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Does group feeding by toxic prey confer a defensive benefit? Aristolochic acid content, larvae group size and survival of pipevine swallowtail (Battus philenor) larvae.

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To the Graduate Council:

I am submitting herewith a thesis written by Lauren Wisner Wilmoth entitled "Does group feeding by toxic prey confer a defensive benefit? Aristolochic acid content, larvae group size and survival of pipevine swallowtail (Battus philenor) larvae.." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ecology and Evolutionary Biology.

James A. Fordyce, Major Professor

We have read this thesis and recommend its acceptance:

Susan E. Riechert, Benjamin M. Fitzpatrick

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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ABSTRACT

Aggregative feeding is widespread in Lepidopteran larvae suggesting that this behavior serves on adaptive function. Many studies of the potential benefits of aggregative feeding in Lepidopteran larvae have been conducted. However, no studies have directly examined the benefits of cryptic larvae being both chemically defended and gregarious. Group feeding occurs disproportionately more in chemically defended larvae than in larvae that have no chemical defense. Most of these larvae are cryptic when they are most highly aggregated and most vulnerable to predation. In this study, the benefits of group feeding in terms of decreased predation were explored in first instar larvae of pipevine swallowtail larvae, *Battus philenor,* a species that exhibits chemical sequestration. Contrary to our expectation, we found that groups of larvae fed a diet with high levels of the toxin aristolochic acid, which they sequester naturally and use as a defense against natural enemies, had significantly lower survivorship due to predation in both the field and in the laboratory experiments compared to groups of larvae fed a diet with low aristolochic acid content. We also found that aristolochic acid does not deter the generalist predator *Hippodamia convergens,* the ladybird beetle, suggesting that this compound is not a universal predator deterrent as previously assumed. Thus, instead of finding a benefit to group feeding and chemical defense in cryptic larvae, we have found a negative impact of group feeding in this population of *B. philenor*. Based on this evidence, we speculate that other benefits of group feeding might be outweighing the negative consequences of increased predation during the first instar. Future research on chemical defense, aposematism, and aggregative feeding should take into consideration that chemical defenses might not be universally effective against all natural enemies.

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INTRODUCTION

Gregarious feeding of larvae has evolved independently in over 20 different Lepidopteran families (Sillen-Tullberg 1988) suggesting that there are evolutionary advantages associated with group feeding. Most butterfly larvae become gregarious feeders passively through the female oviposition behavior of egg clustering (Stamp 1980). Stamp (1980) discussed some potential benefits associated with egg clustering behavior from the perspective of the adult female, the egg, and the larvae. From the adult females' perspective, the goal is to maximize the number of eggs laid during her lifetime. From this view, inclement weather, short life span, high predation, limited number of suitable host plants, and high female egg load would all present a reason to deposit eggs in clusters (Stamp 1980, Damman 1991, Tatar 1991). Clustering might enhance egg survival if clustering decreases the amount of surface area in direct contact with the external environment, thereby decreasing the surface area susceptible to parasitoids and predators. Another benefit of clustering for toxic and aposematic eggs might be enhanced aposematism (Stamp 1980).

Larval Aggregations

Although laying eggs in large clutches might offer advantages during all life stages, the evolutionary advantage of group feeding in larvae life stages is of particular interest, because these are the life stages where the greatest mortality occurs (Zalucki et al. 2002). Many hypotheses concerning potential benefits of group feeding for larvae have been proposed. These hypotheses include thermoregulatory benefits (Porter 1982, Bryant et al. 2000, Ronnas 2010), feeding facilitation (Rathcke and Poole 1975, Young and Moffett 1979, Clark and Faeth 1997, Denno and Benrey 1997, Fordyce and Agrawal 2001), plant manipulation (Fordyce 2003, 2006, Fordyce and Nice 2004), and enhanced aposematism (Gagliardo and Guilford 1993, Alatalo and Mappes 1996, Gamberale and Tullberg 1996,1998, Gamberale-Stille 2000, Tullberg et al. 2000a,

Tullberg et al. 2000b). Feeding facilitation is related to the fact that groups of larvae can better overcome plants with mechanical defenses, such as thick cuticles or trichomes, compared to single individuals (Rathcke and Poole 1975, Young and Moffett 1979, Clark and Faeth 1997, Denno and Benrey 1997, Fordyce and Agrawal 2001). Group feeding by larvae has also been shown to elicit changes in plant quality that facilitate increased larval growth rate (Fordyce 2003, 2006, Fordyce and Shapiro 2003, Fordyce and Nice 2004). Enhanced growth rate leads to an indirect defensive benefit for larvae because quickly growing larvae spend less time in the younger, more vulnerable, life stages (slower-growth / higher-mortality hypothesis; Feeny 1976, Clancy and Price 1987, Denno and Benrey 1997).

Larval Defense: Aposematism

Much emphasis has been placed on the hypothesis that unpalatable, chemically defended larvae have a direct defensive benefit through group feeding due to enhanced aposematism. That is, groups of aposematic larvae display a more apparent signal to predators compared to an individual aposematic larva. The enhanced aposematism hypothesis posits that predators learn to avoid groups of unpalatable aposematic prey more quickly because of the enhanced signal (Gagliardo and Guilford 1993, Alatalo and Mappes 1996, Gamberale and Tullberg 1996,1998, Gamberale-Stille 2000, Tullberg et al. 2000a, Tullberg et al. 2000b). The emphasis researchers have placed on the enhanced aposematism hypothesis is likely due to the fact that a disproportionate number of butterflies that lay eggs in clusters are chemically defended (Stamp 1980, Sillen-Tullberg 1988, Tullberg and Hunter 1996, Ruxton and Sherratt 2006). Most chemically defended butterflies display aposematic coloration to visually signal their unpalatability during at least one life stage. Thus, the enhanced aposematism hypothesis might explain why a disproportionate number of unpalatable larvae feed gregariously.

While the enhanced aposematism hypothesis is intuitively appealing, most chemically defended larvae have yet to exhibit characteristic aposematic coloration during the first instar, the stage when most larvae are most highly aggregated. This is a critically important life stage, because it is generally the stage where larval mortality is highest (Bernays and Cornelius 1989, Fordyce and Agrawal 2001, Zalucki et al. 2002, Fordyce 2003, 2006, Reader and Hochuli 2003, Fordyce et al. 2005, Grant 2007). For many chemically defended larvae, aposematism is exhibited in later instars when groups begin to disperse (Zalucki et al. 2002, Reader and Hochuli 2003, Fordyce et al. 2005). Many of the studies investigating the enhanced aposematism hypothesis have used avian predators, which rely heavily on vision and have the ability to learn (Gagliardo and Guilford 1993, Alatalo and Mappes 1996, Gamberale and Tullberg 1996,1998, Gamberale-Stille 2000, Tullberg et al. 2000a, Tullberg et al. 2000b). However, studies indicate that arthropod predators are more important natural enemies of larvae (Feeny et al. 1985) and they do not rely as heavily on visual cues as avian predators, rather they rely more on chemical and tactile cues (Banks 1957, Evans 1976, Storch 1976). Thus, enhanced aposematism via a larger visual warning signal could be less of a defensive benefit for early instar larvae than suggested. It is plausible, however, that enhanced olfactory aposematism might cue arthropod predators to avoid early instar larvae aggregations (Eisner and Grant 1981).

Any realized defensive benefit of aggregative feeding will depend on how predators find and kill their prey. Prey sampling is a foraging technique used by arthropod predators whereby predators kill one prey item in a group to test out the quality of the group. Predators might, therefore, leave a patch of prey after sampling a toxic prey item leading to higher overall survival for that group of prey. The possibility that predators might leave a group of toxic caterpillars after consuming one or a few individuals borrows from the principle behind the marginal value theorem, which predicts

that a predator will leave a patch of food to search for alternate resources depending on the quality of the food in the current patch and the distance to the next patch of food (Charnov 1976). We predict that chemically defended larvae feeding in groups at early instars are protected, because toxins reduce the quality of the patch, which leads to the predator avoiding the remaining members of the aggregation.

This study examines the direct defensive benefits of aggregative feeding in first instar pipevine swallowtail larvae, *Battus philenor*. We concentrate on first instar larvae because this is the stage where larval aggregations are largest, mortality is the greatest, and larvae are cryptically colored. Specifically, we ask the following questions: i) Do groups of first instar *B. philenor* larvae containing toxins sequestered from their *Aristolochia* host plant have a higher probability of survival due to decreased arthropod predation compared to an individual larva or groups of larvae containing less sequestered toxins both in the field and under controlled laboratory conditions**.** ii) Are aristolochic acids, the toxin sequestered by *B. philenor* from its host plant *Aristolochia*, an effective deterrent against the model generalist predator *Hippodamia convergens* (Coccinellidae)?

METHODS

Study System

We use the pipevine swallowtail*, Battus philenor* (Papilionidae), to investigate the potential benefits of aggregative feeding for cryptic first instar larvae. *Battus philenor* is a specialist herbivore on plants in the genus *Aristolochia* (Racheli and Pariset 1992). *Aristolochia* contain aristolochic acids (AA), toxic alkaloids unique to Aristolochiaceae. *Battus philenor* sequester these toxins as larvae and use them as defense against predators in both their larval and adult stages (Rothschild et al. 1970, Fordyce 2000, Fordyce 2001, Sime 2002, Fordyce and Nice 2008). *Battus philenor* exhibit aposematic coloration to advertise their unpalatability from their second instar on, but are rather cryptic during their first instar (Nice and Fordyce 2006). Mean clutch size for the study population in Eastern Tennessee was 13 with a median of 12 and a range from 1 to 41 (N=100). The larvae usually feed gregariously during early instars with aggregations being most dense during the first instar. Larvae aggregations decrease in size in later instars as larvae disperse (Fordyce and Agrawal 2001). As with many Lepidoptera*, B. philenor* suffer the greatest mortality during the first instar (Zalucki et al. 2002), most likely because they are smaller and contain less sequestered aristolochic acids (AA) compared to later instars (Stamp 1980, Fordyce and Nice 2008). Additionally, first instar larvae are less able to actively defend themselves due to physical constraints in maneuverability (Stamp 1986). Previous studies on other populations of *B. philenor* have shown a positive correlation between larvae toxicity and survival in the field, and an indirect defensive benefit of aggregative feeding through host plant manipulation that facilitates increased larval growth rate (Fordyce and Agrawal 2001, Fordyce 2003, 2006, Fordyce and Nice 2004, 2008).

Survival of aristolochic acid (AA) enhanced versus control larvae in the field

Field experiments were conducted in Eastern Tennessee at Norris Dam State Park (Anderson Co.). All *B. philenor* females, eggs, and larvae were collected from this site. *Aristolochia macrophylla,* a glabrous liana, is the primary host plant for this population of *B. philenor*. During May thru July 2010, wild *B. philenor* females were collected from the field and were permitted to lay eggs on *A. macrophylla* or *Aristolochia tomentosa* in cages in the lab. Eggs were also collected on *A. macrophylla* in the field. All eggs were removed from plants before hatching to ensure that neonates did not begin feeding on the host plant.

Approximately 1300 larvae from eggs of wild *B. philenor* were reared on an artificial diet either with or without additional aristolochic acids (AA). Larvae fed diet with additional aristolochic acid contain more aristolochic acid than first instar larvae in the field, and will hereafter be referred to as AA-enhanced larvae. Larvae fed diet without additional aristolochic acid contain less aristolochic acid than first instar larvae in the field, and will be hereafter referred to as control larvae. Eggs were pooled and neonate larvae were selected haphazardly for diet type treatment to avoid any potential confounding effects of differences in sequestration ability that might vary among families (Fordyce and Nice 2008). The artificial diet used for these larvae followed Fordyce and Nice (2008). To confirm that the diet treatment effectively manipulated larval chemical phenotype, several larvae were analyzed after 48 hours of feeding on artificial diet using high performance liquid chromatography (HPLC) following the protocol described in Fordyce and Nice (2008).

Larvae were placed on young *A. macrophylla* leaves in the field using a 2X2X2 block design with factors being diet type (AA enhanced or control), group size (singleton or groups of 5 individuals), and presence or absence of “crawling” predators to examine the effect of these factors on survivorship. Tanglefoot™ (Tanglefoot Company, Grand

Rapids, MI), a sticky pest barrier, was used to exclude some of the major crawling predators of *B. philenor*. Hereafter, we refer to crawling predators as those predators effectively excluded by the sticky pest barrier. Predators that are not effectively excluded by the sticky pest barrier are referred to as non-crawling predators, which may include many types of spiders, flying arthropods, and arthropods that are able to jump over the barrier. There were 34 replicates of all treatments containing groups of five larvae, and 170 replicates for all treatments containing singletons.

Individual survival was recorded after 48 hours (the approximate amount of time it takes larvae to reach the second instar). Missing larvae were assumed dead as early instars rarely move off of the plant (pers. obs.) Data were analyzed in two ways to ask: 1) if the control group and AA-enhanced group survivorship was different than singleton survivorship; and 2) to determine which factors were important for explaining larval survivorship. To ask whether group survivorship is different than singleton survivorship, the pooled singleton data set was resampled with replacement 10000 times to generate a null distribution of expected survivorship for the larvae in groups of five. Singleton data was pooled, because predators were observed to sample destructively in the field (pers. obs.) meaning that upon discovery of a larva, a predator will kill it. Also, there was no difference in survivorship between singleton control and singleton AA-enhanced larvae (see results). These permutations were conducted in R (R Development Core Team 2009) using code written by the authors (Appendix 2). We analyzed group survival data by comparing mean survival of larvae feeding on each diet type to that of the null distribution created by the pooled singleton data. Additionally, individual survivorship probability was modeled as a generalized linear model with a binomial distribution and logit link function implemented in JMP 8.0 (SAS Institute Inc., Cary, NC) with factors diet type (AA-enhanced or control), group size (one or five), and pest barrier (present or absent).

An additional field experiment that assessed the importance of predators not excluded by the sticky pest barrier was conducted. Larvae were fed either an AA-enhanced diet or control diet for 48 hours prior to being placed in groups of five in the field. All predators were excluded by enclosing the leaf on which larvae were feeding in a mesh bag. Survivorship was recorded after 48 hours in the field just as in the previous experiment. Data were analyzed in JMP using a Wilcoxon rank-sum test to determine whether survival between AA-enhanced groups and control groups was different in this total predator exclusion experiment.

Survival of aristolochic acid (AA) enhanced versus control larvae in the laboratory

A laboratory experiment was performed to assess whether there are differences in survival between larval groups fed AA-enhanced diet versus control diet in a controlled environment. Ladybird beetles, *Hippodamia convergens* (Coccinellidae), were used as model predators for the laboratory experiments. The beetles were obtained from Arbico Organics (P.O. Box 8910, Tuscon, AZ 85738) and were stored in the moist cloth bag they were shipped in and refrigerated until used in experiments. *Hippodamia convergens* typically feed on aphids in the wild, but are known to feed on a diversity of soft-bodied arthropods, including small Lepidopteran larvae.

Groups of five *B. philenor* larvae reared on either the AA-enhanced diet or control diet for 48 hours were placed into a petri dish containing a single *H. convergens*. *Hippodamia convergens* were not permitted to feed for 24 hours prior to the experiment to ensure they would actively forage once placed in the petri dishes. Survival in each experimental arena (N=60) was recorded at 12-hour intervals for 60 hours. 30 arenas contained groups of five AA-enhanced larvae and the other 30 contained groups of five control larvae. The same experimental procedure was also run in a complex environment in which the petri dish contained several *A. macrophylla* leaves (N=30, AA-enhanced=15,control=15). This latter design better mimicked the natural environment in

that it was more challenging for the predator to find the prey. Kaplan-Meier survival analysis was used to assess whether predation rate between AA-enhanced groups and control groups was different. The analysis was conducted in the R statistical environment using functions from the *Survival* and *KMsurv* packages.

Aristolochic acids as a feeding deterrent against *H. convergens*

To determine whether the generalist predator *H. convergens* could detect and was deterred by aristolochic acids, a choice test was conducted using artificial predator diet, Good Bug Power Meal™ obtained from Arbico Organics, with or without the addition of aristolochic acid. Artificial predator diet was used to eliminate the effects of larvae behavior on attraction or deterrence. The artificial diet supplemented with AA (AA+) contained 5 mL of AA solution (22mg AA/L H_20) for every 2 g Good Bug Power Meal™. The artificial diet without AA (control) contained 5 mL H_20 for every 2 g of Good Bug Power Meal™. Because AA does not readily dissolve in water, the AA/water solution was created by combining 25 mL of an AA/ethanol solution (22 mg/50 mL) with 500 mL of distilled water. The ethanol was then boiled off leaving just a solution of 11 mg AA/500 mL of distilled water. HPLC analysis confirmed that this procedure did not affect the stability of the aristolochic acid. To control for the possibility that residual ethanol remained in solution, 25mL of ethanol was also added to and boiled off of 500 mL of distilled water. This water was the water used in the control diet and in the water only treatment.

A gridded petri dish was divided equally into four sections in producing the choice arena. We placed a 1 cm^2 piece of Kimwipe™ (Kimberly-Clark Professional, Roswell, GA) in the center of three of the four sections in establishing the following treatments: no Kimwipe™, a Kimwipe™ soaked in 200 µL of water, a Kimwipe™ soaked in 200 µL of AA+ predator diet and a Kimwipe™ soaked in 200 µL of control predator diet. The treatments within sections were oriented in random directions for each replicate. A

single *H. convergens* was placed into the center of the petri dish, and the position of each predator was recorded every 15 minutes for 4 hours. 40 replicates were completed. A Quade test and posthoc tests, following Conover (1999), was used to determine whether the predators showed an overall preference for one treatment over the others. These data were analyzed in the R statistical environment using code written by the authors that is available in Appendix 2.

RESULTS

Survival of aristolochic acid (AA) enhanced versus control larvae in the field

HPLC analysis confirmed that the diet treatment successfully altered larvae chemistry. Larvae fed the AA-enhanced diet contained approximately 15% more aristolochic acid than larvae fed on the control diet (Wilcoxon one-tailed test: $X^2 = 4.5$, Df=1, P=0.0339).

We examined the effect of group size, chemical defense and predator exclusion on survivorship in the 2X2X2 block field experiment. Survival of all groups and singletons having a pest barrier was significantly greater than survival of groups and singletons without the pest barrier, indicating that crawling predators are an important source of larvae mortality in this system (Table 1) (All tables and graphs found in Appendix 1). Our data further showed that survivorship of groups of larvae fed control diet was not significantly different than the survivorship for AA-enhanced or control singletons in both the pest barrier and no pest barrier treatments (Fig. 1a,b). Our analysis indicated that groups on AA-enhanced diet have significantly lower survival rates than expected based on the null distribution of pooled singleton data in the no pest barrier treatment (Fig. 1a). The mean survival of groups fed the AA-enhanced diet in the pest barrier treatment fell just above the 0.025 quantile of the null expectation based on the resampled pooled singleton data (Fig. 1b).

Analysis of individual larva survival revealed that diet type (AA-enhanced or control) and pest barrier were important for survivorship of larvae. Larvae fed control diet had higher survivorship than those fed AA-enhanced diet, and larvae on plants with pest barrier had higher survivorship than larvae on plants without pest barrier. Group size alone was not a statistically significant predictor of survival (Table 1, Fig. 1a,b). The significant interaction term is driven by groups fed AA-enhanced diet having lower survivorship than singletons and control groups (Table 1, Fig. 2). Results from our total

predator exclusion (mesh bag) experiment showed no difference in survival between individuals fed AA-enhanced diet and the control diet (Fig. 3).

Survival of aristolochic acid (AA) enhanced versus control larvae in the laboratory

The majority of the larvae were consumed after the 60-hour experiment in both the simple and more complex environments. We failed to detect a difference in survival rate between the two groups of larvae (AA-enhanced and control diet) in the simple environment treatment, but we did detect a difference in survival rate between the two groups in the experiment with a complex environment (Fig. 4a,b). Specifically, groups of larvae feeding on AA-enhanced diet were killed at a significantly higher rate than control groups in the complex environment (Fig. 4b).

Aristolochic acids as a feeding deterrent against *H. convergens*

We investigated whether generalist predator *H. convergens* is deterred by aristolochic acid in a choice test. Analysis showed that *H. convergens* was not deterred from aristolochic acid. In fact, *Hippodamia convergens* spent significantly more time in the sections containing artificial predator diet with aristolochic acid compared to the other treatment types ($p=0.0372$) (Fig. 5).

DISCUSSION

Egg clustering is disproportionately common in aposematic, chemically defended Lepidoptera. We hypothesized that groups of toxic first instar larvae would be better defended against predators compared to groups of less toxic larvae, despite being cryptic, because predators would choose to forage elsewhere after consuming toxic individuals. Instead of finding an advantage to group feeding in chemically defended larvae, we found that groups fed AA-enhanced diet had lower survivorship compared to groups fed control diet in both the laboratory and the field experiments. In our field experiment, application of the pest barrier significantly increased larval survivorship, indicating that crawling predators are an important source of larval mortality in this system. However, the pest barrier treatment showed a marginally significant difference between survivorship of groups fed control diet and groups fed AA-enhanced diet where AA-enhanced groups had lower survival. This finding suggests that predation by predators not excluded by the application of the pest barrier are also important sources of larval mortality. Control group survival was not different than our resampled singleton data. This finding suggests that there is no advantage to feeding in a group if members of the group contain little to no chemical defense. Our predator exclusion experiment showed that the differential survival was due to predation, as survivorship of the group treatments when all predators were excluded was not significantly different. Therefore, we suggest that predators not effectively excluded by pest barrier are the cause of the differential survival between the AA-enhanced larvae and control larvae.

The findings from our laboratory experiments testing the predation rate on larvae that consumed AA-enhanced diet versus control diet were consistent with our findings in the field experiments. AA-enhanced groups had lower survival when tested in a complex environment. However, we failed to detect a difference in the simple environment. The failure to detect a difference in survival in the simple environment

might be explained by the possibility that the simple environment made it easier for predators to search and successfully encounter prey resulting in an increased predation rate on both AA-enhanced larvae and control larvae. Due to this increase in predation rate, checking survival at 12-hour intervals might have been too coarse of a grain to detect whether a difference was present.

The results from the *H. convergens* choice test showed that *H. convergens* was not effectively deterred by aristolochic acid at this concentration. In fact, *H. convergens* spent more time in the section that contained artificial predator diet with aristolochic acid than in any of the other three experimental sections. Based on this observation, we speculate that *B. philenor's* consumption of substances containing aristolochic acids might render them more susceptible to some natural enemies. More important, our findings suggest that aristolochic acids are not universal deterrents against *B. philenor's* natural enemies.

Our results show that larval groups fed AA-enhanced diet have lower survival compared to control groups in the lab and in the field that aristolochic acids are not an effective deterrent against *H. convergens*. These findings are in direct conflict with a similar study conducted on another population (Texas) of *B. philenor*, where AA content was shown to be positively correlated with first instar larval survival in the field (Fordyce and Nice 2008). It is possible that these differences are a consequence of different predator communities in the two locations. Also, the host plants and plant communities in the two locations are very different and may affect predator searching behavior and thus larvae survival.

An evolutionary advantage of egg clustering and consequent aggregative feeding for this population, and more generally, an adaptive explanation for the link between egg clustering and toxin sequestration is unclear. Attraction of predators to aristolochic acid or increased predator susceptibility of larvae feeding on diet with high aristolochic acid

content might play a role in the increased mortality of groups fed AA-enhanced diet in the field, but we currently have insufficient evidence to support these or other hypotheses.

A few hypotheses might be entertained to explain the pattern observed in the field experiment. Consistent with the superfluous killing hypothesis (Conover 1966, Johnson et al. 1975), it is possible a predator, upon killing a toxic individual, abandon and move on to the next closest prey item more quickly than it would if the prey had not been toxic. However, *Hippodamia convergens* showed no obvious difference in handling time when feeding on larvae fed either control or AA-enhanced diet in a controlled environment (pers. obs.). It is also possible that larvae eating AA-enhanced diet have lower body weight than individuals eating control diet, resulting in predators consuming more of the toxic individuals to achieve the same level of satiation as eating non-toxic individuals. However, consistent with results from other populations of *B. philenor* (Fordyce 2001), we detected no difference in weight between the control and AA-enhanced groups for this population (unpublished data). Another possible explanation for the increased mortality rates associated with larvae that have fed on a AA-enhanced diet is that consuming the AA-enhanced diet causes the larvae to become more lethargic and, therefore, less likely to defend themselves from predators via thrashing or attempting to escape. However, we did not observe any obvious difference in the behavior during any of our experiments (pers. obs.).

While we have found evidence of one negative consequence of aggregative feeding in this population, there might still be benefits of egg clustering that outweigh the negative effects. For example, previous studies on this species have shown that larger groups of individuals elicit a plant response that facilitates increased larval growth rate (Fordyce 2003). However, there is no evidence for correlated growth as a function of group size on *A. macrophylla* in Tennessee (Appendix 3). Host plant abundance,

quality, and female egg load have also been suggested to play a role in clustering of eggs for *B. philenor* (Damman 1991, Tatar 1991).

While our study focused on the benefits of group feeding in larvae during the first instar because larvae have higher mortality during the first instar than any other life stage (Zalucki et al. 2002), it may be necessary to consider the link between group feeding and chemical defense from another perspective (Stamp 1980). The link between egg clustering and toxin sequestration might be the result of increased survival during the egg stage or later larval instars, or might be due to constraints on the adult female. Perhaps, groups of toxic eggs suffer lower mortality due to predation compared to singletons. While the first instar of *B. philenor* is cryptic, the eggs are orange and generally considered aposematic. Eggs also possess aristolochic acids contributed by the ovipositing female (Fordyce et al. 2005). Later instars of *B. philenor* are also aposematic and still aggregated, though in much smaller groups. Thus, enhanced aposematism might provide a survival benefit at the egg and later instar stages that outweigh the negative consequences of heavy predation during the first instar.

Instead of finding a benefit to aggregative feeding in this study, we found that aggregative feeding in this population resulted in increased predation rates on first instar larvae. More important, this study indicates that sequestered aristolochic acids are not universal deterrents against predators, and may in fact increase larval susceptibility to some predators. Future investigations of chemical defense, aposematism, and aggregative feeding should consider that chemical defenses might not be universally effective against all natural enemies, and that the effectiveness of such defenses might vary across natural enemy communities.

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APPENDICES

APPENDIX 1

Table 1: Generalized linear model with a binomial distribution for survival of larvae in the field for 48 hours. Effect of diet (enhanced AA+ or control), group size (singleton or group of 5), and exclusion of crawling predators (Tanglefoot™ or No Tanglefoot™) was tested.

Source	DF	Chi-Square	Prob>ChiSq
Group Treatment	1	1.5150738	0.2184
Diet Treatment	1	8.8922313	0.0029*
Tanglefoot Treatment	1	98.571896	<.0001*
Group X Diet	1	8.0418036	0.0046*
Group X Tanglefoot	1	5.1781355	0.0229*
Diet X Tanglefoot	1	0.1331049	0.7152
Group X Diet X Tanglefoot	1	0.015812	0.8999

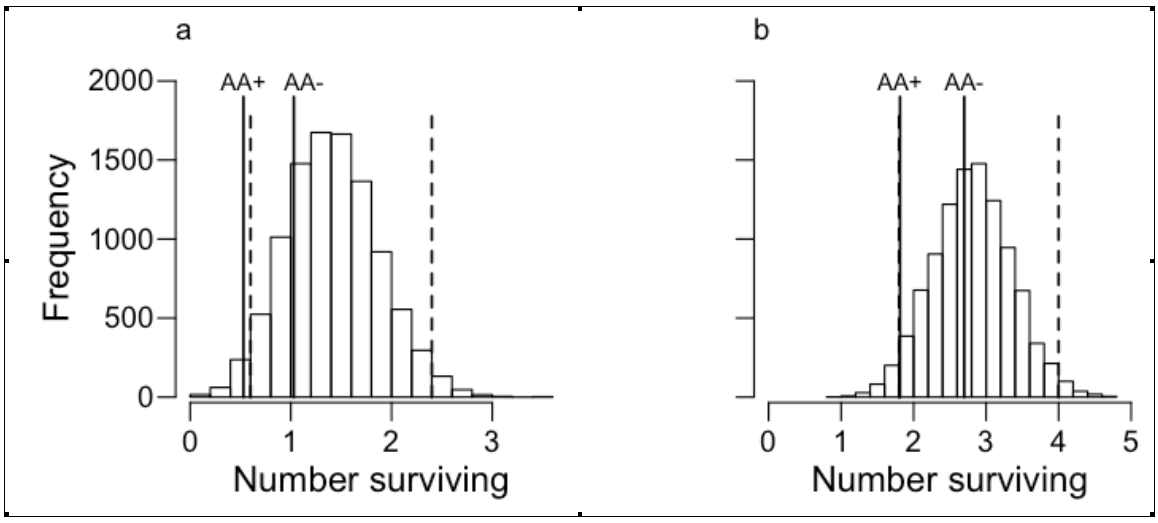

Figure 1: Survival of Singletons versus Groups in the Field. AA+ line indicates the mean survival of larvae for groups of 5 fed AA-enhanced diet for 48 hours prior to being placed in the field. AA- line indicates the mean survival of larvae for groups of five fed control diet for 48 hours prior to being placed in the field. Histogram shows the null expectation of survivorship for groups of five based on 10000 re-samples of single larvae either a) without crawling predator exclusion or b) with crawling predator exclusion. Dotted line indicates the 0.025 and 0.975 quantile.

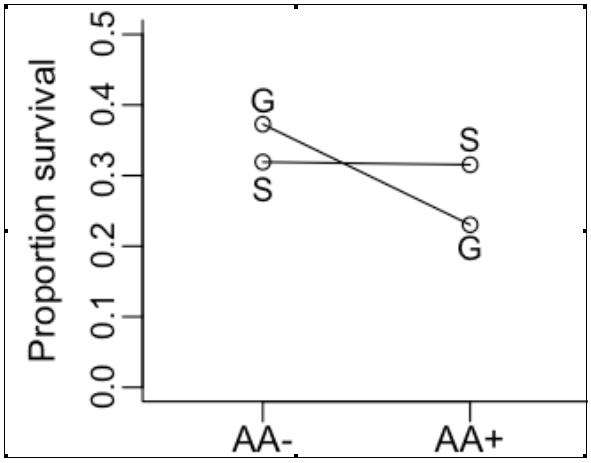

Figure 2: Significant Interaction Term in Field Data. Significant interaction term Tanglefoot™ X Group (p=0.0229) using a generalized linear model with binomial distribution. Effect of diet (enhanced AA+ or control AA-) and group size (singleton S or group of 5 G) was tested.

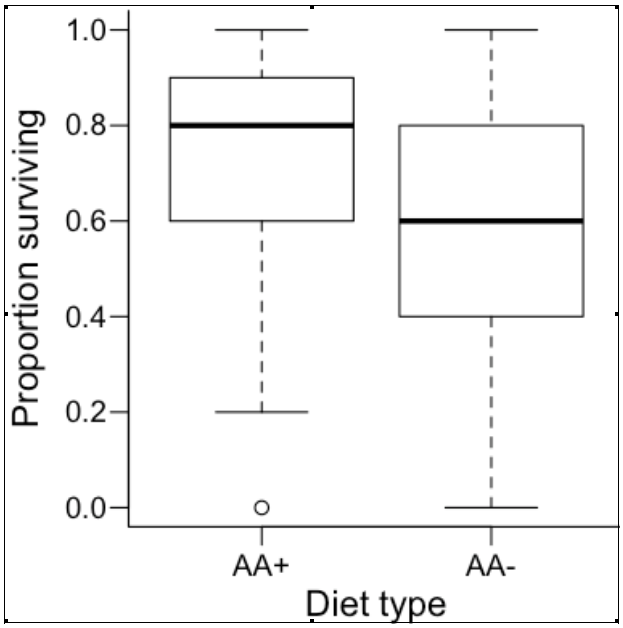

Figure 3: Larvae Survival with Total Predator Exclusion. The effect of diet type on larvae survival with total predator exclusion analyzed using a Wilcoxon test (p=0.2240).

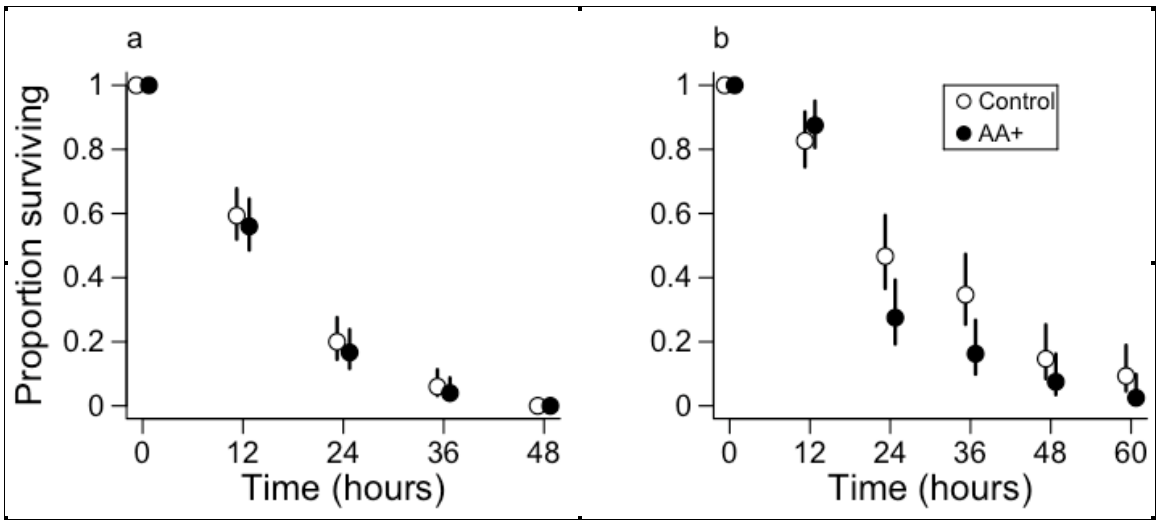

Figure 4: Survival Analysis of Larvae in the Lab. The effect of *B. philenor* larvae diet type on foraging and feeding of *H. convergens*. Survivor plots for groups of larvae placed in a) in a simple environment (Kaplan-Meier X^2=5, df=1, p=0.025*) and b) in a complex environment (Kaplan-Meier X^2=1.6, df=1, p=0.211). Hollow circle is control group, and filled circle is experimental group. Black lines are 95% confidence intervals.

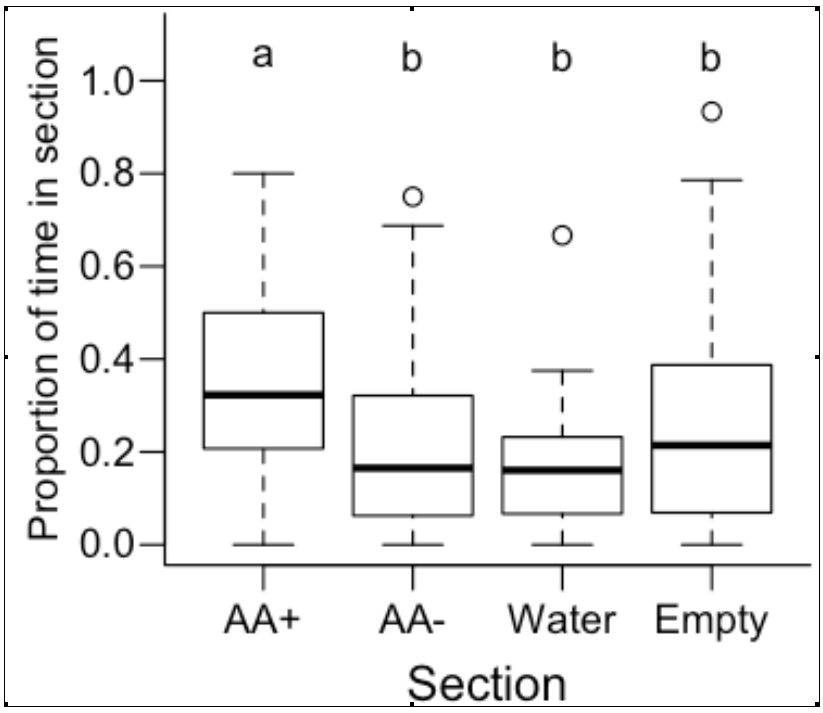

Figure 5: Predator Choice Test. The effects of different predator diets on the orientation of *H. convergens* in a petri dish. Different letters indicated a significant difference at $\alpha = 0.05$. AA+ represents a section containing a piece of Kimwipe™ soaked in artificial predator diet spiked with an aristolochic acid solution. AA- represents a section containing a piece of Kimwipe™ soaked in artificial predator diet moistened with water. The water section contains Kimwipe™ soaked just in water, and the empty section contains nothing. Orientation was checked every 15 minutes for 4 hours. *H. convergens* spent significantly more time in the section AA+ than any of the other sections.

APPENDIX 2

R Code for Null Distribution as illustrated in Figure 1

```
d<-rep(0,257) # without tanglefoot
l<-rep(1,71)

singdata<-c(d,l)

null=NA
for (i in 1:10000){


      null[i]<-sum(sample(singdata,35))

      }

stdnull<-null/5
NTstdnull<-null/5

d<-rep(0,195)  # with tanglefoot
l<-rep(1,139)

singdata<-c(d,l)

null=NA
for (i in 1:10000){

            null[i]<-sum(sample(singdata,35))

      }

stdnull<-null/5
Tstdnull<-null/5

###########FIGURE
quartz(width=6.5,height=3)
par(mfrow=c(1,2),mai=c(0.7,1,0.4,0.05),mgp=c(3,0.6,0))
hist(NTstdnull,las=1,main="",ylab="",xaxt="n",ylim=c(0,2000))
axis(1,at=0:3,labels=c(0,1,2,3),line=-0.2)
mtext("Frequency",2,line=2.8,cex=1.2)
mtext("Number surviving",1,line=1.5,cex=1.2)
#abline(v=quantile(NTstdnull,0.975),lwd=1.5,lty=2)
segments(quantile(NTstdnull,0.975),0,quantile(NTstdnull,0.975),1800,lwd=1.5,lty=2)
segments(quantile(NTstdnull,0.025),0,quantile(NTstdnull,0.025),1800,lwd=1.5,lty=2)
segments(1.030,0,1.030,1900,lwd=1.5)
text(1.130,1990,"AA-",cex=0.8)
segments(0.5294,0,0.5294,1900,lwd=1.5)
text(0.5294,1990,"AA+",cex=0.8)
mtext("a",side=3,line=0.6,adj=0)

hist(Tstdnull,las=1,main="",ylab="",xaxt="n",yaxt="n",ylim=c(0,2000),xlim=c(0,5))
axis(1,at=0:5,labels=c(0,1,2,3,4,5),line=-0.2)
axis(2,at=NULL,labels=FALSE,tick=TRUE)
mtext("Number surviving",1,line=1.5,cex=1.2)
segments(quantile(Tstdnull,0.975),0,quantile(Tstdnull,0.975),1800,lwd=1.5,lty=2)
segments(quantile(Tstdnull,0.025),0,quantile(Tstdnull,0.025),1800,lwd=1.5,lty=2)
segments(2.698,0,2.698,1900,lwd=1.5)
text(2.698,1990,"AA-",cex=0.8)
```

```
segments(1.8125,0,1.8125,1900,lwd=1.5)
text(1.8125,1990,"AA+",cex=0.8)
mtext("b",side=3,line=0.6,adj=0)
```

R code for Quade Test and Post Hoc test following Conover (1999)

```
y<-matrix(c(0.8,  0,    0.13333333, 0.06666667,
0.33333333, 0,    0.66666667, 0,
0.53333333, 0.13333333, 0.13333333, 0.2,
0.66666667, 0.26666667, 0.06666667, 0,
0.2,  0.26666667, 0.06666667, 0.46666667,
0.625,      0.125,      0.1875,     0.0625,
0.26666667, 0.26666667, 0.33333333, 0.13333333,
0.53333333, 0,    0.06666667, 0.4,
0.2,  0.6,  0.13333333, 0.06666667,
0,    0,    0.06666667, 0.93333333,
0.3125,     0.25, 0.375,      0.0625,
0.5625,     0.125,      0.125,      0.1875,
0.125,      0.6875,     0.1875,     0,
0.25, 0.5,  0.1875,     0.0625,
0.1875,     0.75, 0.0625,     0,
0.25, 0.125,      0.125,      0.5,
0.5,  0.0625,     0.1875,     0.25,
0.5625,     0.25, 0,    0.1875,
0.0625,     0.4375,     0.1875,     0.3125,
0.375,      0.125,      0.25, 0.25,
0.5,  0,    0.3125,     0.1875,
0.4375,     0.0625,     0.125,      0.375,
0.3125,     0.125,      0.0625,     0.5,
0.625,      0,    0.1875,     0.1875,
0.375,      0.0625,     0.25, 0.3125,
0.5,  0.1875,     0.25, 0.0625,
0.125,      0.375,      0.1875, 0.3125,
0.0625,     0.5,  0.3125,     0.125,
0.3125,     0.1875,     0.0625,     0.4375,
0.375,      0.125,      0.1875,     0.3125,
0.28571429, 0.21428571, 0.07142857, 0.42857143,
0.42857143, 0.14285714, 0.21428571, 0.21428571,
0.35714286, 0,    0,    0.64285714,
0.28571429, 0.35714286, 0,    0.35714286,
0.21428571, 0.5,  0.07142857, 0.21428571,
0.42857143, 0.21428571, 0.07142857, 0.28571429,
0.07142857, 0.14285714, 0,    0.78571429,
0.35714286, 0.28571429, 0.28571429, 0.07142857,
0.14285714, 0.57142857, 0.21428571, 0.07142857,
0.21428571, 0,    0.28571429, 0.5),
            nrow=40, byrow = TRUE)

colnames(y)<-c("AA+","AA-","Water","Empty")

quade.test(y)

QuadeTest<-function(d=NULL,verbose=TRUE){
      diet=LETTERS[1:length(d[1,])]
      rows<-length(d[,1])
      k<-length(d[1,])
      DIMENSIONS=list(Replicate=as.character(1:rows),Diet=LETTERS[1:k])
      ranks=matrix(nrow=rows,ncol=k,dimnames=DIMENSIONS)

      SampleRange=NA
      for (i in 1:rows){
      ranks[i,]=rank(d[i,])
      temp=sort(as.numeric(d[i,]))
      SampleRange[i]=temp[length(d[1,])]-temp[1]
```

```
      }

      RankQ=rank(SampleRange)
      rankMatrix=cbind(RankQ,ranks)
      SijMatrix=matrix(nrow=rows,ncol=k,dimnames=DIMENSIONS)
      for (i in 1:rows){
      for (j in (1:k)){
            SijMatrix[i,j]=RankQ[i]*(ranks[i,j]-(k+1)/2)
            }
            }
      S=NA

      for (j in (1:k)){
      S[j]=sum(SijMatrix[,j])
      }
      sqrSij=SijMatrix^2

      A.2=sum(sqrSij)
      B=(1/rows)*sum(S^2)
      T.3=((rows-1)*B)/(A.2-B)
      k1=k-1
      k2=(k-1)*(rows-1)

      p.value=1-pf(T.3,k1,k2)
      t.quan=qt(0.975,k2)
      in.brackets=(((2*rows)*(A.2-B))/(k2))^.5
      critdiff=t.quan*in.brackets

 if(verbose==TRUE)cat("\n\n\n******Quade Test*****","\nReplicates
=",rows,"\nk=",diet,"\nS=",S,"\nA2=",A.2,"\nB=",B,"\nT3 = ",T.3,"  num df =",k1," denom
df =",k2,"\np-value = ",p.value,"\n\nMultiple comparisons (alpha=0.05)","\nCritical
difference = ",critdiff,"\nObserved values   ",S,"\n\nObserved values
sorted\n",sort(S),"\n")

      res<-list(p.value,critdiff,S,rankMatrix)
      names(res)<-c("p.value","crit.diff","S","Matrix")
      return(res)
      #return(critdiff)
      }

QuadeTest(y)

quartz("Ladybird beetle Choice Test",3.4,3)
par(mai=c(0.7,0.7,0.05,0.05),mgp=c(3,0.6,0),bty="l")
boxplot(y,xlab="",ylab="",las=1,ylim=c(0,1.1))
mtext("Proportion of time in quadrate",side=2,line=2)
mtext("Quandrate",side=1,line=2,cex=1.2)
text(1,1.05,"a")
text(2,1.05,"b")
text(3,1.05,"b")
text(4,1.05,"b")
```

APPENDIX 3

We placed neonate *B. philenor* on leaves of *A. macrophylla* in the field as a single individual, groups of 10 or groups of 20 to determine whether growth rate was affected by group size. We excluded all predators by enclosing leaves where larvae were fed in a mesh bag and weighed all larvae after 48 hours of feeding in the field. A mean weight for each group type was calculated. An analysis of variance performed in JMP 8.0 (SAS Institute Inc., Cary, NC.) failed to detect an effect of group size on average weight ($p=0.5190$).

VITA

Lauren Wisner Wilmoth was born in Dothan, AL to the parents of Michael and Irene Wisner. She graduated with honors from Huntsville High School in Huntsville, Alabama in 2004. She graduated magna cum laude and Phi Beta Kappa from Hendrix College in Conway, Arkansas in 2008 with a Bachelors of Science degree in Biology and a minor in Secondary Education. Following graduation, she taught high school science for Aurora Public Schools in Aurora, Colorado. In Fall 2009, she accepted a graduate teaching assistantship at the University of Tennessee, Knoxville in the department of Ecology and Evolutionary Biology. Lauren received her Masters of Science degree in Ecology and Evolutionary Biology in May 2011.