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***Passiflora* (Passifloraceae) defenses against *Heliconius cydno* (Nymphalidae: Heliconiinae) oviposition**

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ABSTRACT

The purpose of this study was to determine the relative effectiveness of *Passiflora* defenses: elevated levels of cyanide, egg mimics, and variable leaf shapes, against oviposition from *Heliconius cydno*. The effectiveness of these defenses would reveal *H. cydno*'s preferences for oviposition sites and the primary criteria it uses when evaluating oviposition sites. Forty-two *Passiflora oerstedii* vines from the Monteverde Butterfly Garden, in Costa Rica, were divided into three treatments that added either cyanide, false eggs, or changed the shape of young leaves. Another twelve *Passiflora coccinea* vines were used in a second experiment that added false eggs to the young leaves, tendrils, and meristems. The results show trends that the various treatments deterred oviposition. Out of the 11 total eggs laid on leaves in the cyanide treatment, 82% of the total number of eggs was on the control leaves, then 9% on the methanol leaves and 9% on the methanol-cyanide leaves, indicating that the chemical cues from methanol discourage oviposition by *H. cydno* ($\chi^2 = 11.64$, $df = 2$, $p = 0.003$). For the vines with false eggs, out of a total of 19 eggs, 68% of the eggs were on the control leaves ($\chi^2 = 2.58$, $df = 1$, $p = 0.11$). Lastly, the control leaves of the modified leaf shape experiment had 73% of the total number of eggs, 15, while the modified leaves had 27% ($\chi^2 = 3.23$, $df = 1$, $p = 0.07$). These trends show that *H. cydno* likely relies on chemical cues more than visual cues when choosing oviposition sites. In addition, the lack of evidence for larval cannibalism in *H. cydno* and its willingness to oviposit on multiple *Passiflora* species demonstrate that egg mimics and variable leaf shapes are less significant factors in oviposition decisions.

RESUMEN

El propósito de este estudio fue determinar la efectividad de las defensas de *Passiflora*: altos niveles de cianuro, mimetismo de huevos y diferentes formas de hoja; contra la ovoposición de *Heliconius cydno*. La efectividad de estas defensas se demuestra por los sitios de ovoposición y el criterio que las mariposas toman para elegir los sitios de ovoposición. Cuarenta y dos lianas de *Passiflora oerstedii* del Jardín de Mariposas de Monteverde fueron divididas en tres tratamientos en los que se agregaron cianuro, huevos falsos, o se cambió la forma de la hoja. Otras doce plantas de la especie *P. coccinea* fueron usadas en la segunda parte del experimento en los que se agregaron huevos falsos a las hojas falsas, zarcillos y meristemas. Se muestra una tendencia a que varios tratamientos evitan la ovoposición. De los once tratamientos en los que se pusieron huevos, 82% fueron en las plantas que se pusieron huevos falsos, 9% en las hojas con metanol, 9% en las hojas con metanol-cianuro, indicando que las pistas químicas con metanol ahuyentan la ovoposición por parte de *H. cydno* ($\chi^2 = 11.64$, $df = 2$, $p = 0.003$). De las lianas con huevos falsos, de un total de 19, 68% de los huevos se depositaron en las hojas control ($\chi^2 = 2.58$, $df = 1$, $p = 0.11$). Por último las hojas control de la forma modificada experimentaron un 73% del número de huevos, 15, mientras que las hojas modificadas un 27% ($\chi^2 = 3.23$, $df = 1$, $p = 0.07$). Estas tendencias muestran que *H. cydno* utiliza más señales químicas que visuales cuando eligen los sitios de ovoposición. Además, la falta de evidencia de canibalismo larval en esta especie y la capacidad de ovopositar en varias especies de *Passiflora* demuestra que el mimetismo de huevos y la forma variable de las hojas son menos significativos a la hora de elegir los sitios de ovoposición.

INTRODUCTION

Coevolution is the phenomenon in which two or more species evolve traits in response to another species reciprocally (Futuyma and Slatkin 1983). The co-evolutionary arms race between

Heliconiine butterflies and Passifloraceae vines provides an excellent example of a series of coevolutionary responses (Gilbert, 1983). In spite of the many defenses that Passifloraceae species have evolved to inhibit herbivory, species of Heliconiine still find ways to recognize suitable *Passiflora sp.* on which to lay their eggs.

Many members of Passifloraceae have evolved toxic chemical compounds such as saponins, alkaloids, and phenolic and cyanogenic glycosides, which serve to harm herbivores and thus discourage them from eating these plants (Gibbs 1974, in Benson *et al.* 1975). Certain species have evolved toughened mature leaves to deter herbivory and sharp modified hooked leaf hairs to puncture eggs (Gilbert 1971). Since many members of Heliconiine have evolved to lay their eggs on tendril tips, some *Passiflora* have small tendrils on the meristem, causing eggs to easily fall off the plant, or lose the tendrils and take the eggs with them (Benson *et al.* 1975).

In addition, some *Passiflora* species have evolved glands on the leaves, stipules, or tendril tips that mimic the eggs of specific species of Heliconiine (Benson *et al.* 1975). Some *Passiflora* species' larvae may be cannibalistic (Benson *et al.* 1975). Thus, these egg mimics may discourage oviposition when the adult females lay eggs individually (Benson *et al.* 1975, Gilbert 1975). Morphologically, many Passifloraceae species have diverged greatly in leaf shape since Heliconiine species rely on vision to search for oviposition sites. Some species have even evolved to mimic leaf shapes of other groups of common tropical plants in an attempt to escape detection (Benson *et al.* 1975).

While the *Passiflora* vines have evolved elaborate defenses specifically against Heliconiine oviposition, Heliconiines have also evolved traits to counter these defenses and to continue using these plants. The well-developed vision and learning ability of these butterflies has aided in the evolution of strategies to overcome *Passiflora* defenses (Benson *et al.* 1975). They use chemoreceptors on their antennae, proboscis, and forelimbs to examine the suitability of the host plants for oviposition, including evaluating the authenticity of eggs already present on the plant. Even though chemical compounds synthesized by *Passiflora spp.* are meant to be deterrents, Heliconiines instead use them as recognition symbols, and the larvae and adults can use them to protect themselves against predators (Brower and Brower 1964 in Benson *et al.* 1975).

Previous studies have examined the independent impacts of different plant defenses against oviposition. When given a choice, various Heliconiine species, including *Heliconius sara fulgidas*, *Heliconius charithonia charithonia*, and *Dryas iulia monerata*, prefer to oviposit on leaves with higher concentrations of cyanide (Burkholder 2008). There are several possible reasons for this preference. The larvae could sequester the cyanide and use it for their own defense against predators or remove the nitrogen to simultaneously deactivate the compound and gain a limiting nutrient (Engler *et al.* 2000). In addition, since other herbivores cannot digest or dislike the bitter taste of cyanide, the eggs and larvae benefit from reduced herbivory on the plant and the reduced competition (Engler *et al.* 2000). The presence of egg mimics on *Passiflora* leaves reduces the frequency of oviposition on plant structures. The more closely the leaf glands mimic the Heliconiine eggs, the more effective they are at deterring oviposition on leaves, because adult females would not want to lose their eggs to cannibalism (Biermaier 2008).

While the individual effect of each plant defense is known, their relative effectiveness is not. This project aims to examine the relative effectiveness of cyanide, egg mimics, and leaf shape variability at deterring oviposition. Determining the relative effectiveness of these three plant defenses will elucidate how Heliconiine butterflies prioritize which factors are important in their oviposition site, and which sense they primarily use to help them decide where to lay their

eggs. They are expected to use chemoreception to smell or taste the cyanide on the leaves, both chemoreception and vision to detect the egg mimics, and vision to identify leaf shape (Gilbert and Singer 1975).

Secondly, since *H. cydno* is known to lay eggs on tendrils and meristems in addition to leaves, this study also asks if changes in the leaves will affect oviposition preference on the tendrils. In relation to that, a next logical step in the coevolutionary arms race between *H. cydno* and its host plants is for the host plants to evolve egg mimics on extra-foliar structures, such as tendril tips and meristems. I predicted that vines with false eggs on the leaves, tendril tips, and meristems will deter oviposition.

METHODS

Study One: Relative Effectiveness of Three Plant Defenses

This study was conducted at the Monteverde Butterfly Garden in Monteverde, Costa Rica for 16 days between April 18th and May 7th 2009. *Passiflora oerstedii* and *H. cydno* were studied in a garden enclosed in a mesh material that allows light, precipitation, and wind to penetrate. There were approximately 25 other butterfly species in the garden, but their eggs were laid on different host plants, looked different and/or were laid in groups. The garden represents a moist forest edge habitat at 1,300 meters above sea level. *H. cydno* lays small yellow eggs singly on the tendrils and leaves of a variety of *Passiflora* host plants (Smiley 1978; DeVries, 1987). It is important to use a butterfly species that lays eggs singly in order to test the hypothesis that single egg laying butterflies will be deterred from oviposition by eggs and egg mimics already present on leaves. *P. oerstedii* naturally does not have egg mimics, but it does have extra floral nectaries meant to attract ants to defend the plant against herbivory.

A total of 42 *P. oerstedii* were used in this study. Each vine was randomly divided into the three treatments: addition of cyanide, addition of egg mimics, or change in leaf shape. For each treatment leaf and corresponding tendril, the adjacent leaf and its corresponding tendril (either one above or below) were identified as the control. The relative positions of treatment leaves and tendrils compared to control leaves and tendrils alternated. For instance, if on one plant the first fully-opened leaf and tendril from the meristem were given the treatment, and the second leaf and tendril were the controls, on the next plant the first fully-opened leaf and tendril from the meristem were the controls and the second leaf and tendril received the treatment. Vines were monitored daily for the presence of eggs, and the number of eggs per leaf and tendril were recorded and removed. The eggs were collected daily at the same time. As the plants grew, older and tougher leaves and tendrils were retired and new leaves and tendrils were added.

Fourteen *P. oerstedii* vines were given artificially elevated levels of cyanide. The six youngest fully-opened leaves of each vine were used because *H. cydno* primarily lays eggs on the youngest leaves. There were two replicates of the control, methanol-treated, and methanol-cyanide-treated leaf per vine. Since young leaves have the highest cyanide concentrations, I finely cut up 8.92 grams of new leaf material (of *P. oerstedii*) and mixed it in 100 ml of methanol. Then I painted the leaf extract onto a set of 28 leaves using a small paintbrush. From here on, this treatment is called the cyanide-methanol treatment. A second treatment with only methanol was painted onto a second set of 28 leaves in order to account for the effects of adding any solution to the leaves. The treated leaves were marked on the underside of the leaves. On day eight, I did a second round during which I added leaf extract and methanol only onto the new

fully-opened leaves of each treated vine. I used 2.33 g of new leaf material in 25 ml of methanol for the cyanide-methanol treatment. To add artificial egg mimics to leaves, I used yellow paint pens to paint 1-2 small eggs in random spots on each treated leaf on fourteen vines (Figure 1). The four youngest fully-opened leaves of each vine were used in the experiment: two served as the controls and two had false eggs. On the last set of fourteen vines, I cut treatment leaves so that they had two lobes instead of one, thus changing their leaf shape (Figure 2). The four youngest fully-opened leaves of each vine were included in the experiment: two controls and two modified. The average new surface area was 92% of the original.



FIGURE 1 (left) An example leaf with a *H. cydno* painted false egg.

FIGURE 2 (right) An example leaf with modified shape.

Study Two: Egg Mimics on Vines

Twelve *Passiflora coccinea* vines and the same *H. cydno* population (in the same garden) at the Monteverde Butterfly Garden were used for this study for nine days between April 26th and May 7th 2009. *P. coccinea* naturally has extra floral nectarines and tending ants. I painted yellow egg mimics using a paint marker on the three youngest fully-opened leaves and tendril tips, and the developing leaf cluster on six of the vines. Each plant structure received one egg mimic. The other six served as the control. Each vine was randomly chosen as either the control or treatment.

Additional Observations

Every few days for a total of eight days, I put 2-3 recently collected *H. cydno* eggs into a container with 4-5 con-specific young larvae ranging in size from 0.5-2.5 cm long. In sum, I put in eight eggs into the larvae container. In addition, the larvae were placed on a young *P. oerstedii* vine kept in water. I checked for the presence or absence of the eggs in the larvae container daily. Since it usually takes about 8-10 days for eggs to hatch, if they were absent, they were considered to have been eaten or pushed off the leaf by the larvae.

Data Analysis

For daily counts of eggs on leaves and tendrils, the Wilcoxon sign-rank test was used. For total counts of eggs per treatment and control on the leaves of the first study and the vines of the second study, a chi-squared test was used. To analyze the total counts on the tendrils, I used the multinomial test for the cyanide treatment and the binomial test for the egg mimic and modified leaf shape treatments.

RESULTS

Study One

There were approximately 10-15 *H. cydno* individuals present in the garden. Unfortunately, less oviposition by *H. cydno* occurred than expected, despite relatively favorable weather conditions and availability of desired host plants. Little courtship and mating were observed and no new individuals of *H. cydno* were introduced into the garden during the study period.

In all, cyanide-methanol treated, methanol-treated and control leaves had a total of 11 eggs. For the cyanide treatment, the control leaves had 82% of the total number of eggs while the methanol and the methanol-cyanide leaves each had the same percentage of eggs, 9% (Figure 3). For the total number of eggs, there was a statistically significant difference between the control, and methanol and methanol-cyanide treatments ($\chi^2 = 11.64$, $df = 2$, $p = 0.003$), but no difference was detected between the leaves with methanol and methanol-cyanide. The control leaves had an average of 0.56 eggs per day (SD = 1.75; Figure 4). The average number of eggs per day on the methanol and methanol-cyanide leaves were the same, 0.06 (SD = 0.25; Figure 4). Thus the effect of methanol on the leaves cannot be eliminated, and the effect of increased levels of cyanide on the leaves cannot be tested (signed-rank = -3, $df = 15$, $p = 0.88$ between the control and methanol leaves, and signed-rank = 0, $df = 15$, $p = 0.50$ between the methanol and methanol-cyanide leaves).

For the vines with egg mimics, 68% of a total of 19 eggs was laid on the control leaves, and 32% of the eggs were laid on the leaves with false eggs ($\chi^2 = 2.58$, $df = 1$, $p = 0.11$; Figure 3). On a daily average, 0.81 eggs were laid on the control leaves, while an average of .38 eggs were laid on the experimental leaves, but this difference was not statistically significant (SD = 1.17 and 1.25, respectively, signed-rank = -7.5, $df = 15$, $p = 0.86$; Figure 4). For the modified leaf shape experiment, the control leaves had 73% of a total of 15 eggs while the leaves with a modified shape had 27% of the eggs ($\chi^2 = 3.23$, $df = 1$, $p = 0.07$; Figure 3). The control leaves had an average of 0.69 eggs per day, and the treatment leaves had an average of 0.25 vines per day (SD = 1.74 and 0.44 respectively, signed-rank = -6, $df = 15$, $p = 0.72$; Figure 4). Although these differences between the control and treatment leaves in the total number of eggs and average daily number of eggs were not statistically significant, the p value was close to 0.05, meaning that it is very likely that a trend exists.

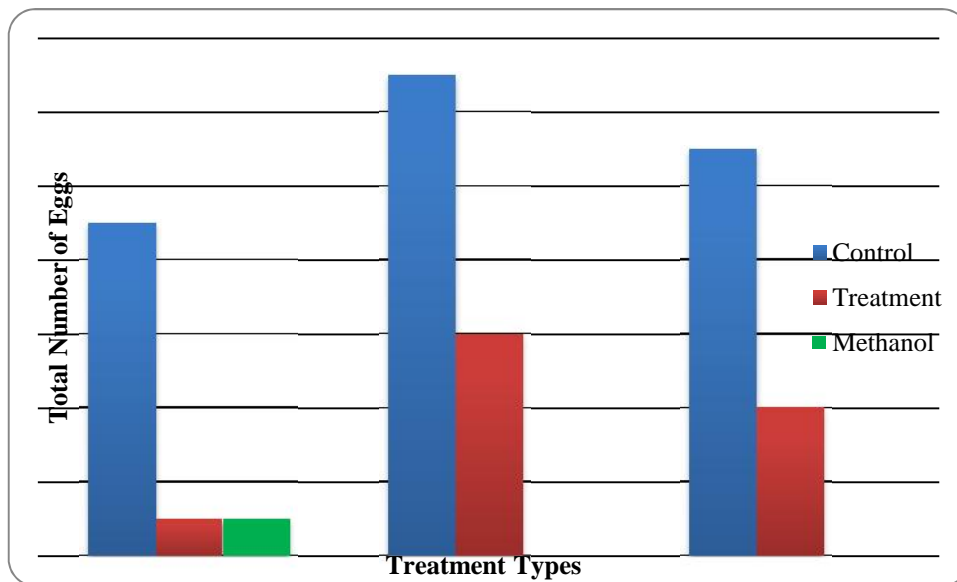


FIGURE 3 Methanol, leaf extract (here denoted as cyanide), egg mimics, and modified leaf shape on *P. oerstedii* negatively affect oviposition by *H. cydno* on those leaves. However, for the cyanide treatment, since there is no difference between the methanol leaves and the leaves with methanol and cyanide, then the effect of methanol cannot be ruled out.

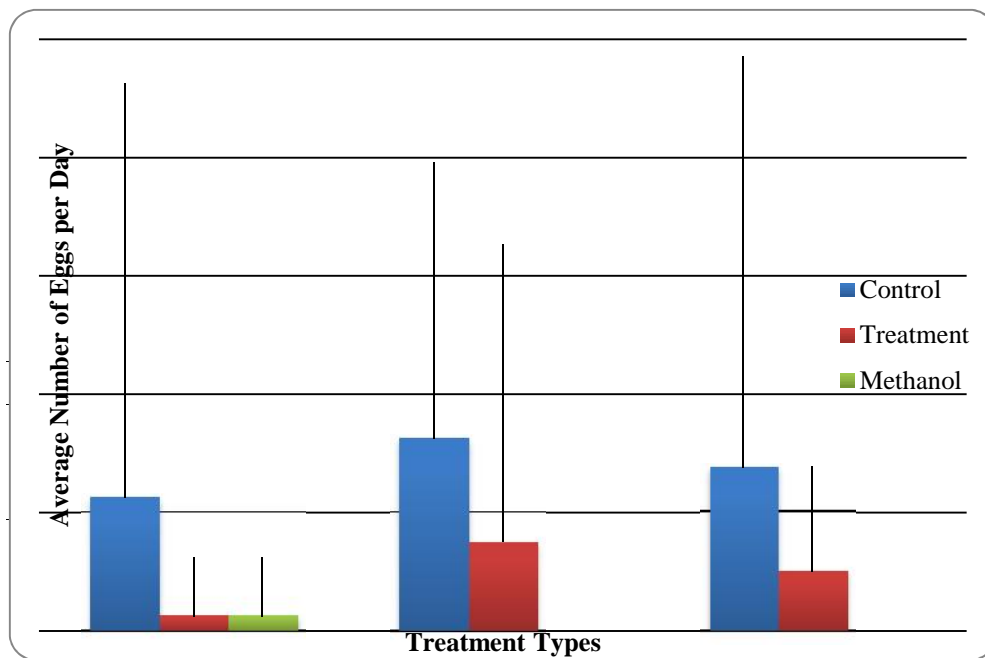


FIGURE 4 This shows trends between the control and treatment leaves of the three treatments showing that the cyanide, egg mimics, and modified leaf shapes inhibit oviposition by *H. cydno* on *P. oerstedii*, but do not completely prevent it.

For the corresponding tendrils, of the vines in the cyanide treatment, the control tendrils had 68% of a total of nine eggs, while the methanol tendrils had 22%, and the tendrils with methanol and cyanide had 11% ($\chi^2 = 4.67$, $df = 2$, $p = 0.10$; Figure 5). As for the daily averages, the control tendrils had 0.75 eggs, the methanol tendrils had 0.25 eggs, and the methanol-cyanide tendrils had 0.125 eggs (SD = 0.88, 0.35, and 0.46 respectively; Figure 6). The control tendrils of the egg mimic experiment had 75% of a total of 16 eggs and the treatment tendrils had 25% of the eggs ($\chi^2 = 4.00$, $df = 1$, $p = 0.05$; Figure 5). The control tendrils averaged 1.5 eggs per day while the experimental tendrils averaged 0.5 eggs per day (SD = 2.07 and 0.53 respectively; Figure 6). For the variable leaf shape experiment, the control tendrils had 86% of a total of seven eggs while the treatment tendrils had 14% of the eggs ($\chi^2 = 3.57$, $df = 1$, $p = 0.06$; Figure 5). Lastly, the daily average for the control tendrils was 0.75 eggs and the daily average for the modified leaf shape tendrils was 0.125 eggs (SD = 0.89 and 0.35 respectively; Figure 6).

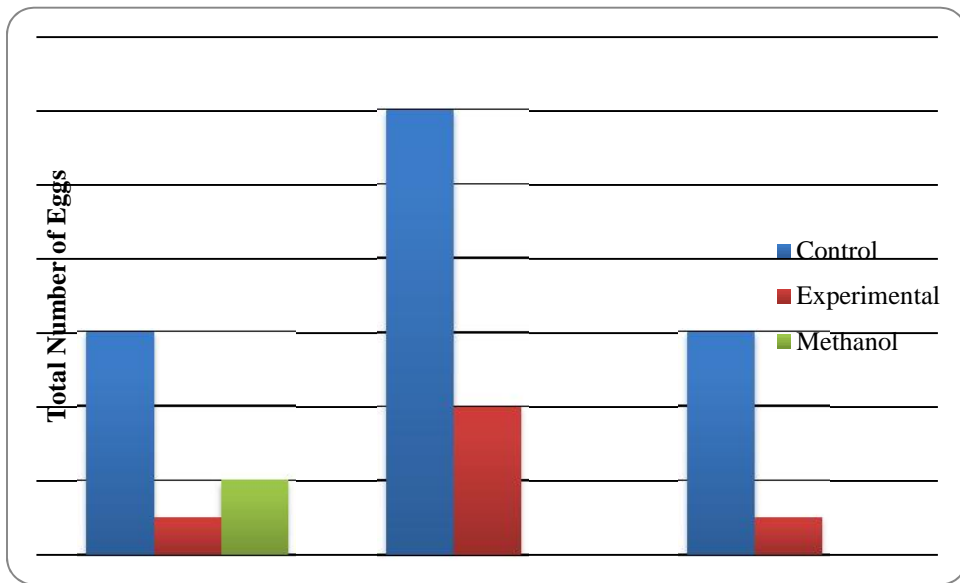


FIGURE 5 The tendrils of *P. oerstedii* show strong trends that the three treatments: cyanide, egg mimics, and different leaf shape on leaves deter oviposition by *H. cydno* on tendrils.

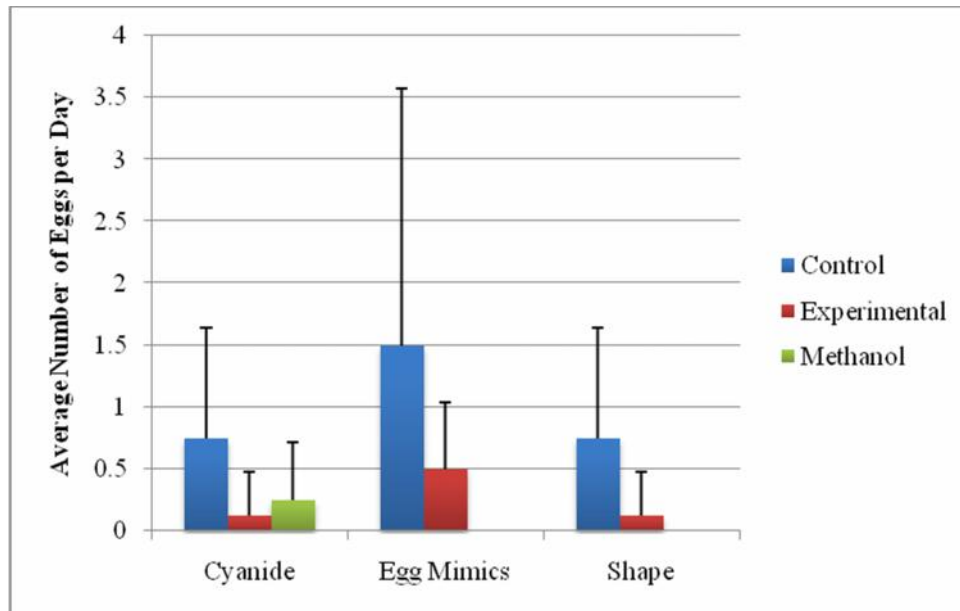


FIGURE 6 This graph shows that there are significant trends between the controls and treatments of each treatment group of the *P. oerstedii* tendrils: cyanide, egg mimics, and different leaf shape. The three treatments deterred oviposition by *H. cydno*, but at similar levels of effectiveness.

Study Two

Of the total 38 eggs that were laid on these vines, the control vines had 58% while the experimental vines had 42% ($\chi^2 = 0.94$, $df = 1$, $p = 0.33$; Figure 7). There was an average of 2.44 eggs per day on the control vines and an average of 1.78 eggs per day on the experimental vines ($SD = 1.01$ and 1.78 respectively; signed-rank = -1.5 , $df = 8$, $p = 0.54$; Figure 8). Because the p values are so high, only a weak trend that the vines with egg mimics deterred oviposition existed.

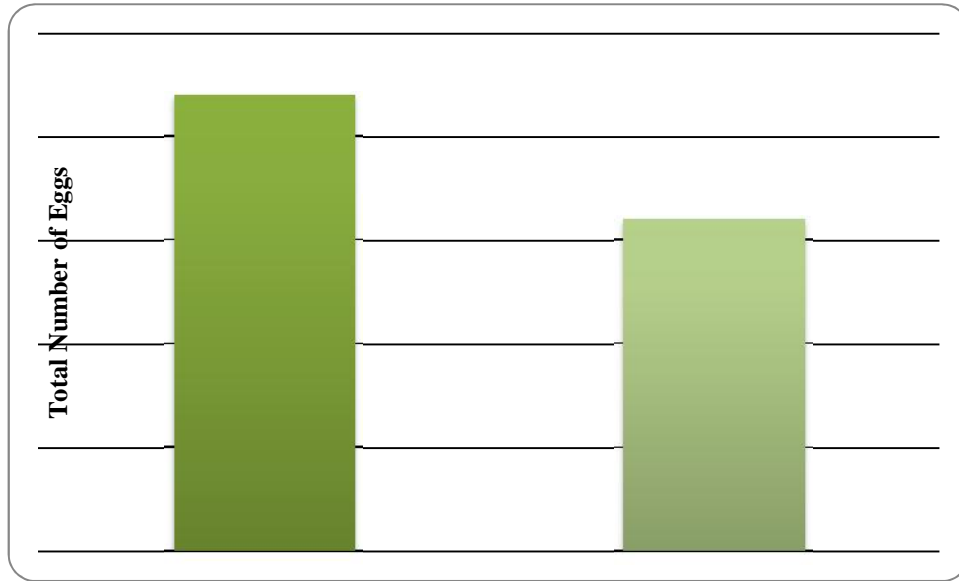


FIGURE 7 There is a trend showing that the presence of egg mimics on the leaves, tendrils, and meristems on *P. coccinea* somewhat deter oviposition by *H. cydno*.

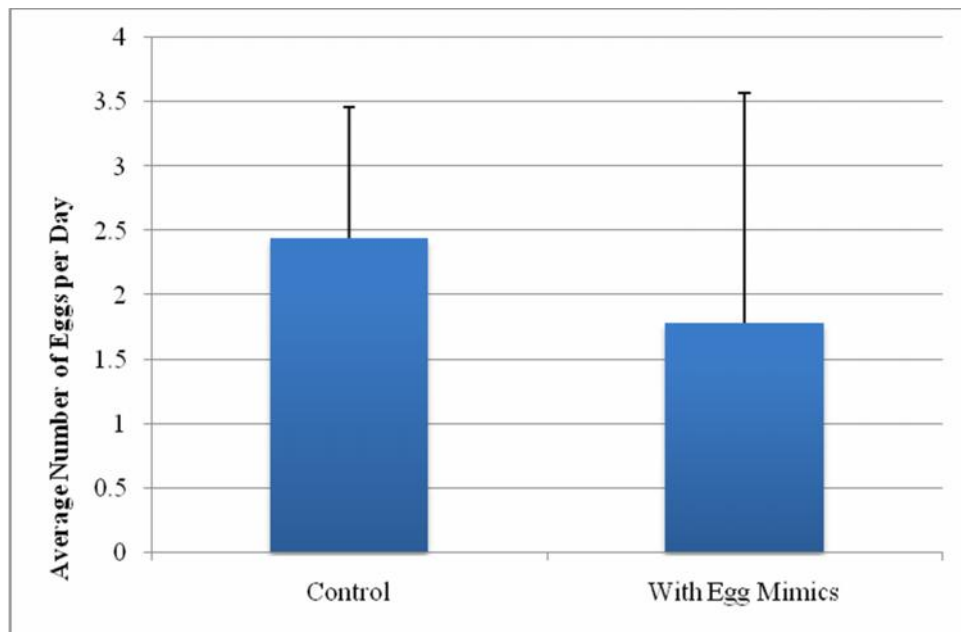


FIGURE 8 The addition of egg mimics on the leaves, tendrils, and meristems to *P. coccinea* vines weakly inhibits oviposition by *H. cydno*.

Additional Observations

The larvae did not eat the recently collected eggs. Every day after I put the new eggs into the container, I found them in their original positions. The 4-5 larvae were observed eating only plant material. At the end of the eight day study period, the eggs were present and remained

unhatched. In addition, while both *P. oerstedii* and *P. coccinea* have extra floral nectaries, only *P. coccinea* was observed to have ants tending the vines.

DISCUSSION

The first study shows that *H. cydno* responds to chemical cues from the cyanide treatment more than visual cues from the egg mimics and variable leaf shape experiments when evaluating oviposition sites. The statistically significant difference between the control, and the two sets of treated leaves, indicates that the *H. cydno* butterflies are able to detect the addition of methanol. Given that there is no visual difference between the control and methanol leaves after the methanol dried, it is deduced that they use chemoreception to recognize the methanol. Therefore, the smell or taste of the methanol is a strong deterrent against *H. cydno* oviposition. Next, the ratios of the numbers of eggs on the control to treatment leaves of the egg mimic and variable leaf shape experiments of the first study are very similar (Figures 3 and 4), thus in this study, it is not possible to determine which of these two leaf characteristics is a better deterrent against *H. cydno* oviposition nor which host plant characteristic, egg mimics or leaf shape, *H. cydno* relies on more when choosing oviposition sites.

The results from the eggs laid on tendrils support the results from the corresponding leaves: that the three treatments deter *H. cydno* from oviposition. Again, the negligible difference between the methanol and the methanol-cyanide leaves of the cyanide treatment indicates that the effect of elevated cyanide levels only is not testable. Many species of *Heliconius* lay on new plant growth and tendril tips to avoid predaceous ants that tend the vine (Turner 1981). However, once the larvae are too large, they will eventually have to move down to the adjacent leaf to feed, thus leaf features for these *Passiflora* spp. are still an important factor in discouraging oviposition. Although these eggs are laid on tendrils, the adult females are still deterred from laying eggs near leaves that have methanol, methanol and cyanide, egg mimics, or a different leaf shape.

A comparison of the cyanide treatment versus the egg mimic and variable leaf shape treatments show that *H. cydno* likely relies more heavily on chemical cues than visual cues when choosing oviposition sites. The results from the second study on *P. coccinea* support this hypothesis. False eggs on leaves, tendril tips, and meristems only mildly deterred oviposition from those vines. While a different leaf shape and the presence of false eggs somewhat reduce oviposition, they do not completely stop it. Possibly, the high leaf shape variation among Passifloraceae species has led *H. cydno* to not heavily rely on leaf shape as an indicator of suitable host plants. Unlike many other *Heliconius* species, *H. cydno* can lay eggs on a variety of *Passiflora* host plants, each of which has a different leaf shape. Examples include *Passiflora vitifolia*, *Passiflora biflora*, *Passiflora oerstedii*, *Passiflora coccinea*, and *Passiflora cyanea*, whose leaves have one to three lobes (Corrales 1996; DeVries 1987). Another study on *H. cydno* showed that although the presence of egg mimics increases the search time for good oviposition sites and reduces oviposition frequency, they do not completely prevent it from occurring on plant structures with egg mimics (Williams and Gilbert 1981). Perhaps in this species, the presence of eggs on leaves or other plant structures are not as important because the larvae are not highly cannibalistic. My observations of the non-cannibalistic larvae support this hypothesis. Ovipositing females that either cannot detect the presence of false eggs or can successfully determine the difference between real and false eggs are rewarded, because their larvae hatch to find themselves on healthy leaves and plant material.

In the future, a longer study with a larger population of ovipositing *H. cydno* would provide more conclusive evidence for *H. cydno*'s preference for oviposition sites and the criteria it primarily uses to evaluate oviposition sites. Furthermore, Passifloraceae species have evolved many other defenses, including extra floral nectaries and trichomes that are also meant to deter oviposition. Testing the relative effectiveness of these defenses in addition to the ones previously studied would add to our understanding of how *Heliconiine* species respond to their host plants. It would also be interesting to determine if the mere presence of extra floral nectaries on a *Passiflora* species but absence of tending ants changes *Heliconius* oviposition behavior in comparison to a *Passiflora* species that has both extra floral nectaries and tending ants.

While this study did not directly answer the initial question of which plant defense is most effective at deterring oviposition, it did reveal several conclusions. *H. cydno* uses its chemoreception more than vision when choosing oviposition sites. This species' willingness to oviposit on multiple host plants and the absence of larval cannibalism relaxes the selection for this species to use leaf shape and presence of eggs as important indicators for desirable oviposition sites. Furthermore, leaf characteristics affect which tendrils *H. cydno* chooses for oviposition. Since this species relies more on chemoreception than vision, this has important implications for its coevolutionary arms race with Passifloraceae plants. Plant species that evolve chemical indications of undesirable oviposition sites are likely to be more effective at deterring oviposition from *H. cydno* than species that evolve visual cues. Changes in leaf characteristics will also affect *Heliconiine* species such as *H. cydno* that evolved to lay their eggs on tendrils.

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