The effects of qualitative and quantitative variation of aristolochic acids on preference and performance of a generalist herbivore

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Abstract
Nearly all plants possess chemicals that are inferred to play a role in anti-herbivore defense or resistance. The effects of various chemical defenses can vary among herbivores. Often, plant defensive compounds are examined in broad, inclusive categories, with an emphasis on total quantity, which might ignore qualitative variation in activity. Aristolochic acids are alkaloids characteristic of plants of the genus Aristolochia (Aristolochiaceae). Although aristolochic acids have been documented as effective herbivore deterrents, it remains unknown whether different kinds of aristolochic acid vary in their efficacy as defense against herbivores. We manipulated the aristolochic acid content of artificial diet to examine the effects of four aristolochic acids on larval preference and performance of the generalist herbivore Spodoptera exigua Hübner (Lepidoptera: Noctuidae). Using choice tests, we observed that the four aristolochic acids tested varied in their deterrent effectiveness, with AA-I having the strongest effect and AA-II having the weakest effect. No-choice tests were used to examine larval performance. The effect on performance varied among the aristolochic acids tested. Higher concentrations of aristolochic acid were generally associated with reduced larval developmental rate and larger size at pupation. These results indicate that various forms of aristolochic acid can vary in their effect on herbivores and that simple aggregate measures of total concentration might not reflect the chemical defensive phenotype of the plant.

Introduction
Nearly all plants possess at least some chemical compounds that putatively serve a defensive function against herbivores (Berenbaum, 1995; Mithöfer & Boland, 2012). The effectiveness of these compounds can vary depending on ecological conditions, such as in the presence of herbivore natural enemies (Price et al., 1980; Clancy & Price, 1987; Williams, 1999; Ode, 2006), or the developmental stage of the herbivore (Zalucki et al., 2002). Plant defensive chemistry is often examined in categories of varying inclusiveness (Mithöfer & Boland, 2012). For example, non-amino acid-based, nitrogen-containing compounds, can broadly be defined as alkaloids. Alkaloids can further be divided into classes of compounds with similar structure, and ultimately chemical species within a class can be considered. Historical development of theory has largely focused on more broadly defined chemical classification. For example, optimal defense theory was developed to explain intra-plant variation in defensive compounds (McCall & Fordyce, 2010). Similarly, chemical similarity among taxonomically related plant groups is believed to play an important role in herbivore host plant breadth (Ehrlich & Raven, 1964; Miller & Feeny, 1989; Janz & Nylin, 1998; Fordyce, 2010). What is less known, however, is how different compounds within a chemical class vary in their influence on herbivore performance, although this is receiving increasing attention (e.g., Theis & Lerdau, 2003; Macel et al., 2005; Gershenzon & Dudareva, 2007).

The most frequently used metric of chemical defense allocation in plants is the pooled sum of all compounds within a target group of compounds, providing a quantitative measure of plant chemistry (e.g., total cardenolides, total iridoid glycosides, total aristolochic acids). Quantitative measures provide a coarse-grain estimate where covariation between total chemical defense and herbivore preference and performance can be examined. An
important assumption of such an approach is that the biological effect of each compound is additive. There are, however, examples of synergistic effects among compounds on herbivore performance (Hay et al., 1994; Richards et al., 2012). Furthermore, it is now known for some systems that different compounds within a chemical class can vary in their effectiveness against herbivores (Zangerl & Berenbaum, 2003; Gershenzon & Dudareva, 2007) and that such qualitative variation can occur among conspecifics and among tissues of the same plant (McCall & Fordyce, 2010; Kleine & Müller, 2011). Thus, understanding the role of qualitative variation in chemical defense might provide a more biologically meaningful metric of defense allocation and host plant defense or susceptibility.

This study examined the effect of qualitative and quantitative variation of aristolochic acids on a model herbivore, Spodoptera exigua Hübner (Lepidoptera: Noctuidae). Aristolochic acids are nitrophanthrene carboxylic acids unique to plants in the Aristolochiaceae (Chen & Zhu, 1987). Previous work has shown that aristolochic acids can effectively deter non-specialist herbivores (Chen & Zhu, 1987; Lajide et al., 1993a,b; Park et al., 1997; Glendinning et al., 2001) and that sequestered aristolochic acids can protect specialist herbivores from natural enemy attack (Brower, et al., 1958; Jeffords et al., 1979; Fordyce, 2000, 2001; Sime et al., 2000; Sime, 2002; Fordyce & Nice, 2008). However, whether the deterrent efficacies of aristolochic acids vary among different forms of the compound is largely unexplored (Lajide et al., 1993b), despite the fact that multiple forms of aristolochic acid are usually found in organisms containing aristolochic acid. Here, we examine the effects of four aristolochic acids (AA-I, AA-II, AA-7-OH, and AA-C) on the behavior and performance of S. exigua. Specifically, we ask: (1) do different aristolochic acids vary in their deterrent efficacy? (2) Is there evidence of synergistic effects between aristolochic acids? And (3) does herbivore performance vary across aristolochic acids?

Materials and methods

Rearing and diet

Spodoptera exigua eggs were obtained from Benzon Research (Carlisle, PA, USA). Neonate S. exigua were placed into 60-ml plastic cups with sealable lids. An artificial, agar-based, Spodoptera diet was purchased from Southland Products (Lake Village, AR, USA) and prepared according to the manufacturer’s instructions. Aristolochic acid content of the diet was manipulated by adding 2 ml of a 95% ethanol solution containing aristolochic acids to the 100 ml of diet while it was still in solution (at ca. 95 °C). The ethanol boiled off, leaving the aristolochic acid in the diet. Control diets received 2 ml of 95% ethanol without aristolochic acid. Aristolochic acid remains stable under this protocol, and the presence of the desired aristolochic acid content was confirmed using high-performance liquid chromatography following previously established methods (Fordyce, 2001; Fordyce & Nice, 2008).

Preference test

To examine the effects of qualitative and quantitative variation of aristolochic acids on preference, we conducted choice tests for each of the four types of aristolochic acid (AA-I, AA-II, AA-7-OH, and AA-C). Each aristolochic acid was independently tested at four concentrations. For each choice test, 50 groups of ca. 10 neonate S. exigua larvae were placed into 60-ml plastic cups with four equal-sized disks of artificial diet cut with a 9-mm cork borer. Each disk had a different concentration [0, 0.01, 0.02, and 0.04 μg mg⁻¹ dry weight (dw)] of a particular aristolochic acid. To test for synergistic effects between AA-I and AA-II, the two aristolochic acids commonly found in many Aristolochia species, larvae were presented with the following four diets: control, AA-I (0.05 μg mg⁻¹ dw), AA-II (0.05 μg mg⁻¹ dw), and a 1:1 mixture of AA-I and AA-II, each at 0.025 μg mg⁻¹ dw). At four 24-h intervals, the observed feeding location of larva was recorded; these observed feeding locations were then summed across four observations (144 h total). We used these observations as a measure of preference at the level of the arena.

We used a hierarchical Bayesian model to estimate preference for each of the diets provided in the choice tests (Fordyce et al., 2011). This approach models the preference within each replicate choice arena as a multinomial distribution with unique parameter values that describe the preference within that arena. We assume that the vector of parameters for each multinomial distribution is drawn from a Dirichlet distribution, the continuous analog to the multinomial distribution, which describes the overall preference across all replicates. Parameter estimation for overall preference for each diet was done using 9 000 Markov chain Monte Carlo (MCMC) generations following a burn in of 1 000 generations. At each step in the MCMC, arena-level preferences (multinomial distributions) inform the overall preference (Dirichlet distribution) and, in turn, the overall-level preferences inform the probability of each arena-level preference. In the end, the Dirichlet parameters are interpreted as an estimate of preference for each of the provided diet items. This approach has the advantage that the parameter describing preference is directly estimated from the data. Thus, the posterior distributions can be interpreted as characterizing the probability distribution that caterpillars in an arena will choose a particular diet.
We used the Deviance Information Criterion (DIC) to compare the fit of the full model, one where each of the possible diet choices has a unique preference parameter value, against a reduced model, where the preference for each diet type is identical. DIC is analogous to Akaike Information Criterion, where the model with the lowest score best fits the data, and is well suited for model comparison in a Bayesian framework when posterior distributions are approximated using MCMC (Spiegelhalter et al., 2002). We further used DIC to compare models where preferences among the concentrations of the various aristolochic acids were compared. That is, we compared whether the preference hierarchy across aristolochic acid concentrations is best modeled as sharing a common vector of preference parameters, or whether they are best modeled as having different preference parameters for each aristolochic acid concentration. The hierarchical Bayesian analysis was implemented using the package BA-YESPREF 1.0 (Fordyce et al., 2011) in the R statistical computing environment (R Development Core Team, 2011).

In addition to directly estimating the posterior distribution for preference among diet choices, we also employed the familiar Quade test for statistical hypothesis testing of differences among diets. The Quade test is a rank-based method that is analogous to a blocked ANOVA with no replication within blocks (Conover, 1999). It is similar to the more familiar Friedman test, except it also ranks and weights each replicate upon the amount of information provided. The Quade test was implemented in R using code written by the authors and post-hoc comparisons followed Conover (1999).

**Performance test**

To examine the effects of AA-I, AA-II, AA-7-OH, and AA-C on *S. exigua* performance, no-choice rearing tests were conducted on diets containing each aristolochic acid. Each experiment consisted of individual larvae placed in 60-ml plastic cups with diet provided ad libitum. Diet treatments consisted of artificial diet containing 0, 0.01, 0.02, or 0.04 µg AA mg⁻¹ diet dw. Each diet treatment was replicated 20 times for each experiment. Three response variables were examined as a measure of larval performance on the diets: days to first molt, days to pupation, and pupal weight. Days to first molt and days to pupation were analyzed using a Kruskal–Wallis test following Conover (1999) in the R statistical computing environment using code written by the authors. Pupal weight was analyzed using ANOVA with AA concentration, sex, and their interaction as factors. Sex determination of pupae followed Scoble (1992). ANOVA analyses were performed in the R statistical computing environment using marginal sum of squares (type III sum of squares) in the package CAR (v1.2-16) (Fox & Weisberg, 2011).

**Results**

**Preference test**

The strength of preference for each concentration of aristolochic acid varied among each of the aristolochic acids tested. In general, higher concentrations of aristolochic acid were avoided in favor of control diets. AA-I was the strongest deterrent, avoided at all concentrations greater than zero (Figure 1). Interestingly, medium concentrations of AA-II (0.02 µg mg⁻¹ dw) apparently had the strongest deterrent effect on larval preference (Figure 1). The lowest concentration of AA-C (0.01 µg mg⁻¹ dw) failed to show a deterrent effect on larval preference, though it was effective at the higher concentrations. For each of the experiments, models allowing preference among concentrations of aristolochic acid to vary (full model) were favored over models that restrict preference to be the same for all aristolochic acid concentrations (reduced model) (Table 1). Modeling the strength of preferences among possible choices for each aristolochic acid separately (DIC = 1 644.99) was a better fit to the data compared to various models that grouped preferences among the aristolochic acids tested (minimum ADIC = 17.39), indicating variation in deterrent effectiveness among aristolochic acids. Inferences based upon examination of the posterior distributions for preference were consistent with the inferences drawn from the Quade tests (Figure 1). There was no obvious difference in preference among the AA-I, AA-II, and 1:1 mixture diets, and larvae consistently preferred the control diet (Figure 2).

**Performance test**

Twenty individual *S. exigua* were raised on each concentration (0, 0.01, 0.02, 0.04 µg mg⁻¹) of each aristolochic acid (AA-I, AA-II, AA-7-OH, AA-C) to examine how quantitative and qualitative variation in this group of compounds affects larval performance. Overall, developmental rate of first instars was reduced when reared on higher concentrations of aristolochic acid-containing diet, with only AA-C not showing a statistically significant trend (Figure 3). We failed to detect an effect of aristolochic acid concentration on days to pupation; AA-I and AA-7-OH were marginally significant at α = 0.05 (T = 4.43, P = 0.092 and T = 4.66, P = 0.084, respectively), whereas AA-II and AA-C were not (T = 1.51, P = 0.23 and T = 0.538, P = 0.22, respectively). There was, however, a positive correlation between days to first molt (where an effect of AA concentration was detected for all AAs, except AA-C) and days to pupation (Spearman correlation:
Figure 1 Estimates of Spodoptera exigua preference parameters for diet content across four aristolochic acids (AAs). Dots indicate the median of the posterior distribution for each preference parameter, bars indicate 95% credibility intervals. Concentration of AA is indicated by H (high, 0.04), M (medium, 0.02), L (low, 0.01), and C (control, 0 μg mg⁻¹ dw). Different letters at the bottom indicate significant differences (α = 0.05) among concentrations of each AA, based upon post-hoc multiple comparisons for the Quade test as recommended by Conover (1999) (n = 50 experimental arenas for each AA).

Table 1 Comparisons of hierarchical Bayesian models examining Spodoptera exigua preference among concentrations of four aristolochic acids. Full models allow preference to vary across concentrations and reduced models constrain preference to be the same across concentrations.

<table>
<thead>
<tr>
<th>Aristolochic acid</th>
<th>Model</th>
<th>DIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA-I</td>
<td>Full</td>
<td>404.39</td>
</tr>
<tr>
<td></td>
<td>Reduced</td>
<td>460.74</td>
</tr>
<tr>
<td>AA-II</td>
<td>Full</td>
<td>367.62</td>
</tr>
<tr>
<td></td>
<td>Reduced</td>
<td>387.22</td>
</tr>
<tr>
<td>AA-7-OH</td>
<td>Full</td>
<td>448.97</td>
</tr>
<tr>
<td></td>
<td>Reduced</td>
<td>457.64</td>
</tr>
<tr>
<td>AA-C</td>
<td>Full</td>
<td>421.65</td>
</tr>
<tr>
<td></td>
<td>Reduced</td>
<td>471.92</td>
</tr>
</tbody>
</table>

DIC, Deviance Information Criterion (see Materials and methods section for explanation).

p = 0.403, P<0.001), suggesting that AA concentration might have an overall effect on developmental rate that we were unable to detect when restricting our examination to time to pupation. The lack of power to detect differences in time to pupation is likely due to the coarse grain (number of days) of the data.

The effect of diet on pupal weight varied across aristolochic acids (Table 2). Female pupae were consistently heavier than males and there were no significant interaction terms between sex and aristolochic acid concentration. Larvae reared on diets containing AA-I or AA-C had significantly higher pupal weights when reared on the highest concentrations (Table 2; Figure 4). We failed to detect an effect of AA-II on pupal weight (Figure 4). The results from AA-7-OH were more idiosyncratic, with medium concentrations resulting in higher pupal weights compared to the lowest concentration. However, no concentration of AA-7-OH was significantly different from the control diet (Figure 4). There was an overall positive correlation between pupal weight vs. days to first molt (Spearman correlation: ρ = 0.283, P<0.001) and pupal weight vs. days to pupation (ρ = 0.591, P<0.001).
Figure 3  Days to first molt for Spodoptera exigua larvae reared on each aristolochic acid (AA)-containing diet (n = 20 replicates per concentration per AA). Concentration of AA is indicated by H (high, 0.04), M (medium, 0.02), L (low, 0.01), and C (control, 0 µg mg⁻¹ dw). Statistics are results from Kruskal–Wallis test for each rearing experiment. Boxes show the interquartiles and whiskers show range of values within 1.5× the interquartile range. Dots indicate outliers (values outside 1.5× the interquartile range). Different letters at the top, within a specific AA, indicate significant differences at α = 0.05 using post-hoc comparison recommended by Conover (1999).

Table 2  Analysis of variance for pupal weights of Spodoptera exigua for each aristolochic acid (AA) rearing experiment

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA-I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration (C)</td>
<td>3</td>
<td>2365.71</td>
<td>3.11</td>
<td>0.032</td>
</tr>
<tr>
<td>Sex (S)</td>
<td>1</td>
<td>14823.01</td>
<td>19.49</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C*S</td>
<td>3</td>
<td>591.36</td>
<td>0.26</td>
<td>0.86</td>
</tr>
<tr>
<td>Error</td>
<td>67</td>
<td>760.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA-II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>503.78</td>
<td>0.62</td>
<td>0.61</td>
</tr>
<tr>
<td>S</td>
<td>1</td>
<td>19278.39</td>
<td>23.58</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C*S</td>
<td>3</td>
<td>575.58</td>
<td>0.70</td>
<td>0.55</td>
</tr>
<tr>
<td>Error</td>
<td>67</td>
<td>817.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA-7-OH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>3114.47</td>
<td>4.36</td>
<td>0.007</td>
</tr>
<tr>
<td>S</td>
<td>1</td>
<td>5559.37</td>
<td>7.79</td>
<td>0.007</td>
</tr>
<tr>
<td>C*S</td>
<td>3</td>
<td>1375.85</td>
<td>1.93</td>
<td>0.13</td>
</tr>
<tr>
<td>Error</td>
<td>67</td>
<td>713.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>2005.68</td>
<td>3.15</td>
<td>0.031</td>
</tr>
<tr>
<td>S</td>
<td>1</td>
<td>10025.31</td>
<td>15.72</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C*S</td>
<td>3</td>
<td>171.13</td>
<td>0.27</td>
<td>0.85</td>
</tr>
<tr>
<td>Error</td>
<td>69</td>
<td>637.64</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discussion

We used a model generalist herbivore to examine the effects of four types of aristolochic acid on caterpillar performance and preference. Our intent was not to explore the consequences of aristolochic acid on S. exigua per se, but rather to explore whether different forms of aristolochic acid have the potential to vary in their effectiveness against herbivores in general. The aristolochic acid chemistry of Aristolochia varies qualitatively and quantitatively among species, among members of the same species, and can vary substantially within individual plants (Fordyce, 2000; McCall & Fordyce, 2010; Dimarco et al., 2012). The concentrations of aristolochic acid tested in this study are at the lower end of the spectrum of concentrations measured among Aristolochia species, and some species can have orders of magnitude higher concentrations of aristolochic acids than those tested here (Chen & Zhu, 1987; Fordyce, 2001; Fordyce & Nice, 2008; Dimarco et al., 2012). Chemically, each of the aristolochic acids tested vary in their polarity, or lipophilicity, ranging from the most polar, AA-C, through AA-7-OH and AA-II, to the most lipophilic, AA-I. It has been proposed that the polarity of defensive compounds affects their defensive efficacy because more-lipophilic compounds can pass more easily across gut membranes and attack receptor sites in cells (Fordyce & Malcolm, 2000). However, we failed to detect a strong pattern in this study consistent with this hypothesis for aristolochic acids. AA-1 is the most lipophilic of the compounds we tested, and had the strongest effect on preference and performance. However, AA-II is the second most lipophilic aristolochic acid we tested and did not have a strong effect on either preference or performance. Our results do show, however, that there is significant variation among these four aristolochic acids in terms of herbivore deterrence and effects on herbivore growth, indicating that qualitative and quantitative variation are important determinants for the overall effectiveness of these compounds as anti-herbivore defenses.

The deterrent effectiveness varied across the aristolochic acids tested. On the basis of DIC scores, the preferences among concentrations of each aristolochic acid were best modeled separately, indicating variation in S. exigua response to each toxin.
The most effective deterrent was AA-I, one of the most common aristolochic acids found in Aristolochia (Urzua & Priestap, 1985; Fordyce, 2000; Sime et al., 2000). For each of the aristolochic acids tested, the 95% credibility interval for preference for the control did not include 0.25, indicating that each has some deterrent qualities. For both AA-II and AA-C, the lowest concentrations of aristolochic acid were preferred similar to the control diet, suggesting that for these compounds higher concentrations might be required to effectively deter herbivores. We failed to detect a synergistic effect in deterrence between AA-I and AA-II. Given the strong deterrent effect of AA-I (Figure 1), the deterrent effect of the mixture was likely driven by AA-I. Future investigations of possible synergistic effects between these two aristolochic acids will require a much finer grained gradient of both compounds, as it is possible that synergism might occur when AA-I is at much lower concentrations.

The effect of aristolochic acid on larval development similarly varied across the aristolochic acids examined. The consistent trend observed across aristolochic acids was an increase in time to molt to the second instar. It is not known whether the decreased early developmental rate observed for caterpillars feeding on aristolochic acid-containing diet was directly due to a physiological cost associated with dealing with these toxins, or rather was a consequence of reduced feeding rate. Thus, we are unable to disentangle the deterrent effect and the toxic effect of aristolochic acid on larval growth rate. The effect of aristolochic acid on pupal weight was more idiosyncratic among the various aristolochic acids and concentrations. The overall positive correlation between pupal weight and early larval developmental rate is interesting, especially if increased pupal weight is positively related to fitness. Pinto et al. (2009) found a similar effect of total aristolochic acids on larval weight of the specialist herbivore Battus polydamas archidamas Boisduval. If increased larval or pupal weight is positively correlated with fitness, the presence of aristolochic acids might pose a paradox for the plant. However, under field conditions this might be resolved for the plant if reduced early developmental rate increases the probability of larval mortality through either natural enemy attack or vulnerability to abiotic factors (Clancy & Price, 1987; Benrey & Denno, 1997; Williams, 1999; Fordyce & Shapiro, 2003).

We found that various aristolochic acids vary in their effects against the model generalist herbivore, S. exigua. In nature, herbivory on Aristolochia is restricted largely to Papilionidae, in particular the tribes Troidini and Zerynthiini, though recently a pyralid moth, Mapeta xanthomelas Walker, has been discovered that similarly uses Aristolochia and sequesters aristolochic acids (Durán et al., 2012). Although this study focused on the deterrent and toxic effects of various aristolochic acids, it is important to note that other plant characteristics, such as leaf toughness, might be a more important determinant for the performance of Aristolochia specialist herbivores (Dimarco et al., 2012). Interestingly, AA-I had the most consistent effect on S. exigua. AA-I is commonly observed in Aristolochia species, and is the compound that is primarily present in sequestering specialists of the plant. However, AA-II is also a common constituent of Aristolochia chemistry and we failed to detect an effect of this compound on preference or performance. Our results indicate that future investigations on the ecological role these alkaloids play should pay special attention to qualitative variation in aristolochic acids, in addition to quantitative variation.
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