty in our estimate. Predicting the response of toxicity to selection in the wild could also
438 be complicated by the observed skewed distribution of toxicity, as skewed breeding values could generate an
439 asymmetrical selection response. Furthermore, we cannot rule out that some of the observed among-brood
440 variance is explained by common environment effects other than maternal effects and the measured effect of
441 diet, e.g. differences in group size during larval development. However, the demonstrated negligible plastic
442 responses to diet, common-garden rearing, and the temporal spread of egg-laying of each brood (eggs laid

443 singly over a time period of up to two weeks per brood) are expected to reduce the likelihood of such
444 common environment effects among broods.
445
446 **Implications of evolutionary potential in chemical defenses for aposematic and mimetic systems.**
447 Studies of mimicry often focus on color pattern, i.e. the warning signal (Benson, 1972; Jiggins, 2017; Kapan,
448 2001; Mallet & Barton, 1989; Merrill et al., 2015). However, it is important to note that contrasting selective
449 agents could be acting on the defense trait associated with the warning signal, both traits equally required for
450 aposematism and mimicry. The selection pressures acting on chemical defense traits can be expected to be
451 complex and dynamic (Briolat et al., 2019; Jones et al., 2016; Speed et al., 2012). The role of predators as the
452 major selective agent is clearly a key component of possible adaptive evolutionary processes herein (Briolat
453 et al., 2019; Ruxton et al., 2018). Even high evolutionary potential will only lead to evolutionary changes if
454 the defense trait variation influences predation rates (Speed et al., 2012). In general, empirical data on the
455 impact of variation in defenses and its signaling on predators is lacking, but there is evidence for variation
456 among predators in traits including perceptual abilities, individual motivation and tolerance to chemical
457 defenses (Briolat et al., 2019; Halpin et al., 2017). Furthermore, prey toxicity level does not necessarily
458 correlate with actual unpalatability, the ultimate cue for predator choices (Winters et al., 2018). Predator-
459 mediated selection is therefore expected to be dynamic and difficult to estimate. In addition, bottom-up
460 selection related to co-evolutionary relationships with host plants, as well as selection on energy optimization
461 may also play a role (Turner, 1984; Ruxton et al., 2018).
462 Species may only respond adaptively to such selection pressures given heritable genetic
463 variation in these traits. Evolutionary potential in chemical defenses could allow species to respond to the
464 expected dynamic top-down and bottom-up selection, resulting in diverse patterns of adaptive evolution in
465 chemical defense traits. This could have important consequences for the evolutionary trajectories of
466 aposematic and mimetic species, and ultimately play a role in the evolution of warning signal diversity. This
467 is because evolutionary changes in chemical defense levels could alter mimetic relationships among groups
468 of species across geographic space, including shifts between Müllerian, Batesian and quasi-Batesian
469 relationships, which could in turn influence advergent vs. convergent selection and contribute to the
470 observed diversity in mimicry systems (Briolat et al., 2019; Mallet & Joron, 1999; Speed, 1999). With

471 regards to *Heliconius,* the combination of a locally dynamic selective environment, flexible genetic
472 mechanisms for generating warning signals (Van Belleghem et al., 2017; Wallbank et al., 2016), and
473 evolutionary potential in toxicity could explain the rapid origin of inter- and intraspecific variation. In this
474 light, the ability of *Heliconius* to biosynthesize defense compounds could allow for increased genetic control
475 of chemical defenses compared to most other chemically defended Lepidoptera, which rely solely on host
476 plant compound sequestration (Bowers, 1992; Nishida, 2002; Zvereva & Kozlov, 2016). Because *Heliconius*
477 can sequester cyanogenic compounds of only some *Passiflora* species (de Castro et al., 2019; Engler et al.,
478 2000), selection would be targeted toward *de novo* synthesized defenses particularly in environments lacking
479 such host species. Furthermore, previous investigations both confirmed that the cyanogenic glucoside
480 compounds in *Heliconius* deter vertebrate predators (Cardoso, 2020) and that predator avoidance learning
481 rate increases when comparing low to moderate levels of cyanogenic compound concentrations (Chouteau et
482 al., 2019). Thus, the level of cyanogen defenses is likely to be a direct target of selection by predators in
483 some circumstances.

484 By altering the nature of mimetic relationships or selection for increased defense levels on a
485 new warning signal form, evolvability of defense traits could drive the evolution and spread of new color
486 patterns and the reproductive isolation that may follow, potentially even leading to the formation of new
487 species (Jiggins et al., 2001; Mallet et al., 1998). A phylogenetic signal in the cyanogenic glucoside chemical
488 profile in the Heliconiini tribe supports this idea (de Castro et al., 2019; Sculfort et al., 2020) and
489 underscores the potential for adaptive evolution in chemical defense traits to influence evolutionary
490 trajectories in mimicry systems. However, more empirical data is called for in future studies to estimate
491 levels of evolutionary potential and patterns of selective responses in defensive traits in aposematic and
492 mimetic species.
493

494 **Evolutionary processes shaping chemical defense variation: the role of automimicry**

495 A further complication in chemical defense evolution is the interplay between individual and public good
496 (Jones et al., 2016). In this respect, our data demonstrating a broad-sense genetic component in the defense
497 trait coupled with patterns of natural variation of cyanogen levels in *Heliconius erato* populations are
498 consistent with balancing selection related to automimicry, in which automimic "cheaters" exploit the

499 protection given by their warning coloration with investing less in chemical defenses themselves.
500 Automimicry may occur if producing and maintaining chemical defenses is costly (Brower et al., 1970;
501 Speed et al., 2012), leading to selection reducing individual-level toxicity (Bowers, 1992; Ruxton et al.,
502 2018; Zvereva & Kozlov, 2016), and if there are also individual benefits of investing in chemical defenses
503 (e.g. Ruxton & Speed, 2006; Svennungsen & Holen, 2007). Previous studies indicate that *de novo* synthesis
504 of defenses may generally be more costly than the sequestration of plant compounds (Zvereva & Kozlov,
505 2016), which may make automimicry more likely in species with biosynthesized defenses. The occurrence of
506 automimicry could lead to a decrease in population average unpalatability, which could theoretically dilute
507 the protection conveyed by the warning signal (Gibson, 1984; Jones et al., 2016; Speed et al., 2012), as in
508 Batesian and quasi-Batesian mimicry, and influence the evolutionary trajectories of the species sharing the
509 warning signal. Thus, empirical data on within-population defense variation and automimicry can give
510 important insights into the diversity of mimetic systems (Jones et al., 2016).
511 Our results show that all three butterfly populations along the studied rainfall gradient
512 exhibited similar skewed distributions of biosynthesized cyanogens. The majority of individuals in these
513 populations had relatively low, although not zero, levels of toxicity (24% of individuals had less than 0.5%
514 concentration of biosynthesized cyanogenic compounds from an overall range of 0.1 - 2.9%), whereas a
515 smaller proportion had substantially higher levels, and the pattern was surprisingly similar in all populations.
516 Such a pattern could be explained by age distributions, as previous studies suggest that *Heliconius*
517 accumulate cyanogenic toxins with age (de Castro et al., 2020; Nahrstedt & Davis, 1985), or by individual
518 differences in the balance between sequestration and biosynthesis of cyanogenic compounds (de Castro et
519 al., 2019; Engler-Chaouat & Gilbert, 2007; de Castro et al., 2020). However, as many individuals in the
520 study populations had near zero levels of biosynthesized toxins, the pattern could also be indicative of
521 automimicry. In *Heliconius*, the costs of chemical defense remain poorly known, but some cases of
522 automimicry have previously been reported (Arias et al., 2016). Here, toxicity and mass were not associated,
523 which could imply that potential costs of cyanogen biosynthesis are not related to or mediated by a trade-off
524 with body size. In terms of benefits of chemical protection, requirements for automimicry are likely to be
525 met, as empirical data on *Heliconius* imply direct benefits (surviving predator attacks) for chemically
526 defended individuals (Boyden, 1976; Pinheiro, 1996; Pinheiro & Campos, 2019). Furthermore, an increase in

527 protection level is found especially when comparing low to moderate cyanogenic toxicity levels (Chouteau et
528 al., 2019), applicable to the observed cyanogen levels of *H. erato* (Arias et al., 2016; de Castro et al., 2019).
529 However, future studies on natural defense variation and automimicry in *Heliconius* should measure the
530 entire range of cyanogens (both sequestered and biosynthesized) and other chemical defense compounds
531 potentially interacting to produce the unpalatability level perceived by relevant predators (Jiggins, 2017;
532 Winters et al., 2018). In more general terms, future research in this framework should investigate the costs of
533 defensive compound biosynthesis (see also Zvereva & Kozlov, 2016) and its role in the evolution of
534 automimicry, as well as automimicry-originating defense variation as a potential source introducing diversity
535 into mimetic systems.
536
537 **The roles of phenotypic plasticity and ecological sources of defense variation in wild populations**
538 It has been suggested that the often wide variation in defense toxins could be explained by the non-
539 adaptiveness of toxicity profiles, in which case the variation would stem from chance effects and plastic
540 responses to the environment (Speed et al., 2012). Empirical data on chemical defense variation in wild
541 populations e.g. along environmental gradients could help to evaluate ecological vs. evolutionary origins of
542 chemical defense variation, and the expected consequences for diversity in aposematic and mimetic systems
543 (Briolat et al., 2019). Although the large amount of unexplained variance in our quantitative genetics model
544 may suggest the presence of unknown environmental sources of variation in biosynthesized toxicity, we
545 found very little evidence for a role of known ecological factors in explaining variation in toxicity profiles
546 along our studied environmental gradients, despite the steepness of these gradients. For example, the
547 chemical profiles were very similar in the Panamanian Wet and Dry populations, which experience a five-
548 fold difference in rainfall. Furthermore, despite water availability inducing substantial differences in the
549 cyanogen content and quality indicators of *P. biflora*, both our field and experimental data indicate that
550 variation within the host plant species does not contribute to variation in *H. erato* biosynthesized toxicity.
551 This is in contrast to our expectations of dietary quality as a major source of plastic chemical defense
552 variation.
553 However, there were striking differences in the chemical profiles between the two distinct
554 warning color forms of *H. erato* that we sampled from geographically distant populations in Panama and

555 Ecuador. Compared to their Panamanian conspecifics sampled along the gradient of rainfall at sea level, the
556 Ecuadorian populations inhabit a very different and geographically distant biotope along the eastern slope of
557 the Andes (Montejo-Kovacevich et al., 2020). These environments differ in larval host plant and
558 nectar/pollen availability, the community of predators and of co-mimics, which are far more diverse in
559 Ecuador. Lower levels of cyanogen biosynthesis in Ecuadorian populations of *H. erato* may suggest that
560 these populations have specialized on a *Passiflora* host which provides more cyanogens for sequestration (de
561 Castro et al., 2019; Engler-Chaouat & Gilbert, 2007; de Castro et al., 2020). Alternatively, the more diverse
562 mimetic ring in Ecuador could also provide greater protection by more toxic model species, relaxing
563 selection on defenses in co-mimics (Mallet, 1999; Speed, 1993, 1999; Turner, 1987).
564 In this light, our data may provide an example of how ecological variation could lead to
565 differential selective environments between conspecific populations, thereby leading to local adaptation of
566 the acquisition mechanisms and levels of defensive compounds. Such adaptive radiations would be
567 facilitated by evolutionary potential in defense traits. More data on different aposematic and mimetic systems
568 will be needed to confirm whether ecological variation may have a greater influence on chemical defense
569 variation through introducing spatially and temporally varying selection pressures, rather than by introducing
570 "ecological noise" through plastic responses in defense traits.
571
572 **CONCLUSION**
573 The role of ecological and evolutionary processes in the origins and maintenance of the wide variation in
574 chemical defenses, and the consequences for the evolution of aposematism and mimicry holds many
575 unanswered questions. Our analysis of biosynthesized cyanogenic toxicity in *Heliconius erato,* studied along
576 natural environmental gradients and in a common garden, shows that this important chemical defense trait
577 varies substantially within and among populations. We found no evidence for a role of dietary variation
578 (within host species) or steep environmental gradients in explaining toxicity profiles, despite a large
579 component of unexplained variance in common-garden reared broods. Instead, the results suggest that
580 adaptive processes originating from e.g. larval host species availability, mimicry environment, and
581 individual-level energy optimization may play important roles in the variation of cyanogenic defenses. This
582 is in line with our data indicating substantial broad-sense evolvability in cyanogenic toxicity, although more

583 data will be needed to confirm the origins of the observed among-brood variance and shed light on how the
584 indicated maternal effects will affect the overall selection response. Our study suggests that adaptive
585 evolution could be an important force driving variation in defensive traits. Such adaptive processes could
586 play a part in explaining the incredible diversity in aposematic and mimetic systems.
587

588 **ACKNOWLEDGEMENTS**

589 Personnel and researchers at the Smithsonian Tropical Research Institute (STRI Panama) and Ikiam at
590 Universidad Regional Amazónica (Ecuador) are acknowledged for help with permits and the field collection
591 of the study species. We are especially grateful to Marleny Rivera (STRI) for reliable and friendly assistance
592 in field sampling. Samples were processed at the Molecular Ecology Laboratory at the University of Helsinki
593 (UH), and analysed at the NMR facility at the Institute of Biotechnology (UH), which is supported by
594 Biocenter Finland and Helsinki Institute of Life Science (HiLIFE). Søren Bak and David Pattison
595 (Department for Plant and Environmental Sciences, University of Copenhagen) are acknowledged for
596 collaboration on the LC-MS analyses of *Passiflora* samples. Juha-Matti Pitkänen, Tuomas Niemi-Aro and
597 Annukka Ruokolainen are thanked for technical assistance, and Guillaume Minard, Anne Duplouy, Richard
598 Wallbank, Richard Merrill, Oscar Paneso, Krzysztof Kozak and Sophia Gripenberg for advice on study
599 design and lab and field work. Mika Zagrobelny and Marcio Cardoso are thanked for advice on cyanogenic
600 assays and Mika Zagrobelny also for providing reference samples of the CNglcs compounds. Research and
601 collecting permits were granted by the Ministerio de Ambiente, Republica de Panama (Permit SE/AP-21-16)
602 and the Ministerio del Ambiente, Ecuador (under the Contrato Marco MAE-DNB-CM-2017-0058). Financial
603 support was provided by the Academy of Finland (Grant no. 286814 to A.M.), ERC (SpeciationGenetics
604 339873 to J.C.), NERC Doctoral Training Partnership (NE/L002507/1 to G.M.K.), and Spanish Agency for
605 International Development Cooperation (AECID, grant number 2018SPE0000400194 to C.B.).
606

607 **AUTHOR CONTRIBUTIONS**

608 A.M., M.S. and C.J. conceived the study. A.M., M.S. and G.M.K. conducted fieldwork. A.M. conducted
609 experimental work. A.M. and E.C. conducted laboratory work. A.M. and Ø.O. collated the dataset and

conducted the analyses. W.M. and C.B. provided facilities and materials. A.M., C.J. and W.M. wrote the manuscript. All authors read and approved the final manuscript.

DATA ACCESSIBILITY

All data generated and analysed during this study will be deposited in a digital data repository (Dryad data repository) at acceptance for publication.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

iii. REFERENCES

Arias, M., Meichanetzoglou, A., Elias, M., Rosser, N., de-Silva, D. L., Nay, B., & Llaurens, V. (2016). Variation in cyanogenic compounds concentration within a Heliconius butterfly community: Does mimicry explain everything? *BMC Evolutionary Biology*, *16*(1), 272. https://doi.org/10.1186/s12862-016-0843-5

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2014). *Fitting Linear Mixed-Effects Models using lme4*. https://arxiv.org/abs/1406.5823v1

Benson, W. W. (1972). Natural Selection for Miillerian Mimicry in Heliconius erato in Costa Rica. *Science*, *176*(4037), 936–939. https://doi.org/10.1126/science.176.4037.936

Benson, W. W., Brown, K. S., & Gilbert, L. E. (1975). Coevolution of Plants and Herbivores: Passion Flower Butterflies. *Evolution*, *29*(4), 659–680. https://doi.org/10.1111/j.1558-5646.1975.tb00861.x

Boggs, C. L., & Freeman, K. D. (2005). Larval food limitation in butterflies: Effects on adult resource allocation and fitness. *Oecologia*, *144*(3), 353–361. https://doi.org/10.1007/s00442-005-0076-6

Bowers, M. D. (1992). The evolution of unpalatibility and the cost of chemical defense in insects. In *Insect Chemical Ecology: An Evolutionary Approach* (pp. 216–244). Chapman Hall.

Bowman, A. W., & Azzalini, A. (2014). *R package "sm": Nonparametric smoothing methods (version 2.2-5.5)*. http://www.stats.gla.ac.uk/~adrian/sm

Boyden, T. C. (1976). Butterfly Palatability and Mimicry: Experiments with Ameiva Lizards. *Evolution*, *30*(1), 73–81. JSTOR. https://doi.org/10.2307/2407673

Briolat, E. S., Burdfield-Steel, E. R., Paul, S. C., Rönkä, K. H., Seymoure, B. M., Stankowich, T., & Stuckert, A. M. M. (2019). Diversity in warning coloration: Selective paradox or the norm? *Biological Reviews*, *94*(2), 388–414. https://doi.org/10.1111/brv.12460

Brower, L. P., Pough, F. H., & Meck, H. R. (1970). Theoretical Investigations of Automimicry, I. Single Trial Learning. *Proceedings of the National Academy of Sciences*, *66*(4), 1059–1066. https://doi.org/10.1073/pnas.66.4.1059

Burdfield-Steel, E., Brain, M., Rojas, B., & Mappes, J. (2019). The price of safety: Food deprivation in early life influences the efficacy of chemical defence in an aposematic moth. *Oikos*, *128*(2), 245–253. https://doi.org/10.1111/oik.05420

Burdfield-Steel, E., Pakkanen, H., Rojas, B., Galarza, J. A., & Mappes, J. (2018). De novo Synthesis of Chemical Defenses in an Aposematic Moth. *Journal of Insect Science*, *18*(2). https://doi.org/10.1093/jisesa/iey020

Butler, D. G., Cullis, B. R., Gilmour, A. R., Gogel, B. G., & Thompson, R. (2017). *ASReml-R Reference Manual Version 4*. VSN International Ltd, Hemel Hempstead, HP1 1ES, UK.

Camara, M. D. (1997). Predator Responses to Sequestered Plant Toxins in Buckeye Caterpillars: Are Tritrophic Interactions Locally Variable? *Journal of Chemical Ecology*, *23*(9), 2093–2106. https://doi.org/10.1023/B:JOEC.0000006431.34359.c2

Cardoso, M. Z., & Gilbert, L. E. (2013). Pollen feeding, resource allocation and the evolution of chemical defence in passion vine butterflies. *Journal of Evolutionary Biology*, *26*(6), 1254–1260. https://doi.org/10.1111/jeb.12119

Cardoso, Márcio Zikán. (2020). The effect of cyanogenic glucosides and their breakdown products on predation by domestic chicks. *Chemoecology*, *30*(3), 131–138. https://doi.org/10.1007/s00049-020-00304-6

Cardoso, Márcio Zikán, & Gilbert, L. E. (2007). A male gift to its partner? Cyanogenic glycosides in the spermatophore of longwing butterflies (Heliconius). *Naturwissenschaften*, *94*(1), 39–42. https://doi.org/10.1007/s00114-006-0154-6

Cerovic, Z. G., Masdoumier, G., Ghozlen, N. B., & Latouche, G. (2012). A new optical leaf-clip meter for simultaneous non-destructive assessment of leaf chlorophyll and epidermal flavonoids. *Physiologia Plantarum*, *146*(3), 251–260. https://doi.org/10.1111/j.1399-3054.2012.01639.x

Chouteau, M., Dezeure, J., Sherratt, T. N., Llaurens, V., & Joron, M. (2019). Similar predator aversion for natural prey with diverse toxicity levels. *Animal Behaviour*, *153*, 49–59. https://doi.org/10.1016/j.anbehav.2019.04.017

de Castro, É. C. P., Demirtas, R., Orteu, A., Olsen, C. E., Motawie, M. S., Zikan Cardoso, M., Zagrobelny, M., & Bak, S. (2020). The dynamics of cyanide defences in the life cycle of an aposematic butterfly: Biosynthesis versus sequestration. *Insect Biochemistry and Molecular Biology*, *116*, 103259. https://doi.org/10.1016/j.ibmb.2019.103259

de Castro, É. C. P., Zagrobelny, M., Cardoso, M. Z., & Bak, S. (2018). The arms race between heliconiine butterflies and Passiflora plants – new insights on an ancient subject. *Biological Reviews*, *93*(1), 555–573. https://doi.org/10.1111/brv.12357

de Castro, É. C. P., Zagrobelny, M., Zurano, J. P., Cardoso, M. Z., Feyereisen, R., & Bak, S. (2019). Sequestration and biosynthesis of cyanogenic glucosides in passion vine butterflies and consequences for the diversification of their host plants. *Ecology and Evolution*, *9*(9), 5079–5093. https://doi.org/10.1002/ece3.5062

Eggenberger, F., & Rowell-Rahier, M. (1992). Genetic component of variation in chemical defense ofOreina gloriosa (Coleoptera: Chrysomelidae). *Journal of Chemical Ecology*, *18*(8), 1375–1387. https://doi.org/10.1007/BF00994363

Engler, H. S., Spencer, K. C., & Gilbert, L. E. (2000). Preventing cyanide release from leaves. *Nature*, *406*(6792), 144. https://doi.org/10.1038/35018159

Engler-Chaouat, H. S., & Gilbert, L. E. (2007). De novo Synthesis vs. Sequestration: Negatively Correlated Metabolic Traits and the Evolution of Host Plant Specialization in Cyanogenic Butterflies. *Journal of Chemical Ecology*, *33*(1), 25–42. https://doi.org/10.1007/s10886-006-9207-8

Fordyce, J. A., & Nice, C. C. (2008). Antagonistic, Stage-Specific Selection on Defensive Chemical Sequestration in a Toxic Butterfly. *Evolution*, *62*(7), 1610–1617. https://doi.org/10.1111/j.1558-5646.2008.00388.x

Gardner, D. R., & Stermitz, F. R. (1988). Host plant utilization and iridoid glycoside sequestration byEuphydryas anicia (Lepidoptera: Nymphalidae). *Journal of Chemical Ecology*, *14*(12), 2147–2168. https://doi.org/10.1007/BF01014022

Gibson, D. O. (1984). How is Automimicry Maintained? In *The Biology of Butterflies* (pp. 163–165). Academic Press.

Gleadow, R. M., & Woodrow, I. E. (2002). Defense chemistry of cyanogenic Eucalyptus cladocalyx seedlings is affected by water supply. *Tree Physiology*, *22*(13), 939–945. https://doi.org/10.1093/treephys/22.13.939

Halpin, C. G., Skelhorn, J., Rowe, C., Ruxton, G. D., & Higginson, A. D. (2017). The Impact of Detoxification Costs and Predation Risk on Foraging: Implications for Mimicry Dynamics. *PLoS ONE*, *12*(1). https://doi.org/10.1371/journal.pone.0169043

Hansen, T. F., & Houle, D. (2008). Measuring and comparing evolvability and constraint in multivariate characters. *Journal of Evolutionary Biology*, *21*(5), 1201–1219. https://doi.org/10.1111/j.1420-9101.2008.01573.x

Hansen, Thomas F., Pélabon, C., & Houle, D. (2011). Heritability is not Evolvability. *Evolutionary Biology*, *38*(3), 258. https://doi.org/10.1007/s11692-011-9127-6

Hayden*, K. J., & Parker, I. M. (2002). Plasticity in cyanogenesis of Trifolium repens L.: Inducibility, fitness costs and variable expression. *Evolutionary Ecology Research*, *4*(2), 155–168.

Hay-Roe, M. M., & Nation, J. (2007). Spectrum of Cyanide Toxicity and Allocation in Heliconius erato and Passiflora Host Plants. *Journal of Chemical Ecology*, *33*(2), 319–329. https://doi.org/10.1007/s10886-006-9234-5

Hereford, J., Hansen, T. F., & Houle, D. (2004). Comparing strengths of directional selection: How strong is strong? *Evolution; International Journal of Organic Evolution*, *58*(10), 2133–2143. https://doi.org/10.1111/j.0014-3820.2004.tb01592.x

Holloway, G. J., de Jong, P. W., & Ottenheim, M. (1993). The Genetics and Cost of Chemical Defense in the Two-Spot Ladybird (Adalia bipunctata L.). *Evolution*, *47*(4), 1229–1239. JSTOR. https://doi.org/10.2307/2409988

Jiggins, C. D. (2017). *The Ecology and Evolution of Heliconius Butterflies* (1 edition). Oxford University Press.

Jiggins, C. D., Naisbit, R. E., Coe, R. L., & Mallet, J. (2001). Reproductive isolation caused by colour pattern mimicry. *Nature*, *411*(6835), 302–305. https://doi.org/10.1038/35077075

Jones, R. S., Speed, M. P., & Mappes, J. (2016). Parameterising a public good: How experiments on predation can be used to predict cheat frequencies. *Evolutionary Ecology*, *30*(5), 825–840. https://doi.org/10.1007/s10682-016-9851-6

Joron, M., & Mallet, J. L. (1998). Diversity in mimicry: Paradox or paradigm? *Trends in Ecology & Evolution*, *13*(11), 461–466. https://doi.org/10.1016/S0169-5347(98)01483-9

Kapan, D. D. (2001). Three-butterfly system provides a field test of müllerian mimicry. *Nature*, *409*(6818), 338–340. https://doi.org/10.1038/35053066

Kruuk, L. E. B. (2004). Estimating genetic parameters in natural populations using the 'animal model.' *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *359*(1446), 873–890. https://doi.org/10.1098/rstb.2003.1437

Lande, R., & Price, T. (1989). Genetic correlations and maternal effect coefficients obtained from offspring-parent regression. *Genetics*, *122*(4), 915–922.

Liang, W.-S. (2003). Drought stress increases both cyanogenesis and β-cyanoalanine synthase activity in tobacco. *Plant Science*, *165*(5), 1109–1115. https://doi.org/10.1016/S0168-9452(03)00306-6

Lindstedt, C., Suisto, K., Burdfield-Steel, E., Winters, A. E., & Mappes, J. (2020). Defense against predators incurs high reproductive costs for the aposematic moth Arctia plantaginis. *Behavioral Ecology*. https://doi.org/10.1093/beheco/araa033

Lindstedt, C., Talsma, J. H. R., Ihalainen, E., Lindström, L., & Mappes, J. (2010). Diet Quality Affects Warning Coloration Indirectly: Excretion Costs in a Generalist Herbivore. *Evolution*, *64*(1), 68–78. https://doi.org/10.1111/j.1558-5646.2009.00796.x

Lynch, M., & Walsh, B. (1998a). Maternal Effects. In *Genetics and Analysis of Quantitative Traits* (pp. 687–714). Oxford University Press.

Lynch, M., & Walsh, B. (1998b). Resemblance Between Relatives. In *Genetics and Analysis of Quantitative Traits*. Oxford University Press.

Mallet, J. (1999). Causes and Consequences of a Lack of Coevolution in Müllerian mimicry. *Evolutionary Ecology*, *13*(7), 777–806. https://doi.org/10.1023/A:1011060330515

Mallet, J., & Barton, N. H. (1989). Strong Natural Selection in a Warning-Color Hybrid Zone. *Evolution*, *43*(2), 421–431. https://doi.org/10.1111/j.1558-5646.1989.tb04237.x

Mallet, J., & Joron, M. (1999). Evolution of Diversity in Warning Color and Mimicry: Polymorphisms, Shifting Balance, and Speciation. *Annual Review of Ecology and Systematics*, *30*(1), 201–233. https://doi.org/10.1146/annurev.ecolsys.30.1.201

Mallet, J., McMillan, W. O., & Jiggins, C. D. (1998). Mimicry and warning color at the boundary between microevolution and macroevolution. In *Endless Forms: Species and Speciation*. Oxford University Press.

Massad, T. J., Fincher, R. M., Smilanich, A. M., & Dyer, L. (2011). A quantitative evaluation of major plant defense hypotheses, nature versus nurture, and chemistry versus ants. *Arthropod-Plant Interactions*, *5*(2), 125–139. https://doi.org/10.1007/s11829-011-9121-z

Merrill, R. M., Dasmahapatra, K. K., Davey, J. W., Dell'Aglio, D. D., Hanly, J. J., Huber, B., Jiggins, C. D., Joron, M., Kozak, K. M., Llaurens, V., Martin, S. H., Montgomery, S. H., Morris, J., Nadeau, N. J., Pinharanda, A. L., Rosser, N., Thompson, M. J., Vanjari, S., Wallbank, R. W. R., & Yu, Q. (2015). The diversification of Heliconius butterflies: What have we learned in 150 years? *Journal of Evolutionary Biology*, *28*(8), 1417–1438. https://doi.org/10.1111/jeb.12672

Merrill, R. M., Naisbit, R. E., Mallet, J., & Jiggins, C. D. (2013). Ecological and genetic factors influencing the transition between host-use strategies in sympatric Heliconius butterflies. *Journal of Evolutionary Biology*, *26*(9), 1959–1967. https://doi.org/10.1111/jeb.12194

Montejo-Kovacevich, G., Martin, S. H., Meier, J. I., Bacquet, C. N., Monllor, M., Jiggins, C. D., & Nadeau, N. J. (2020). Microclimate buffering and thermal tolerance across elevations in a tropical butterfly. *Journal of Experimental Biology*, *223*(8). https://doi.org/10.1242/jeb.220426

Müller, C., Zwaan, B. J., de Vos, H., & Brakefield, P. M. (2003). Chemical defence in a sawfly: Genetic components of variation in relevant life-history traits. *Heredity*, *90*(6), 468–475. https://doi.org/10.1038/sj.hdy.6800265

Nahrstedt, A., & Davis, R. H. (1983). Occurrence, variation and biosynthesis of the cyanogenic glucosides linamarin and lotaustralin in species of the Heliconiini (Insecta: Lepidoptera). *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *75*(1), 65–73. https://doi.org/10.1016/0305-0491(83)90041-X

Nahrstedt, A., & Davis, R. H. (1985). Biosynthesis and quantitative relationships of the cyanogenic glucosides, linamarin and lotaustralin, in genera of the Heliconiini (Insecta: Lepidoptera). *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *82*(4), 745–749. https://doi.org/10.1016/0305-0491(85)90519-X

Nishida, R. (2002). Sequestration of Defensive Substances from Plants by Lepidoptera. *Annual Review of Entomology*, *47*(1), 57–92. https://doi.org/10.1146/annurev.ento.47.091201.145121

Opitz, S. E. W., & Müller, C. (2009). Plant chemistry and insect sequestration. *Chemoecology*, *19*(3), 117. https://doi.org/10.1007/s00049-009-0018-6

Pinheiro, C. E. G. (1996). Palatablility and escaping ability in Neotropical butterflies: Tests with wild kingbirds (Tyrannus melancholicus, Tyrannidae). *Biological Journal of the Linnean Society*, *59*(4), 351–365. https://doi.org/10.1111/j.1095-8312.1996.tb01471.x

Pinheiro, C. E. G., & Campos, V. C. (2019). The responses of wild jacamars (Galbula ruficauda, Galbulidae) to aposematic, aposematic and cryptic, and cryptic butterflies in central Brazil. *Ecological Entomology*, *44*(4), 441–450. https://doi.org/10.1111/een.12723

Prudic, K. L., Timmermann, B. N., Papaj, D. R., Ritland, D. B., & Oliver, J. C. (2019). Mimicry in viceroy butterflies is dependent on abundance of the model queen butterfly. *Communications Biology*, *2*(1), 68. https://doi.org/10.1038/s42003-019-0303-z

R Core Team. (2018). *R: A language and environment for statistical computing. R Foundation for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Reudler, J. H., Lindstedt, C., Pakkanen, H., Lehtinen, I., & Mappes, J. (2015). Costs and benefits of plant allelochemicals in herbivore diet in a multi enemy world. *Oecologia*, *179*(4), 1147–1158. https://doi.org/10.1007/s00442-015-3425-0

Ritland, D. B., & Brower, L. P. (1991). The viceroy butterfly is not a batesian mimic. *Nature*, *350*(6318), 497. https://doi.org/10.1038/350497a0

Ruxton, G. D., & Speed, M. P. (2006). How can automimicry persist when predators can preferentially consume undefended mimics? *Proceedings of the Royal Society B: Biological Sciences*, *273*(1584), 373–378. https://doi.org/10.1098/rspb.2005.3238

Ruxton, G. D., William Allen L., Sherratt, T. N., & Speed, M. P. (2018). *Avoiding Attack: The Evolutionary Ecology of Crypsis, Aposematism, and Mimicry* (Second Edition). Oxford University Press.

Sculfort, O., Castro, E. C. P. de, Kozak, K. M., Bak, S., Elias, M., Nay, B., & Llaurens, V. (2020). Variation of chemical compounds in wild Heliconiini reveals ecological factors involved in the evolution of

chemical defenses in mimetic butterflies. *Ecology and Evolution*. https://doi.org/10.1002/ece3.6044

Smilanich, A. M., Dyer, L. A., Chambers, J. Q., & Bowers, M. D. (2009). Immunological cost of chemical defence and the evolution of herbivore diet breadth. *Ecology Letters*, *12*(7), 612–621. https://doi.org/10.1111/j.1461-0248.2009.01309.x

Speed, M. P. (1993). Muellerian mimicry and the psychology of predation. *Animal Behaviour*, *45*(3), 571–580. https://doi.org/10.1006/anbe.1993.1067

Speed, M. P. (1999). Batesian, quasi-Batesian or Müllerian mimicry? Theory and data in mimicry Research. *Evolutionary Ecology*, *13*(7), 755–776. https://doi.org/10.1023/A:1010871106763

Speed, M. P., Ruxton, G. D., Mappes, J., & Sherratt, T. N. (2012). Why are defensive toxins so variable? An evolutionary perspective. *Biological Reviews*, *87*(4), 874–884. https://doi.org/10.1111/j.1469-185X.2012.00228.x

Stoffel, M. A., Nakagawa, S., & Schielzeth, H. (2017). rptR: Repeatability estimation and variance decomposition by generalized linear mixed-effects models. *Methods in Ecology and Evolution*, *8*(11), 1639–1644. https://doi.org/10.1111/2041-210X.12797

Svennungsen, T. O., & Holen, Ø. H. (2007). The evolutionary stability of automimicry. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1621), 2055–2063. https://doi.org/10.1098/rspb.2007.0456

Turner, J. R. G. (1984). Mimicry: The palatability spectrum and its consequences. In *The Biology of Butterflies* (pp. 141–161). Academic Press.

Turner, J. R. G. (1987). The evolutionary dynamics of batesian and muellerian mimicry: Similarities and differences. *Ecological Entomology*, *12*(1), 81–95. https://doi.org/10.1111/j.1365-2311.1987.tb00987.x

Van Belleghem, S. M., Rastas, P., Papanicolaou, A., Martin, S. H., Arias, C. F., Supple, M. A., Hanly, J. J., Mallet, J., Lewis, J. J., Hines, H. M., Ruiz, M., Salazar, C., Linares, M., Moreira, G. R. P., Jiggins, C. D., Counterman, B. A., McMillan, W. O., & Papa, R. (2017). Complex modular architecture around a simple toolkit of wing pattern genes. *Nature Ecology & Evolution*, *1*(3), 1–12. https://doi.org/10.1038/s41559-016-0052

Vandegeer, R., Miller, R. E., Bain, M., Gleadow, R. M., & Cavagnaro, T. R. (2013). Drought adversely affects tuber development and nutritional quality of the staple crop cassava (Manihot esculenta Crantz). *Functional Plant Biology*, *40*(2), 195–200. https://doi.org/10.1071/FP12179

Villemereuil, P. de, Morrissey, M. B., Nakagawa, S., & Schielzeth, H. (2018). Fixed-effect variance and the estimation of repeatabilities and heritabilities: Issues and solutions. *Journal of Evolutionary Biology*, *31*(4), 621–632. https://doi.org/10.1111/jeb.13232

Wallbank, R. W. R., Baxter, S. W., Pardo-Diaz, C., Hanly, J. J., Martin, S. H., Mallet, J., Dasmahapatra, K. K., Salazar, C., Joron, M., Nadeau, N., McMillan, W. O., & Jiggins, C. D. (2016). Evolutionary Novelty in a Butterfly Wing Pattern through Enhancer Shuffling. *PLOS Biology*, *14*(1), e1002353. https://doi.org/10.1371/journal.pbio.1002353

Walsh, B., & Lynch, M. (2018a). Selection in the presence of heritable maternal effects. In *Evolution and Selection of Quantitative Traits* (pp. 537–547).

Walsh, B., & Lynch, M. (2018b). *Evolution and Selection of Quantitative Traits*. Oxford University Press.

Wilson, A. J., Réale, D., Clements, M. N., Morrissey, M. M., Postma, E., Walling, C. A., Kruuk, L. E. B., & Nussey, D. H. (2010). An ecologist's guide to the animal model. *Journal of Animal Ecology*, *79*(1), 13–26. https://doi.org/10.1111/j.1365-2656.2009.01639.x

Winters, A. E., Wilson, N. G., van den Berg, C. P., How, M. J., Endler, J. A., Marshall, N. J., White, A. M., Garson, M. J., & Cheney, K. L. (2018). Toxicity and taste: Unequal chemical defences in a mimicry ring. *Proceedings of the Royal Society B: Biological Sciences*, *285*(1880), 20180457. https://doi.org/10.1098/rspb.2018.0457

Yezerski, A., Gilmor, T. P., & Stevens, L. (2004). Genetic Analysis of Benzoquinone Production in Tribolium confusum. *Journal of Chemical Ecology*, *30*(5), 1035–1044. https://doi.org/10.1023/B:JOEC.0000028465.37658.ae

Zvereva, E. L., & Kozlov, M. V. (2016). The costs and effectiveness of chemical defenses in herbivorous insects: A meta-analysis. *Ecological Monographs*, *86*(1), 107–124. https://doi.org/10.1890/15-0911.1

SUPPLEMENTARY FILES

Supplementary File 1. A. Full results table of between-population variation of cyanogenic glucoside compounds in natural populations of *Heliconius erato* and *Passiflora biflora*. **B.** Full results table of REML animal models.

Supplementary File 2. Summary table of *Heliconius erato* broods.

Supplementary File 3. Supplementary results: Cyanogenic toxicity and overall quality of host plant *Passiflora biflora* watering treatment groups.

Supplementary File 4. Supplementary Methods. Detailed information about methods used in the study.