1. GENERAL INFORMATION

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Title: High evolutionary potential in the chemical defenses of an aposematic *Heliconius* butterfly

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2. ABSTRACT

Chemical defences against predators underlie the evolution of aposematic coloration and mimicry, which represent classic examples of adaptive evolution. Yet, unlike color patterns, little is known about the evolutionary potential of chemical defences. Neotropical *Heliconius* butterflies exhibit incredibly diverse warning color patterns and widespread mimicry. Their larvae feed exclusively on cyanogenic *Passiflora* vines, can metabolize and sequester host plant toxins, as well as biosynthesize defensive cyanogenic toxins themselves. Here, we investigate variation in biosynthesized toxicity both in wild populations along environmental gradients and in common-garden broods and feeding treatments in *Heliconius erato*, together demonstrating considerable intraspecific variation and evolutionary potential in this important chemical defense trait. Toxicity varied markedly among wild populations from Central and South America. Within wild populations, the distribution of toxicity was consistently skewed, indicative of automimic "cheaters" that may exploit, and consequently deplete, the protection of the warning coloration. In a common-garden rearing design comprising more than 300 butterflies across 20 broods, variation in host-plant nutritional quality or cyanogen levels did not translate into differences in toxicity of butterflies feeding on these plants. Instead, toxicity had a significant heritable genetic component, in part explained by maternal inheritance. The evolvability of toxicity was high (e_{μ}=1.55%), suggesting that toxicity can evolve rapidly. Through its link with the evolution of warning color pattern mimicry, the high evolutionary potential of cyanogenic toxicity may have facilitated diversification and ecological speciation in *Heliconius*, highlighting the importance of understanding the evolution of chemical defense in aposematic and mimetic species.

3. 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 [1–3]. 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 [4]. This can also lead to mimicry among co-occurring prey species, which can be either Batesian mimicry, where a palatable mimic exploits an unpalatable model, or Müllerian mimicry, in which unpalatable species copy one another for mutual benefit (reviewed by [5]). However, because palatability can vary along a continuum [6], the distinction between these forms of mimicry is not always clear-cut, and theory suggests that an intermediate form of Müllerian mimicry, known as quasi-Batesian mimicry, may also occur [7]. Quasi-Batesian mimicry occurs when a less well protected species acts in a parasitic manner, diluting the protection of the better defended species [8,9]. Such a situation is expected to be less common than simple Müllerian mimicry [10], but empirical evidence from laboratory and field experiments supports its existence [11,12]. These different mimicry relationships depend largely on predictable differences in toxicity among mimetic species and individuals, and yield different predictions about the origins of diversity in warning coloration and mimicry [8,13,14]. Although mimicry is one of the earliest and best studied examples of Darwin´s theory of evolution by natural selection and some of evolution´s oldest mathematical models [15], many open questions remain concerning the evolution of chemical defenses underlying aposematism and mimicry.

A key factor in defining mimicry relationships is the relative protection levels of individuals and species, which varies with unpalatability and abundance [5,9,16]. This is because the effectiveness of a warning signal depends on how often the predators encounter the signal [17], and how memorable these encounters are, i.e. the level of toxicity or unpalatability. Turner [6] and Speed [8] highlight the importance of studying the geography of the palatability spectrum because of its high relevance in defining mimicry relationships. Extensive variation in defensive toxins within and among prey populations has repeatedly been demonstrated [18], supporting the prediction that chemical defenses vary widely along a continuum [8,18–20]. The levels of unpalatability of mimic species can range from equal to very uneven [1,19,21], and species may be protected by different chemical compounds [20,22]. Such variation has important implications for the dynamics of mimicry, through e.g. leading to alternation between the different types of mimicry, which could in turn

influence convergent vs. advergent selection (where selective pressures cause phenotypic convergence of one species on another, but not vice versa [9]), and contribute to the observed diversity in mimicry systems [8,10].

Chemical defense also varies within species and populations. 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 [6]. At the individual level, there may be selection for optimizing the allocation of energy and resources between defense and other functions because of the potentially high costs of production and maintenance of chemical defenses. This could lead to energetic trade-offs [23,24] and automimicry (the occurrence of palatable "cheaters" in a chemically defended population [18,25]). These "cheaters" (automimics) benefit from the protection of their warning coloration, without investing in energetically expensive chemical defenses [18]. A decrease in population average unpalatability due to the occurrence of automimics or weakly defended individuals could dilute the protection conveyed by the warning signal [18,26,27], as in Batesian and quasi-Batesian mimicry. Host-plant chemistry is likely to drive intraspecific variation in sequestered defensive compounds [1,2], as demonstrated in butterflies [28,29]. For species that biosynthesize defensive compounds *de novo*, the origin of variation in defense levels is less clear. Sources of variation may include biotic factors such as the energy and resources available for the expensive compound synthesis, as well as interactions (and potential trade-offs) with life-history traits such as age, reproductive stage or immunological status [1,30]. An important mechanism underlying defense variation is probably variation in the biochemical efficiency of sequestration and biosynthesis of defensive chemicals, a trait that could itself evolve. However, little is known about whether such variation is genetic or environmental in origin. Despite the central role of chemical defenses in the evolution of aposematic and mimetic species, we know little about the evolutionary potential of these traits.

Neotropical *Heliconius* butterflies, with their incredible diversity in wing coloration, are a well-known example of aposematism and mimicry [31,32]. *Heliconius* larvae feed exclusively on passion vines (*Passiflora* genus), with which they have radiated and speciated through a coevolutionary arms race [33,34]. *Passiflora* are cyanogenic, and contain one or several of about 30 different cyanogenic glucoside (CNglc) compounds [3]. Some of these CNglcs found in *Passiflora* can be sequestered by the larvae of most *Heliconius* species [3,35], and the chemical content of host plants can influence the level of sequestered defenses of the

butterfly [29]. However, the *Heliconius* genus represents a minority among Lepidopterans additionally capable of biosynthesizing defensive compounds *de novo* [36]. *Heliconius* larvae and adult butterflies can biosynthesize the CNglcs linamarin and lotaustralin from aliphatic amino acids [37], but the sources of variation in biosynthesis are less well known than those in host-plant dependent compound sequestration. The capacity of cyanogen biosynthesis in *Heliconius* is thought to be related to the evolution of pollen feeding. This is because pollen feeding allows the exploitation of amino acid sources during adulthood for reproduction and the uniquely long adult life-span of these butterflies, and could thus free larval-acquired resources for purposes such as defense [38–41]. Substantial variation in the amounts and types of cyanogens between different *Heliconius* species has been documented, both within and between mimicry rings [3,19,41,42]. Some of this is explained by the level of diet specialization, which is a major determinant of the relative roles of plant-CNglcs sequestration and *de novo* biosynthesis, shown to be negatively correlated traits among *Heliconius* species [3,42]. In general, the more specialized the species, the larger the role of sequestration and the higher the total level of CNglcs, while the more generalist species acquire their defense compounds mostly from biosynthesis, and have on average lower total cyanogen levels [3]. However, we know little about how cyanogenic toxicity varies within *Heliconius* species or within populations, how it is influenced by environmental variation, and what is its potential to evolve.

Here, we analyzed the evolutionary potential of biosynthesized cyanogenic toxicity in *Heliconius erato* by estimating genetic variance components and evolvabilities of this important chemical defense trait using a common-garden breeding design. The pedigree data consisted of 20 broods and altogether more than 300 individuals. In addition, we analyzed natural variation in the cyanogenic content of the host plant *Passiflora biflora*, and the contribution of larval diet (*P. biflora* with varying cyanogenic content and overall nutritional quality) to the variation in butterfly biosynthesized cyanogenic toxicity. Finally, we studied variation in toxicity within and among natural populations of *H. erato* along environmental clines and among populations originating from two regions of South and Central America.

4. RESULTS

Common-garden pedigree data comprising more than 300 *Heliconius erato* individuals revealed a significant heritable genetic component and high evolvability in biosynthesized cyanogenic toxicity (Fig. 1D). In

contrast, nutritional quality and cyanogenic toxicity in the host plant *Passiflora biflora*, which varied significantly in response to full-factorial water availability treatments, had negligible impacts on the biosynthesized toxicity level of the butterflies feeding on these plants. The distribution of biosynthesized cyanogenic toxicity in natural *H. erato* populations sampled at wet, intermediate, and dry sites along a rainfall gradient in Panama was consistently skewed, so that a large proportion of individuals had a noticeably low level of toxicity compared with a smaller proportion of high-toxicity individuals (Fig. 1C). Furthermore, a comparison of geographically distant natural populations of *H. erato* from Panama and Ecuador showed that populations of the same species can have substantially different levels of biosynthesized toxicity (Fig. 1B). Together, these results demonstrate the high level of intraspecific variation and evolutionary potential in this important chemical defense trait of an aposematic and mimetic *Heliconius* butterfly.

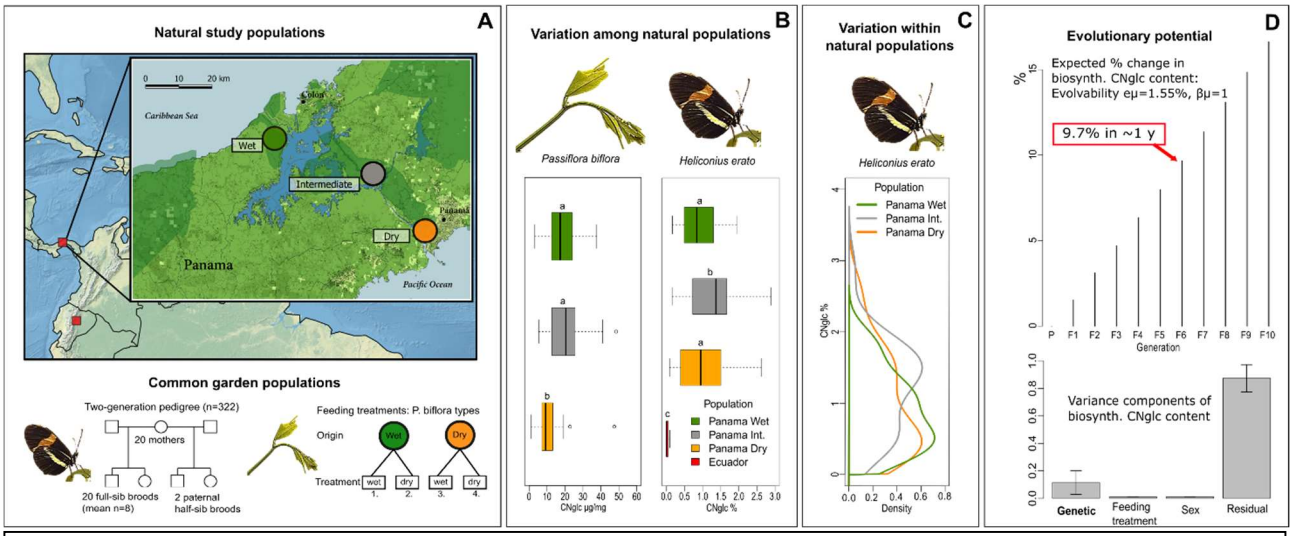

Figure 1. Natural variation and evolutionary potential of biosynthesized cyanogenic toxicity in *Heliconius erato*. **A)** Top panel: Locations of the natural study populations in Central and South America (Panama and Ecuador, respectively), with a more detailed map of study areas along the rainfall 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: Expected % change in *H. erato* biosynthesized cyanogenic toxicity through generations assuming evolvability e_{μ} =1.55% (measured value) and mean-scaled selection gradient β_{μ}=1. Bottom panel: Variance components of *H. erato* biosynthesized cyanogenic toxicity (with S.E.) based on pedigree data (see panel A).

Genetic variance in toxicity suggests high evolvability

The level of biosynthesized cyanogenic toxicity (concentrations of linamarin and lotaustralin, the two cyanogenic compounds biosynthesized by *Heliconius* larvae and adults, analyzed with ^{1}H-NMR from samples of adult individuals), varied two-fold among the 20 full-sib 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 CNglcs concentration level (Fig. 1D, Table 1, Table S1), corresponding to a broad-sense heritability (H^2) of 0.115. The genetic variance component was larger and statistically significant for the concentration of linamarin compared to lotaustralin (H^2 = 0.139 for linamarin, H^2 = 0.067 for lotaustralin; Table 1, Table S1). Heritabilities are useful measures of the resemblance between offspring and parents, but are poor standardized measures of evolutionary potential [43]. As a measure of evolutionary potential, we therefore computed mean-scaled evolvabilities (e_μ) as $e_\mu = V_G/\mu^2$, where μ is the trait mean, and which gives the expected percent change in the trait mean per generation under a unit strength of selection [43,44]. The broad-sense evolvability of total CNglcs concentration was e_μ=1.55%, meaning that the trait mean is expected to change by 1.55% per unit strength of selection in a generation (Fig. 1D). Evolvability was higher for the concentration of linamarin compared to that of lotaustralin (e_μ= 1.89% vs. e_μ= 1.04%; Table 1, Table S1).

					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 1. Summary of variance components of *Heliconius* biosynthesized cyanogenic toxicity estimated with REML animal models. V_{mat}, V_{trm} and V_{sex} are the variance components for maternal effects, host plant feeding treatment and sex, respectively. See Table S1 for full model results.

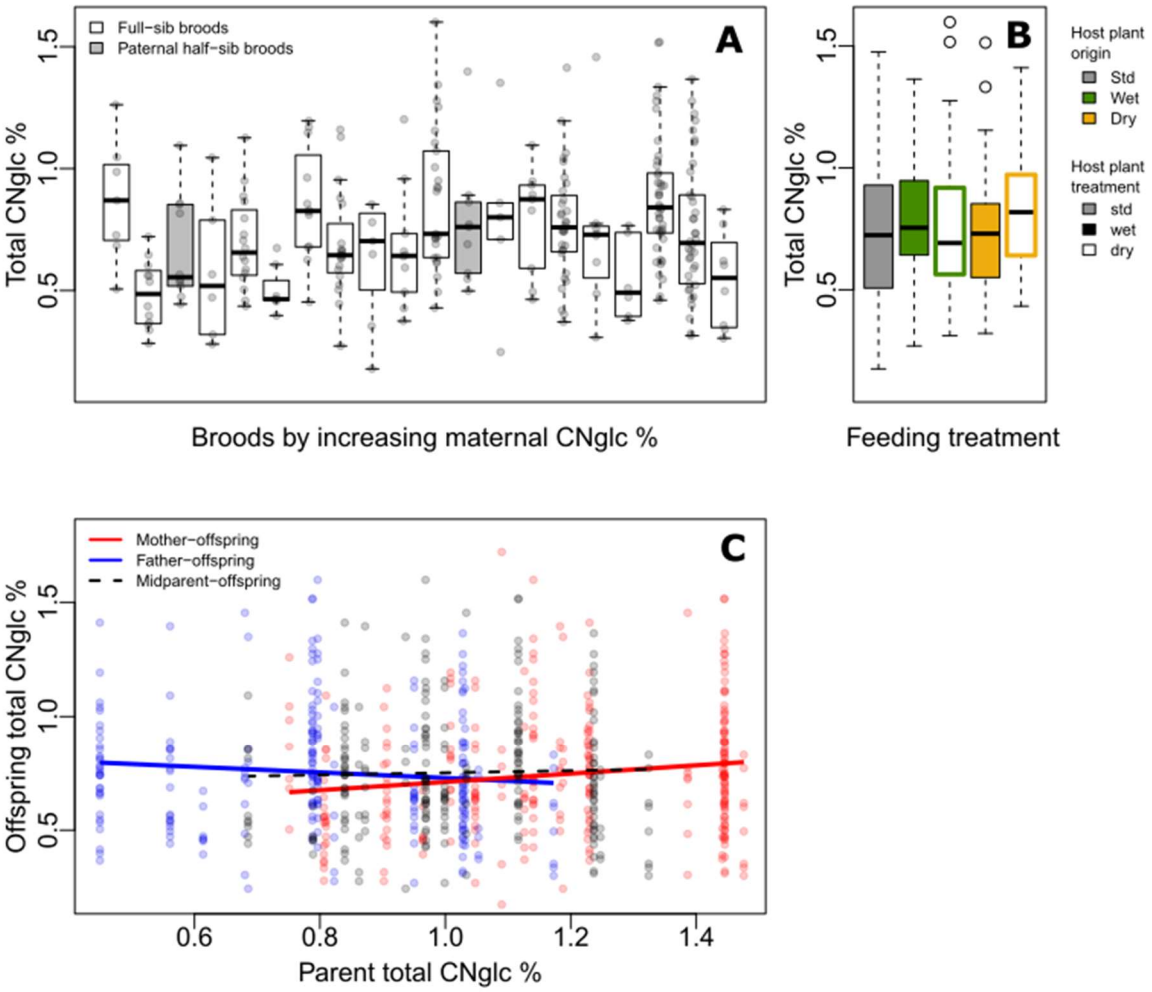

Figure 2. A) Variation in total biosynthesized CNglcs concentration (% dry mass) among full-sibling broods of *Heliconius erato* by increasing maternal CNglc % (one pair of full-sib broods are paternal half-siblings, in grey) **B)** Total biosynthesized CNglcs concentration (% dry mass) in newly-emerged 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.

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-family variance; Table 1, Table S1). Parent-offspring regressions revealed that offspring CNglc concentration was positively associated with the CNglc concentration of the mother (slope = 0.181 ± 0.071, t = 2.539, P = 0.012 for total CNglc concentration; Fig. 2C), while the association between fathers and offspring was negative and poorly supported statistically (slope = -0.122 ± 0.083, t = -1.469, P = 0.143 for total CNglc concentration). In turn, the midparent-offspring

regression was nearly flat (slope = 0.046 ± 0.107, t = 0.435, P = 0.664 for total CNglc concentration; Fig. 2C). These regression slopes (accounting for parent identity as a random factor) translate into a maternal-effect coefficient for total CNglc concentration of m = 0.261 (95% confidence interval = -0.099, 0.614), which implies the existence of considerable positive maternal effects in biosynthesized cyanogenic toxicity. The maternal effect coefficient was higher for linamarin level (mean m = 0.288, 95% C.I. -0.094, 0.687) than lotaustralin level (mean m = 0.193, 95% C.I. -0.135, 0.520).

Butterflies reared on different host plant types (cultivated in a full-factorial combination of plant origins and water availability treatments, see Fig. 1A) as larvae did not differ in biosynthesized cyanogenic toxicity (Fig. 2B), and the host plant type explained a negligible proportion of variance in toxicity level (V_{trm}/V_P = 0.011; Fig. 1D, Table 1, Table S1). This is despite the host plant types showing highly significant differences in cyanogenic toxicity and plant quality indices representing nutritional values (e.g. carbon-nitrogen balance; see Supplementary S3), which were measured because larval-acquired nitrogenous resources have been proposed as an important source for cyanogen biosynthesis (Cardoso and Gilbert 2013). The minor effect of the larval diet compared to the effect of family is also evident from a simple linear model of total CNglcs concentration explained by maternal identity, sex, plant treatment, and their two-way interactions, in which the effect of plant treatment was not statistically significant ($F_{3, 228}$ = 1.335, P = 0.26), whereas the effect of maternal identity was ($F_{19, 228}$ = 2.996, P<0.001). Sex was also a negligible source of variation in toxicity (V_{sex}/V_P = 0.011; Fig. 1D, Table 1, Table S1). Despite the common-garden conditions that all individuals experienced throughout their life cycle, the residual (unexplained) variance component of CNglcs concentration remained large (V_{res}/V_P = 0.874 for total CNglcs, Fig. 1D, Table 1, Table S1). This large amount of unexplained variance is also demonstrated by a significant difference in toxicity level between the parental and offspring generation ($F_{1, 320}$ = 27.42, P < 0.001 for total CNglcs), despite the two generations sharing common garden conditions.

Wide natural variation in toxicity of butterflies and host plants

The distribution of biosynthesized CNglcs levels in the natural populations of *H. erato* sampled along an environmental rainfall gradient in Panama was similar in the Dry, Intermediate and Wet populations (Fig. 1C): a large proportion of individuals had toxicity values in the low end of the toxicity range (e.g. 40% of

individuals had total CNglc 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 CNglc concentrations within the highest 25% of toxicity values). Total CNglcs concentration and concentration of linamarin did not differ detectably among the three Panamanian populations. However, 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.

The three populations along the altitudinal gradient in Ecuador differed markedly in biosynthesized cyanogenic toxicity from their Panamanian conspecifics: most Ecuadorian samples lacked both linamarin and lotaustralin. 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), which is below the minimum level observed in the Panamanian populations.

The CNglc levels of the host plant *Passiflora biflora* differed significantly among the natural populations along the rainfall gradient in Panama ($F_{2, 48} = 3.65$, $P = 0.034$; Fig. 1B). However, the cyanogen level in *Passiflora* varied in a contrasting pattern to that of *H. erato*, in which individuals from the Intermediate population were most toxic on average (Fig. 1B). In the plants, the Dry population had substantially lower cyanogen levels compared with both the Intermediate and Wet populations. Passibiflorin was the main compound (93-95% of total CNglcs; Fig. 3) in all populations, with the remaining proportion made up mainly of passibiflorin triglycoside (5-6% of total CNglcs; Fig. 3). In addition, 15% of the samples from the Dry, and 9% from the Intermediate populations contained traces of the CNglc compound tetraphyllin A, which was not present in the Wet population (Fig 3).

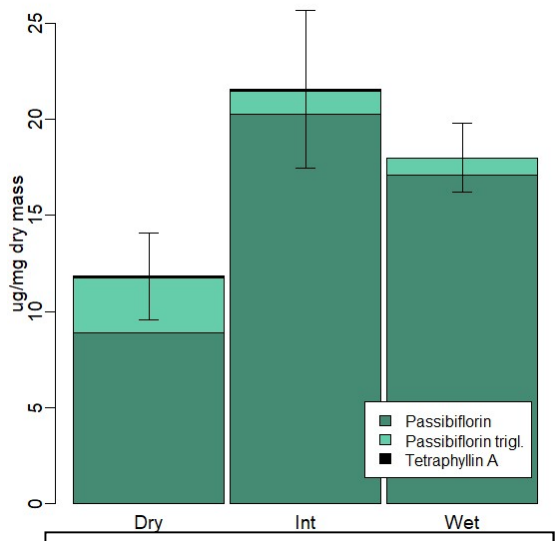

Figure 3. Average CNglc compound compositions in natural Dry, Intermediate and Wet populations of the host plant *Passiflora biflora* in Panama. Standard error bars are shown for the total CNglcs concentration.

5. DISCUSSION

High evolutionary potential of biosynthesized cyanogenic toxicity and its implications

We have shown for the first time that there is high evolvability in the biosynthesized chemical defenses of an aposematic *Heliconius* butterfly. Our heritability estimate of biosynthesized cyanogenic toxicity (0.115 ± 0.086) is close to the average heritability value of physiological traits (0.12 ± 0.05) in a meta-analysis of a wide range of traits and taxa [43]. Concerning evolvability, however, the estimated value for cyanogenic toxicity is high (1.55 ± 0.89%) compared to the average value of physiological traits (0.49 ± 0.14%), as well as the median evolvability of all studied traits across taxa (0.26 ± 0.03%; see [43]). The evolvability of e_{μ}=1.55% means that the mean cyanogenic toxicity can be expected to change by 1.55% per unit strength of selection per generation. This high estimate of evolvability implies that the trait has the potential to evolve rapidly, especially in conditions of strong selection, also taking into account the short generation time (usually one to two months) of these tropical butterflies. For example, assuming the species has six generations per year, and increased cyanogenic toxicity is selected for with selection as strong as direct selection on fitness (β_{μ}= 100%, see [45]), the trait mean has the potential to increase by 9.7% in just one year.

Studies of mimicry typically focus on the warning signal, namely the color pattern [31,32,46–48]. However, contrasting selective agents could be acting on the defense trait associated with the warning signal, both equally required for aposematism and mimicry. The complex selection regimes acting on chemical defense, together with heritable genetic variation, could result in diverse patterns of adaptive evolution in chemical defense traits, and influence the evolutionary trajectories of aposematic and mimetic species. This is because evolutionary changes in chemical defense levels could alter mimetic relationships among groups of species, including shifts between Müllerian, Batesian and quasi-Batesian relationships, which could in turn influence advergent vs. convergent selection and contribute to the observed diversity in mimicry systems [8,10,14].

Heliconius butterflies are well known for rapid inter- and intraspecific diversification in their color patterns [31,32]. Co-mimetic species have evolved numerous geographic races or sub-species [49], of which *H. erato* and *H. melpomene* present one of the most striking examples of the diversity of region-specific color-pattern races. The origins of such diversity, and the role of Müllerian or other forms of mimicry in creating this diversity, remain poorly understood [10,32,50–52]. The evolvability of cyanogenic toxicity

demonstrated here suggests that local adaptation in chemical defense could have altered the nature of relationships between species across geographic space, which may have played a role in the evolution of wing pattern diversity. The capacity of *Heliconius* for defense compound biosynthesis could allow for increased genetic control (and thus, increased evolvability) of chemical defenses compared with most other chemically defended Lepidoptera which rely solely on host plant compound sequestration [36]. Furthermore, evolutionary shifts in color pattern mimicry have been shown to result in reproductive isolation and speciation in *Heliconius* [53,54]. Through influencing the evolution of color pattern mimicry, evolutionary changes in chemical defenses could play a part in facilitating such ecological speciation.

Our results also indicate a central role for maternal effects in the inheritance of cyanogenic toxicity. These effects could have a genetic and/or environmental basis in the mother. However, mothers were raised in common garden conditions, and environmental variance is thus expected to be low. The maternal effects could originate from e.g. epigenetic effects, direct transfer of toxins from mother to offspring in eggs [37], or other types of maternal influence on the offspring phenotype, beyond the contribution of maternally transmitted genes [55–57]. Paternal effects were not observed, even though *Heliconius* males are known to transfer a nuptial gift containing cyanogenic compounds to females at mating [58]. It is noteworthy that the occurrence of a maternal effect of the type estimated here, defined simply as an effect of the mothers phenotype on the offspring's phenotype (as in Falconer´s dilution model [55,56]), could significantly strengthen the response of the trait to selection. Walsh and Lynch [59] simulated the predicted selection response with maternal effects under Falconer´s model, and demonstrated that for a trait with a maternal effect coefficient of $m = 0.25$, which is comparable to the $m = 0.261$ found here for cyanogenic toxicity, the cumulative response to selection would over time exceed that of a trait with $m = 0$ by as much as about 50% (with heritability values remaining equal). This effect however is expected to dilute over generations and is not straightforward to predict. Nevertheless, maternal effects could accelerate the high evolutionary potential of cyanogenic toxicity, highlighting the potentially important role of chemical defense evolution in the diversification and speciation of *Heliconius*.

Complex selection pressures in chemical defense: explaining defense variation in wild populations?

We found considerable variation in biosynthesized cyanogenic toxicity both in common-garden laboratory and wild population butterfly individuals. The extensive variation in host plant quality and toxicity had a negligible influence on the cyanogenic toxicity level of the *H. erato* butterflies feeding on these plants in the common garden. The relationship between larval diet and the level of sequestered compounds has been demonstrated in many taxa, including *Heliconius* [29,42]. Here, we focused solely on the biosynthesized cyanogenic compounds linamarin and lotaustralin, for which the sources of variation are less clear. *Heliconius erato* is not capable of sequestering the cyanogenic compounds found in *Passiflora biflora*, its major natural host plant and the host plant used in our experiment, because of the modified structure of those compounds [3,35], and we therefore expected only *de novo* biosynthesis of cyanogens in our common-garden samples. The lack of a relationship between larval diet and biosynthesized cyanogens is still somewhat surprising, as previous studies on *Heliconius* suggest that general larval nutrient intake (amino acids derived from host plants) may be important for adult cyanogen biosynthesis [41]. In contrast, we found no significant impact of host plant quality on butterfly biosynthesized cyanogenic toxicity, despite the extensive variation in overall nutritional values of the plants (e.g. carbon-nitrogen ratio).

Toxicity levels varied significantly among wild butterfly populations both between regions and along environmental clines. The rainfall gradient along which populations were sampled is substantial, and the habitat types vary from deciduous dry tropical forest to rainforest. The Ecuadorian populations, on the other hand, inhabit a very different and geographically distant biotope along the eastern slope of the Andes. These environmental clines are likely to differ in multiple biotic and abiotic factors potentially relevant for chemical defense, including host plant and nectar/pollen availability and the community of predators, a key candidate for a strong and dynamic selective force driving local adaptations in chemical defense [14,18]. The field and experimental data indicate that quality variation within the main host plant species *P. biflora* does not contribute to plastic responses in *H. erato* toxicity. However, in the case of wild-collected samples the larval and adult diet is unknown (*H. erato* feeds on *P. biflora*, *P. coriaceae* and to a lesser extent *P. auriculata* in the Canal Zone of Panama [60], while the preferred host plants in Ecuador are not well known). Some natural intraspecific variation found in biosynthesized toxicity could therefore originate from variation in host plant species use, potentially influenced by a negative correlation between biosynthesis and sequestration, which appears to occur from interspecific to individual levels [3,42,61]. *Heliconius erato* cannot

sequester *Passiflora* cyanogens from *P. biflora*, but can do so from some of its other possible host plants [3]. This could also help to explain the very low biosynthesized toxin levels in *H. erato* in Ecuador, where host plant availability may differ. These populations could have specialized on a particular *Passiflora* host, selecting for a major decrease in biosynthesis, and leading to local adaptation in the biosynthesized toxicity level, in line with the demonstrated high evolutionary potential of this trait. Cases of zero levels of biosynthesized cyanogens in *Heliconius* are rare, but previous observations of such species provide evidence for their high levels of host plant specialization and plant defense compound sequestration [3,62]. Such adaptive radiations of *Heliconius* driven by host plant niche use could also have been facilitated by the high evolutionary potential in biosynthesized cyanogenic toxicity leading to local adaptations in the sequestration-biosynthesis balance.

In the case of the host plant *Passiflora biflora*, it is particularly notable that watering treatments induced substantial differences in the cyanogen content and overall quality of the plants, and corresponding variation in cyanogen content was observed also in the wild populations of *P. biflora*. This adds to growing evidence of water availability as a major factor influencing toxin levels of cyanogenic plants, many of which are of great economic importance (e.g. *Eucalyptus* [63], cassava [64], white clover [65]). Although here the cyanogen variation in *P. biflora* did not influence the studied chemical defense traits of *H. erato*, water availability-induced variation in *Passiflora* and other cyanogenic plant species could have important consequences for these plants as well as their associated species community, especially herbivores feeding on them. This is particularly relevant when acknowledging that changes in precipitation are expected to exert the most powerful selective forces in relation to global climate change [66].

Patterns of within-population variation in toxicity: maximizing the proportion of "cheaters"?

All three butterfly populations along the rainfall gradient exhibited similar skewed distributions of biosynthesized cyanogens. The majority of individuals in these populations had relatively low levels of toxicity, whereas a smaller proportion had substantially higher levels, and the pattern was surprisingly similar in all populations. Such a pattern could arise from e.g. age distributions, as some studies suggest that *Heliconius* may accumulate cyanogenic toxins with age [40]. However, many individuals in the study populations had near zero levels of toxicity, and thus, the pattern is also indicative of automimicry.

Automimic "cheaters" exploit the protection given by their warning coloration, without investing in chemical defenses themselves [18,25].

An important assumption for automimicry to occur is that chemical defenses must be costly, and there is thus selection for reducing individual-level toxicity, possibly through trade-offs with other important energy-expensive traits. Costs of chemical defense in *Heliconius* are poorly known. There is considerable evidence demonstrating costs of defense in other chemically-defended arthropods [18,24]. For the "cheaters" not to take over the whole population, there must also be benefits of investing in chemical defense (e.g. [67,68]). These benefits are not always simple to demonstrate, as unpalatability may not be a linear correlate of toxicity (e.g. [69]), and it often varies widely particularly based on the predator community [14]. However, empirical data on *Heliconius* imply direct benefits (surviving predator attacks) for chemically defended individuals [70–72], and indicate an increase in protection level especially when comparing low to moderate cyanogenic toxicity levels [69], applicable to observed cyanogen levels of *H. erato* [3,19].

Theory suggests that the frequency of automimics in a population can reach an equilibrium where the fitness of the "cheaters" (which pay reduced costs of defense but are more likely to be injured or killed by predators) is equal to the fitness of the defended individuals (which pay more costs for defense but have higher probability of surviving predator attacks) [67,68]. This could help explain the observed patterns of variation in cyanogenic toxicity within natural populations of *Heliconius erato*, which may be indicative of selection maximizing the frequency of automimics based on individual-level costs and benefits of chemical defense. The occurrence of automimicry in *Heliconius* (also observed by [19]) indicates that individual-level costs of producing and maintaining cyanogenic toxicity could lead to decreases in population average unpalatability. Future studies should investigate the entire range of both CNglcs and other chemical defense compounds potentially interacting with biosynthesized CNglcs in producing the unpalatability level perceived by relevant predators. An automimicry-related decrease in unpalatability could theoretically dilute the protection conveyed by the warning signal against predators [18,26,27], similarly as in Batesian and quasi-Batesian mimicry, and influence the evolutionary trajectories of the species sharing the warning signal.

6. CONCLUSION

We have shown in this analysis of biosynthesized cyanogenic toxicity in a *Heliconius* butterfly that this important chemical defense trait varies substantially within and among populations. This variation was unrelated to nutritional quality and toxicity variation in its main *Passiflora* host. Instead, the results suggest that larval host species availability and individual-level energy optimization may play more important roles as selective agents acting on biosynthesized toxicity. We show that biosynthesized cyanogenic toxicity has a significant heritable genetic basis and substantial evolutionary potential, indicating that it can evolve rapidly in response to the prevailing selection pressures. Despite the key role of chemical defense in mimicry dynamics, adaptive changes in chemical defense levels are often somewhat overlooked as a mechanism of evolution in aposematic and mimetic species, including the widely studied *Heliconius* butterflies. This study is to date the most comprehensive analysis of the patterns of intraspecific chemical defense variation in wild *Heliconius* populations, and the first to shed light on the evolutionary potential of this important trait. Our results highlight the implications of adaptive evolution in chemical defense traits for the evolution of *Heliconius* and other aposematic and mimetic species.

7. METHODS

Study sites and field collection of butterflies and host plants

Heliconius erato (Lepidoptera: Nymphalidae) is a widespread neotropical butterfly typically occurring at the edges of primary and secondary tropical rainforests. *Heliconius erato demophoon* butterflies and its typical host plant *P. biflora* were collected from three populations in Panama along a steep rainfall gradient (Wet, Intermediate and Dry study sites; Fig 1A, S4), and *H. erato lativitta* from High and Low altitude sites on the Eastern slope of the Andes in Ecuador (Fig. 1A, S4). Butterflies were caught using a hand net and transported live to the laboratory. All individuals caught from the Panamanian study areas (n = 92) were sexed and weighed, and were preserved in 1 ml 100% MeOH (body excluding wings and one half of the thorax) on the day of capture (males) or after allowing to oviposit in an insectary (females, see S4). At the Ecuadorian sites, fertilised females were brought to a common environment, and the thorax of 21 F1 individuals from each altitude were preserved in 1 ml 100% MeOH.

Passiflora biflora leaf samples (one to four leaves pooled per plant individual, see S4) were collected from the Panamanian Dry (n = 20), Int (n = 11) and Wet (n = 20) study areas (Fig. 1A), and preserved fresh in 1ml 100% MeOH. In addition, 50 cm cuttings, all from different plant individuals, were sampled from the Dry (n = 12) and Wet (n = 12) study areas for greenhouse cultivation.

Host plant greenhouse cultivation and treatments

Passiflora biflora were cultivated in the greenhouses at the University of Helsinki, Finland (75% relative humidity; 8-20 hrs: 25°C, light; 20-8 hrs: 20°C, dark) in a soil mixture (50% compost, 20% coir, 15% perlite, 15% sand or gravel)(see S4 for details on cultivation methods and watering treatments). Standard (std)-treated *P. biflora* were used as oviposition plants for the *H. erato* parental generation and larval diet for the F1 families not included in feeding treatments. The *P. biflora* cuttings collected from the Dry and Wet study areas in Panama were cultivated in a full-factorial combination of origins and water treatments, resulting in the four host plant treatment types used in feeding treatments of *H. erato* (next section). Plants in the dry treatment were kept at 75% relative humidity and watered two to three times weekly with about 1/3 of the water volume compared to the wet treatment (constant water availability, relative humidity up to 100%), such that the surface soil was allowed to dry and the leaves slightly droop between watering events. The dry treatment thus aimed to mimic conditions of drought stress.

Butterfly rearing and breeding design

Laboratory *H. erato* populations descendent of the wild Dry population (transported live from Panama as eggs, larvae and adult butterflies) were established at the University of Helsinki greenhouses (see S4 for details on transport and rearing methods). Larvae were reared on std-type *P. biflora*. All butterflies were individually marked, and were allowed to fly, mate, and oviposit freely in 2×2×2 m mesh cages with potted std-type *P. biflora* and a standardized sugar solution diet provided *ad libitum*. The butterfly cages were observed throughout the day for mating pairs, and mated females were moved to individual 2×2×2 m mesh cages and allowed to oviposit on std-type *P. biflora*. Eggs of 14 females (eight mated with a known father, average no. of full-sibs/family = 8, see Table S2) were collected, and hatched larvae fed with std-type *P. biflora* until pupation. The offspring of six mated pairs (both parental identities known, average no. of full-

sibs/family = 29, Table S2) 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. F1 individuals were collected within three days following emergence (unmated and unfed), were 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 S4 for details on sample extraction and ^{1}H-NMR methods). The data were processed and analyzed using Bruker TopSpin software. The peaks of CNglcs 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 [32]), 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 CNglcs [42,60], and the CNglc 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 [3,42].

The values of CNglc concentrations in the wild-collected samples were normalized with a square-root-transformation before applying ANOVA to test for population differences in R 3.4.4 [73]. 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 [74]. For the analyses using common-garden data, untransformed values were used.

Host plant toxicity and quality analyses

The cyanogenic content of the *Passiflora biflora* samples was analyzed using liquid chromatography-mass spectrometry (LC-MS/MS) with Agilent 1100 Series LC (Agilent Technologies, Germany) hyphenated to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, Germany) following the procedure of

Castro et al. [3](see S4 for details on sample extraction, spectrometry and analyses). Mass spectral data were analyzed with the native data analysis software (Compass DataAnalyses, Bruker Daltonics). The *P. biflora* CNglc compounds were detected and quantified following Castro et al. [3].

Plant quality traits representing nutritional values of the greenhouse-cultivated *P. biflora* treatment types were measured (average n = 63) with Dualex® Scientific+ leaf clip meter (Cerovic et al. 2012; S4). ANOVA including plant origin, treatment and their interaction was applied in R 3.4.4 [73] to test for differences in CNglcs and plant quality traits between the treatment groups.

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

The concentration of the two tested biosynthesized CNglcs, linamarin and lotaustralin were strongly correlated with each other (r = 0.897, P<0.001), and with the combined total concentration of biosynthesized CNglcs (r = 0.94 for Lotaustralin, r = 0.99 for Linamarin, P<0.001). Toxicity did not depend on adult mass in either sex (F = 0.52, P = 0.471 for total CNglcs concentration; mass data available for n = 196 individuals). Adult mass also did not differ between the feeding treatments (F=0.88, P= 0.480; model including feeding treatment, sex and their interaction), and was not included as a variable in further analyses.

Variance components of *Heliconius erato* biosynthesized cyanogenic toxicity were analyzed by fitting linear mixed models applying the "animal model" framework [76,77], in which the full pedigree (relatedness matrix) is incorporated as an individual-level random effect. The models were fitted using ASReml-R Version 4 [78], an R package that fits linear mixed models using Residual Maximum Likelihood (REML) estimation. The two-generation pedigree consisted of 20 broods and their parents, and included mother-offspring, father-offspring, full-sibling and paternal half-sibling relationships (n = 322 individuals, Table S2). Of the 20 broods, six were included in the feeding treatments receiving four different *P. biflora* types as larval diet (Table S2). Individuals of the parental generation were assumed to be unrelated. Because our breeding design resulted mostly in full-sib families, we have limited power to distinguish additive-genetic from other genetic variance components (dominance, epistasis, maternal/paternal effects). We thus consider our genetic-variance estimate as a 'broad-sense' genetic variance, although the estimate approaches

the narrow-sense estimate more than a simple full-sib design due to the parent-offspring and half-sib relations in the breeding design [79].

Animal models were fitted separately for the three biosynthesized cyanogenic toxicity traits (concentration of linamarin, lotaustralin and total CNglcs). The primary models (referred to as "Animal Model1") included the individual breeding value as a random effect, and the fixed effects of sex and feeding treatment (variance explained by fixed effects computed using the method of Villemereuil et al. [80]; see also S4). "Animal Model2" included additionally the random effect of maternal identity. We computed broad-sense heritabilities (H^2) as the proportion of the total phenotypic variance (V_P) explained by the (broad-sense) genetic variance component (V_G). When calculating heritabilities we included the variance due to sex in the total phenotypic variance, but we did not include the variance due to the treatment because this component of the variance is unlikely to represent natural variation [80].

The role of maternal effects in the evolution of cyanogenic toxicity was explored by estimating maternal-effect coefficients following Falconer´s dilution model, which assumes that offspring phenotype is a direct, linear function of maternal phenotype, beyond the contribution of maternally transmitted genes [55–57]. Thus, the interpretation of this 'maternal effect' is related to specific measured traits (here, cyanogenic toxicity), and is different from the typical formulation of 'maternal environmental effects' (influence of the maternal environment on offspring traits). We estimated maternal effect coefficients (m) as the difference in slope between a mother-offspring (mo) and a father-offspring (fo) regression, i.e. $m = \beta_{mo} - \beta_{fo}$ [55,56]. We fitted the parent-offspring regressions as linear mixed models with the *lme4* R package [81], including family as a random factor and maternal or paternal toxicity phenotype as explanatory variables of offspring toxicity phenotype.

Data availability

Datasets used in this study will be made available online at acceptance for publication.

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10. AUTHOR INFORMATION

Contributions

A.M., M.S. and C.J. conceived the study. A.M., M.S. and G.M.K. conducted fieldwork. A.M. conducted experimental work. A.M. and E.C. conducted laboratory work. A.M. and Ø.O. collated the dataset and conducted the analyses. A.M. and C.J. wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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11. SUPPLEMENTARY ITEMS

S1. Table S1: Full results table of REML animal models.

S2. Table S2: Summary table of *Heliconius erato* broods.

S3. Cyanogenic toxicity and overall quality of *Passiflora biflor*a host plant treatment groups.

S4. Supplementary Methods