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Preferential oviposition by Heliconiinae (Nymphalidae) butterflies on *Passiflora biflora* (Passifloraceae) leaves with higher cyanide concentrations

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ABSTRACT

Passiflora spp. produces cyanogenic glycosides to prevent herbivory. The butterfly subfamily Heliconiinae (Nymphalidae) has broken through this defense with the ability to ingest the cyanogenic compounds. A coevolutionary arms race of adaptations and counter-adaptations followed, in which it is believed that *Passiflora spp.* evolved a series of counter-adaptive defenses, like egg-mimics, leaf shape, and extrafloral nectarines, to specifically combat heliconiines. While sometimes overcoming these adaptations, heliconiines still consider them for oviposition. Additionally, the role of cyanide may also have an effect on oviposition. It has been suggested that while detrimental to larvae, cyanide provides protection that promotes oviposition. There are also numerous studies suggesting defensive and nutritional benefits of CN when Heliconiinae is able to sequester cyanogenic compounds. Many times there are trade-offs in the defenses of young leaves, which might suggest that cyanide indicates fewer defenses. This study examines the role of cyanide (CN) concentrations in *Passiflora biflora* on oviposition by Heliconiinae. Two studies were performed on cyanide preference. First, an analysis of cyanide concentration in similar leaves with and without eggs was conducted. Second, leaves had their cyanide concentrations artificially increased with CN/methanol extract and were then monitored for oviposition. When analyzing the cyanide concentrations of similar leaves with and without eggs, a trend of preferential oviposition on leaves of higher cyanide concentration was observed. There also seemed to be a two-fold difference, on average, between leaves with and without eggs, 0.50 μ g and 0.25 μ g CN respectively. The second study showed a preference for leaves with CN/methanol extract to leaves with extract alone and leaves with no alteration with eggs totaling nine, one, and one, respectively. This study suggests the importance of cyanide concentration for oviposition by heliconiines. This behavior might be explained by the protection provided from non-heliconiine herbivores or by possible nutritional and chemical benefits associated with the sequestration of CN. Additionally, if trade-offs are present, cyanide might indicate fewer non-cyanide defenses.

RESUMEN

Passiflora spp. produce glucósidos de cianuro para prevenir la herbivoría. La subfamilia de mariposas Heliconiinae (Nymphalidae) ha traspasado esta defensa con la habilidad de ingerir compuestos con cianuro. Una carrera armamentista coevolucionaria de adaptaciones y contra adaptaciones seguidas, en la cual se cree que *Passiflora spp.* evoluciona una serie de defensas adaptativas, como mimetismo de huevos, forma de la hoja, y nectarios extraflorales, para combatir específicamente a estas mariposas. Mientras algunas veces las mariposas sobrepasan estas adaptaciones considerando estas plantas para ovipositar. Adicionalmente, el rol del cianuro puede tener un efecto en la ovoposición. Se ha sugerido que en lugar de destruir la larva, el cianuro provee una protección impulsando la ovoposición. Existen también numerosos estudios que sugieren beneficios nutricionales y defensivos del CN cuando Heliconiinae es capaz de tomar compuestos cianogénicos. Algunas veces existen compensaciones en la defensa de las hojas jóvenes, lo que

puede indicar menos defensas. Este estudio examina el rol de concentraciones del cianuro (CN) en *Passiflora biflora* en la ovoposición por Heliconiinae. Dos estudios fueron realizados en la preferencia del cianuro. El primero, un análisis de la concentración de cianuro en hojas similares con y sin huevos. Segundo, se incrementó artificialmente la concentración de cianuro en hojas con extractos de CN/methanol que fueron monitoreadas para ver ovoposición. Cuando se analizó las concentraciones de cianuro en hojas similares con y sin huevos, existe una tendencia a preferir ovipositar en hojas con altas concentraciones de cianuro. También parece haber una diferencia, en promedio, entre hojas con y sin huevos en concentraciones de 0.50µg y 0.25µg CN respectivamente. El Segundo estudio muestra una preferencia por hojas con el extracto de CN/etanol sobre hojas con solo el extracto y hojas sin alteración, con un total de nueve, uno y uno huevos respectivamente. Este estudio sugiere que la importancia de las concentraciones de cianuro en la ovoposición por Heliconiinae. Este comportamiento puede ser explicado por la protección provista para herbívoros diferentes a estas mariposas o por posibles beneficios químicos y nutricionales asociados con la secuenciación del CN. Adicionalmente, si hay una compensación presente, el cianuro puede indicar menores defensas no relacionadas con el cianuro.

INTRODUCTION

Many plants have evolved secondary compounds to combat herbivory, including *Passiflora spp.* vines which produce noxious cyanogenic glycosides that are converted to HCN during consumption (Adersen and Adersen 1993; Benson *et al.* 1975; Levin 1976). This cyanogenic defense deters most herbivorous insects except for nymphalid butterflies in the subfamily Heliconiinae. Heliconiines have a similar cyanogenic defense against predators, and are able to synthesize the chemicals *de novo* (Engler *et al.* 2000; Feuillet 2004). These butterflies are able to ingest CN containing compounds, which has been suggested as a result of their defense against auto-toxicity (Benson *et al.* 1975). Thus, the Heliconiinae, unlike most herbivores, overcame the CN defense of *Passiflora spp.* It is widely believed that this, in turn, caused *Passiflora spp.* to evolve defenses solely against Heliconiinae – egg-mimics, hairs, leaf shape –, which heliconiines have sometimes overcome. The series of adaptations and counter-adaptations has been termed a coevolutionary arms race (Benson *et al.* 1975; DeVries 1987; Ehrlich and Raven 1964; Futuyma 1986; Turner 1981).

Female heliconiine butterflies consider many of the adaptations of *Passiflora spp.* when ovipositing. For example, they avoid leaves with eggs or egg-mimics, as heliconiine larvae are cannibalistic (Gilbert and Singer 1975). Additionally, they prefer young growth that is easier for small caterpillars to consume (Coley and Aide 1991). While it has been shown that egg mimics are able to deter females from oviposition, Heliconiinae has developed tremendous eyesight to distinguish the egg-mimics and have been known to probe them to test for authenticity (Benson *et al.* 1975; DeVries 1987; Williams and Gilbert 1981). Additionally, *Heliconius sara* will often oviposit in the presence of eggs or egg-mimics (Benson *et al.* 1975). *Passiflora spp.* has also changed its leaf shape in order to escape oviposition. Leaf shape is often irregular to avoid recognition and sometimes similar to leaves of common species to avoid detection (Benson *et al.* 1975). The female overcomes the changing leaf shape of *Passiflora spp.* because she has chemoreceptors on her forelegs (Benson *et al.* 1975), and the sensing of secondary compounds, like cyanogenic glycosides, is frequently used to recognize host plants when visual cues are unavailable (Ehrlich and Raven 1964).

Here, I investigated whether or not a female butterfly prefers to oviposit on leaves with higher cyanide concentration than those without. Leaves with more cyanide are less likely to be attacked by non-heliconiine herbivores, may offer a higher nutritional reward

as CN contains nitrogen, which is generally limiting in plant material, and provide precursors for the larval and/or adults own defenses. In addition, there might be trade-offs, in that more cyanide may mean fewer other defenses affecting oviposition. This is especially true in young leaves, which have not yet developed persistent mechanical defenses that compete against secondary compounds during production (Koricheva *et al.* 2004).

Higher cyanide concentration may deter potential competition and predation, offering protection to the larvae. It has been demonstrated that certain butterfly species, including heliconiine, oviposit preferentially for protection, even if it means forfeiting efficiency and nutrition (Karban and Agrawal 2002). For example, butterfly species *Pieris napi* (F. Pieridae) oviposits off its larval food plant to avoid parasitoid wasps (Ohsaki and Sato 1994). Additionally, the heliconiine species *Dryas iulia* has been observed ovipositing on adjacent plants, dead leaves, or other objects away from host plants to avoid predation by ants attracted by EFNs (Benson *et al.* 1975). Occasionally, higher cyanide concentrations have been shown to be detrimental to some species of heliconiine. For example, larval mortality of *Heliconius erato* increases significantly when fed high CN leaves (Hay-Roe and Nation 2007). So the question is why endure increased mortality for higher CN concentration. Cyanide provides protection against both competition against non-heliconiine herbivores and mortality from large generalist herbivores and omnivorous mammals. Many non-heliconiine herbivores cannot ingest CN and are unable to compete for larval food sources (Aderson and Aderson 1993; Benson *et al.* 1975; Levin 1976). Whereas many large herbivores and omnivorous mammals are able to metabolize small quantities of cyanogenic compounds, their bitter taste often deters ingestion (Gleadow and Woodrow 2002). Moreover, the absence of these animals might reduce direct harm on eggs or larvae, which could occur from the consumption of leaves.

A number of species in the Heliconiinae have developed the ability to sequester cyanogenic compounds for nutrition by exchanging the nitrogen with a thiol group, thereby releasing nitrogen for its own use while deactivating the noxious compound (Engler *et al.* 2000; Nishida 2002). In addition, several studies suggest sequestered CN can be used directly for chemical defense and that higher a sequestered concentration in the larval stage directly relates to the concentration in the adult stage (Engler *et al.* 2000; Gleadow and Woodrow 2002; Karban and Agrawal 2002; Nishida 2002). Moreover, if reduced *de novo* CN synthesis translates to increased energy, then sequestering CN might allow energy to be redirected to larval growth and development.

Therefore, I expect that female heliconiine butterflies will oviposit with greater frequency on leaves with higher cyanide concentrations to give their larvae access to more nitrogen for use in both growth and their own chemical protection. Additionally, if decreased larval growth and development from CN-related toxicity occurs, I still expect to find a higher frequency of oviposition due to the protective benefits that CN provides. However, if the detrimental effects lower frequency it would still indicate that CN concentration plays a significant role in oviposition. There is also the case as with *Heliconius erato favorinus* that neither suffers or benefits by CN (Hay-Roe and Nation 2007). If there is indeed a trade-off in *P. biflora* defenses, then higher CN concentrations might indicate to the butterfly that there are fewer defenses. Thus, a heliconiine like *H. erato favorinus* could potentially prefer a higher CN leaf.

MATERIALS AND METHODS

Study Site

Cyanide preference was analyzed using two studies. The first study compared concentrations of cyanide on similar leaves with and without eggs, and the second study increased the concentration of CN on leaves and recorded the frequency of oviposition. Both studies were performed at the butterfly-rearing garden owned by Amabelis Arguedas in Cementerio de Santa Elena de Monteverde, Costa Rica, at 1300 meters. The garden measured 18 x 24 x 8 m. Nearly a quarter of the area composed of *P. biflora* to rear heliconiine larvae.

Cyanide Concentration

P. biflora leaves were collected throughout the garden. Eggs found on leaves were from one of several possible heliconiine species, based on reports of their host plant specificity (Benson *et al.* 1975; DeVries 1987; Smiley 1978). Possible species included: *Heliconius sara fulgidas*, *Heliconius charithonia charithonia*, *Heliconius clysonymus montanus*, *Dryas iulia monerata*, and *Dione moneta poeyii*. When a leaf with an egg was located, the leaf was taken along with the nearest leaf of similar age and size. When possible, this leaf was taken from an adjacent vine, and when not possible, half of the time this leaf was the next youngest and half of the time this leaf was the next oldest. Age was determined by the distance from the meristem. If herbivore damage was found on either leaf the pair was excluded to keep paired weights and sizes approximately equal. The pairs were analyzed for cyanide concentration.

Whole leaf samples were first weighed and then crushed to determine cyanide concentration. Cyanide was detected using the Sodium Picrate Test (Seigler 1991) using three drops of toluene as the solvent. Each sample was allowed to react for ten minutes at room temperature (20-22°C). The resulting picrate filter paper was soaked in three mL of distilled water for 30 seconds and the water analyzed in a spectrophotometer (MRC, UV-200-RS) at a wavelength of 540 nm. Recorded percent transmittance was converted to absorbance, and the results were plotted against the standard curve in order to obtain concentration. The standard curve was created using serial dilutions of potassium cyanide and analyzed using the same conditions above, see sub-section: Standard Curve.

Statistical analysis performed employed the paired t-test for significance. Additionally, mean cyanide concentrations of leaves with eggs vs. without were analyzed.

Artificial Increase of Leaf Cyanide Concentration

Leaf cyanide concentrations were artificially increased. A cyanide leaf extract was made with 10.5 g of ground leaf material from *Passiflora* collected in the garden and 20 mL of methanol. Young leaves, described in this study as within the first five open leaves of the meristem, were used to ensure CN was present, as youngest leaves have the highest cyanide concentrations (Hay-Roe and Nation 2007; Webber and Woodrow 2008). A blank extract was made with only methanol. Leaves of *P. biflora* were tagged with a

small green mark, slightly darker than natural leaf color, in one of three locations, bottom-left edge, bottom-right edge, or petiole, indicating cyanide/methanol extract, extract alone, or control, respectively. Edge marks were green and short to make them unlike eggs or egg-mimics, thus not interfering with oviposition. Petiole marks were short and along the underside to avoid detection. Young leaves were painted with cyanide/methanol extract and extract alone using a small paintbrush. The entire upper side of the leaf was painted with extract, and shortly after application no noticeable difference was observed between painted and unpainted leaves. Painted and unpainted leaves were randomly distributed within small patches consisting of 50 to 100 leaves as well as on single vines throughout the garden. Leaves that had eggs were excluded. Two separate paintings were conducted, one at the beginning of the experiment and one eight days later. The leaves were monitored for oviposition everyday with eggs being removed when found. Leaves were recorded as either with or without eggs.

Standard Curve

The standard curve for cyanide concentration was created using serial dilutions of potassium cyanide starting at 100 $\mu\text{g}/\text{mL}$ and ending at 0.1 $\mu\text{g}/\text{mL}$. Dilutions were made with a micropipette. Technique was the same as leaf cyanide determination, but 100 μL of the potassium cyanide concentrations were tested in lieu of whole leaf. After obtaining readings from the spectrophotometer a curve of absorbance vs. concentration (μg cyanide) was constructed. A blank utilizing all methods without the addition of cyanide or plant material was used to zero the instrument before beginning daily analysis.

RESULTS

Cyanide Concentration

A total of 38 pairs of similar leaves with and without eggs were analyzed. The observed trend is that Heliconiinae butterflies oviposited more frequently on leaves with higher cyanide concentrations (paired t-test, $t = -2.43$, $df = 37$, $p = 0.02$). The average cyanide concentration for leaves of similar type with and without eggs were 0.50 $\mu\text{g}/\text{g}$ leaf material and 0.25 $\mu\text{g}/\text{g}$ leaf material respectively (figure 1).

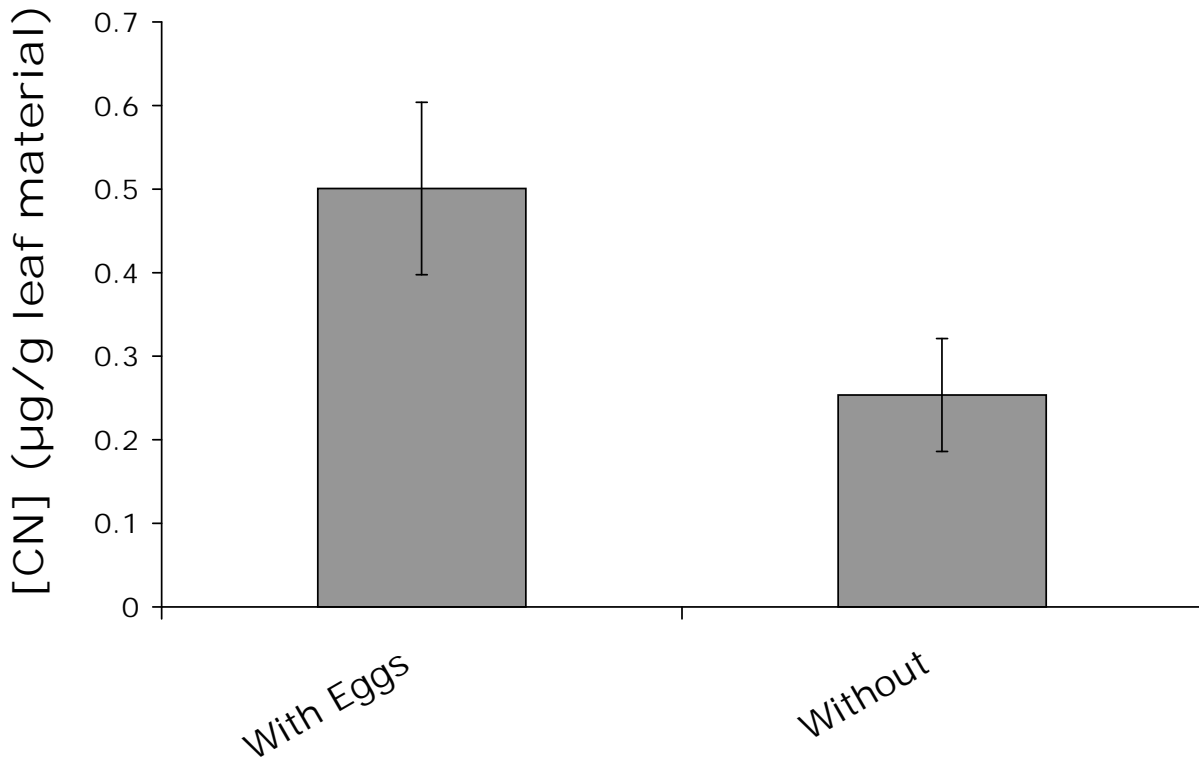


Figure 1. Average cyanide concentration for *P. biflora* leaves with and without heliconiine eggs. Standard error bars are shown. 38 pairs of similar leaves were tested for a total of 76 analyses. Leaves with eggs were found by inspecting vines in a butterfly-rearing garden; leaves without eggs were of similar age and size on the nearest vine or adjacent to leaves with eggs, alternating one leaf older or younger.

Cyanide Manipulation

237 leaves were monitored for oviposition. Of 101 leaves painted with cyanide/methanol extract, nine eggs were found. One egg was found on the 102 methanol alone leaves, and one egg was found on the 34 control leaves (Figure 2). There was a non-random distribution with more eggs found on the leaves with cyanide/methanol extract than expected, indicating preference (g-test, $g = 7.47$, $df = 2$, $p = 0.017$).

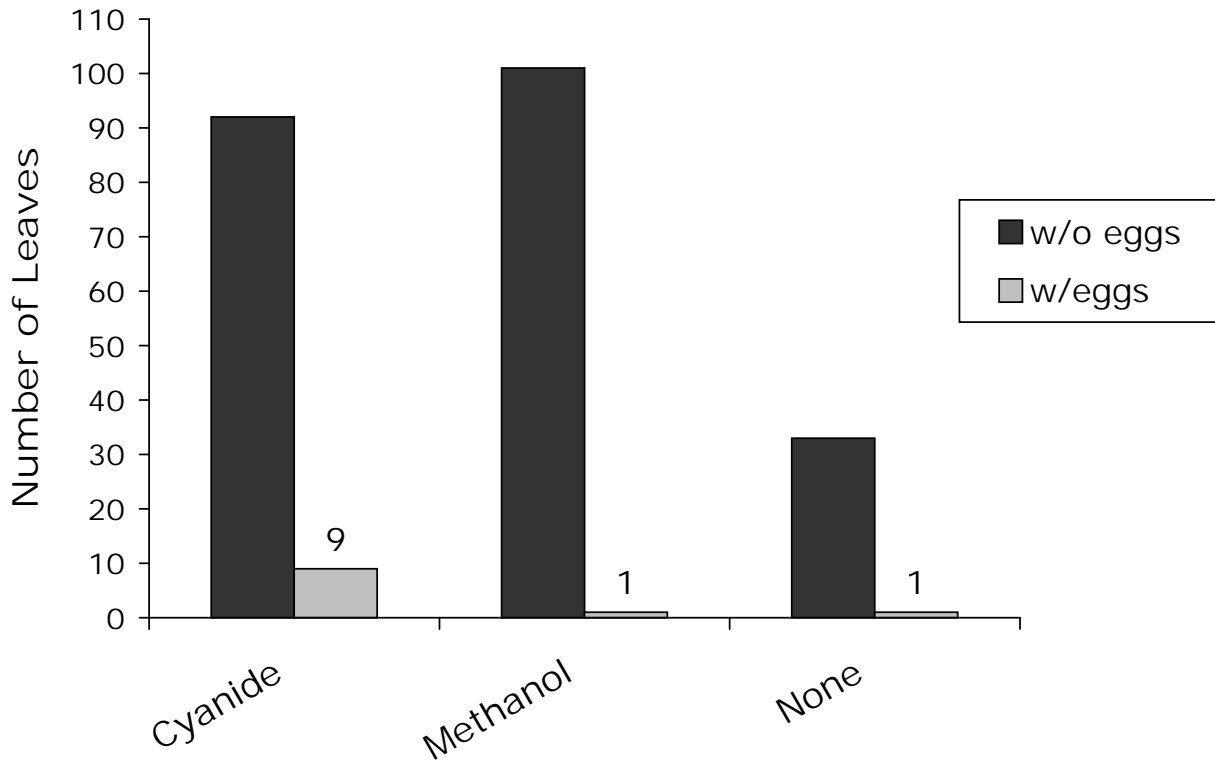


Figure 2. Frequency of heliconiine butterfly eggs on *P. biflora* leaves with cyanide/methanol extract, methanol extract alone, and control leaves with no alterations.

Standard Curve

The following standard curve (figure 3) was constructed to analyze percent transmittance data. There was a very high confidence in the accuracy of resulting function ($R^2 = 0.9805$) used to obtain cyanide concentrations from the recorded absorbencies.

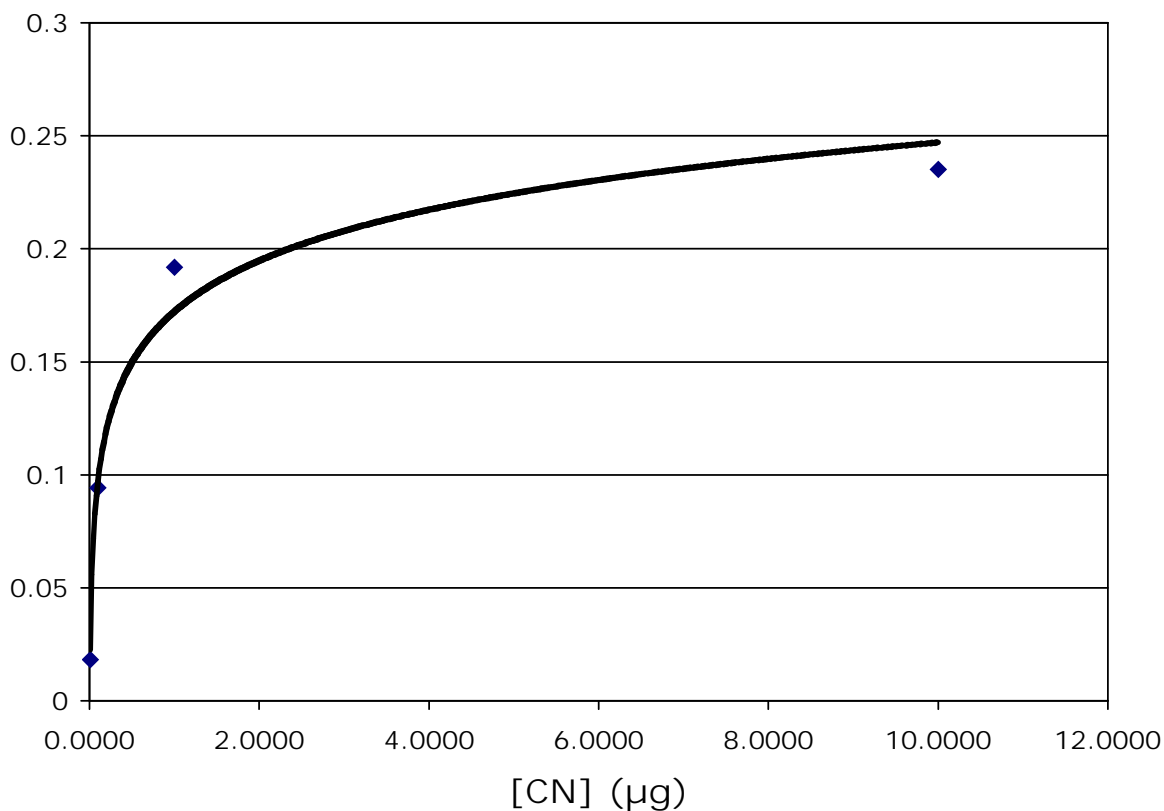


Figure 3. Cyanide concentration standard curve for testing *Passiflora* leaves ($y = 0.0325\ln(x) + 0.1722$). The standard was a solution of potassium cyanide.

DISCUSSION

This study shows that heliconiine butterflies preferentially oviposit on *P. biflora* leaves with higher cyanide concentrations. Unaltered *P. biflora* leaves with eggs had higher CN concentrations, on average, than similar and adjacent leaves. Likewise, leaves with artificially increased CN were chosen more often by ovipositing heliconiines. These trends indicate that heliconiine butterflies choose to lay eggs on more highly concentrated leaves.

Heliconiine butterflies seem to prefer leaves with more cyanide for oviposition. There are many benefits that might be associated with higher CN concentrations. Even if CN-related toxicity occurs in larvae, the added protection provided might still promote oviposition. Because cyanogenic glycosides contain nitrogen, which is often limiting in plant material, there are possible nutritional benefits connected with more CN.

Additionally, CN could provide the precursors to the heliconiine's own defense. Because of potential trade-offs, more cyanide might indicate fewer defenses, in which case Heliconiinae might oviposit more frequently even if there are no direct benefits.

One of the first benefits associated with higher CN is the added protection from herbivores that could possibly harm the larvae by food depletion or direct damage. Cyanide acts as a deterrent against most forms of non-heliconiine herbivory, including herbivory from omnivorous mammals (Adersen and Adersen 1993; Benson *et al.* 1975; Levin 1976). Many of these non-heliconiine herbivores are unable to ingest CN so they cannot compete for larval resources. Additionally, they are able to sense, or "smell", cyanide, which indicates unpalatable or dangerous leaves (Ehrlich and Raven 1964). Large herbivores and omnivorous mammals, which are able to ingest CN in small quantities, are deterred by the bitter taste of cyanogenic compounds (Gleadow and Woodrow 2002). Large herbivores can harm larvae and eggs by the removal of whole leaves during feeding. With higher cyanide concentrations, leaves might have a more prominent taste and "smell" thereby reducing non-heliconiine herbivory. However, some heliconiine larvae are vulnerable to CN-related toxins. Studies on *Heliconius erato* showed that ingestion of high levels of cyanogenic glycosides increases larval mortality (Hay-Roe and Nation 2007). Nevertheless, this may not deter females from ovipositing on leaves with high cyanide. In effect, this may substantiate the trend as numerous studies indicate that several species of butterfly, including those in Heliconiinae, forfeit nutrition and larval growth efficiency for protection (Benson *et al.* 1975; Karban and Agrawal 2002; Ohsaki and Sato 1994). Thus, it is possible that the butterflies in the garden benefit from the higher CN leaves and choose them for oviposition even if it causes them some harm.

Alongside added protection, the garden heliconiines could have chosen higher CN leaves for the purpose of nutrition or chemical defense. Although heliconiines are known for their *de novo* synthesis of CN (Engler *et al.* 2000; Feuillet 2004), at least some species have more recently been shown to sequester CN from their *Passiflora* host plants (Gleadow and Woodrow 2002; Karban and Agrawal 2002; Nishida 2002). If this is the case for the heliconiines in the garden, selecting high CN leaves may mean better chemical protection for the developing larvae. Further, if *de novo* synthesis is facultative, high CN leaves may save energy that can be diverted to growth. Engler *et al.* (2000) explored the effects of cyanide on *Heliconius sara* and discovered, for the first time, that it was able to metabolize the cyanogenic glycosides deactivating it while at the same time releasing nitrogen for nutritional use. While *H. sara* was not the only heliconiine present in the garden, the other species may also gain nutrition from CN. Thus, higher CN leaves may offer a leaf of higher nutritional value.

The use of plant-derived cyanogenic compounds by *Heliconiinae* creates an interesting paradox for host plants like *P. biflora*. *P. biflora* has attempted to deter heliconiines from oviposition through egg-mimics and extrafloral nectaries (EFNs), but research, however, has shown that heliconiines are not always deterred by these modifications (Benson *et al.* 1975; DeVries 1987; Feuillet 2004; Williams and Gilbert 1981). Furthermore, the young leaves of *Passiflora* vines are more susceptible to herbivory (Coley and Aide 1991) as well as more highly affected by trade-offs for defense. Young leaves have not yet developed persistent mechanical defenses, such as toughness, pubescence, or egg-mimics, all which compete against secondary compounds

for resources (Koricheva *et al.* 2004). Thus, young leaves with more cyanide might indicate fewer other defenses. This might explain the trend in the garden for higher CN preference, especially if the butterflies function like *H. erato*, as cyanide itself also benefits larval development. Nonetheless, there are butterflies that neither benefit nor suffer from CN in any studied concentration (Hay-Roe and Nation 2007). These butterflies might also prefer higher cyanide leaves given the possible reduced defenses associated with trade-offs.

However, some species of *Passiflora* have solved the problem of multiple defenses and limited energy pools. *P. adenopoda* has developed hooked trichomes, which provides an absolute defense against butterfly larvae (Gilbert 1971). Currently only three species of *Passiflora* have developed this modification and it has been suggested that this is the next step for *Passiflora*-Heliconiinae coevolution (Gilbert 1971). If hooked trichomes provide an absolute defense against damage from larvae, a trade-off would only need to occur between cyanide and pubescence as long as cyanide alone was powerful enough to deter other potential herbivores. Currently, though, most *Passiflora* vines do not have hooked trichomes and are unable to reduce CN as a defense because it is needed for non-heliconiine herbivory. Additionally, because *Passiflora* may always use CN as its main generalist herbivory defense, it will continue to play an important role in Heliconiinae oviposition.

Future Studies

Having seen trends in preferential oviposition on leaves with higher cyanide concentration (this study) and the deterrent effect of egg-mimics (DeVries 1987; Williams and Gilbert 1981), a suggested study would be the artificial increase in cyanide concentrations on leaves with egg-mimics via extract or other method in order to compel Heliconiinae to oviposit on leaves with egg-mimics rather than those without. This might help to better understand which deterrent impacts oviposition more. In addition, further studies could improve upon this study by utilizing known cyanide concentration manipulations in order to discover optimum cyanide concentrations for oviposition. A more telling approach to this study would be to monitor every leaf available for oviposition rather than small portions. If cyanide does play a significant role in oviposition, this study would possibly have more pronounced trends, as butterflies would almost always be faced with a choice between painted and unpainted leaves. In regards to the metabolic developments of *H. sara*, studies similar to Engler *et al.* (2000) should be performed on many species of Heliconiinae to further understand their metabolism as well as learn how common it is among this subfamily of butterflies.

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