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A novel trade-off of insect diapause affecting a sequestered chemical defense

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Abstract Diapause allows insects to temporally avoid conditions that are unfavorable for development and reproduction. However, diapause may incur a cost in the form of reduced metabolic energy reserves, reduced potential fecundity, and missed reproductive opportunities. This study investigated a hitherto ignored consequence of diapause: trade-offs involving sequestered chemical defense. We examined the aristolochic acid defenses of diapausing and non-diapausing pipevine swallowtail butterflies, *Battus philenor*. Pipevine swallowtail larvae acquire these chemical defenses from their host plants. Butterflies that emerge following pupal diapause have significantly less fat, a female fitness correlate, compared to those that do not diapause. However, butterflies emerging from diapaused pupae are more chemically defended compared to those that have not undergone diapause. Furthermore, non-diapausing butterflies are confronted with older, lower quality host plants on which to oviposit. Thus, a trade-off exists where butterflies may have greater energy reserves at the cost of less chemical defense and sub-optimal food resources for their larvae, or have substantially less energetic reserves with the benefit of greater chemical defense and plentiful larval food resources.

Keywords Chemical defense · Diapause · Life-history evolution · Trade-off · Sequestration

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Introduction

Diapause is a state of dormancy widely observed in the insects where metabolic processes are substantially reduced, permitting individuals to persist in a quiescent state for months or years. Diapause has been observed in all life-history stages of insects and can be obligate or facultative (Danks 1994). Numerous studies have examined the evolutionary and ecological consequences of diapause in a cost/benefit framework and important trade-offs have been described for this life history trait (Tauber et al. 1986; Danks 1987). The propensity to diapause can vary within and among populations, and such variation provides the opportunity to examine trade-offs associated with this life-history trait and the underlying evolutionary mechanisms maintaining this variation (Tauber et al. 1986; Mousseau and Roff 1989; Hopper 1999; Wiklund et al. 1992; Danks 1994; Nylin and Gotthard 1998; Soula and Menu 2003).

Many studies have concluded that one important benefit of diapause is that it allows insects to temporally avoid unfavorable conditions, such as hostile climatic conditions, insufficient or low quality food resources, and risks imposed by natural enemies (Danks 1987; Lill 2001). Diapause can, however, impose a cost through the depletion of stored energy reserves and a reduction in fecundity, increased vulnerability to natural enemies, and lost reproductive opportunities (Danks 1987; Tauber and Tauber 1992; Ishihara and Shimada 1995; Saunders 2000; Ellers and Van Alphen 2002). The defensive benefits described in association with diapause have been largely indirect, realized through the avoidance of activity during periods of high natural enemy threat or by synchronizing activity when resource quality facilitates accelerated growth, thereby reducing the time developing juveniles remain in more vulnerable stages (Feeny 1976; Clancy and Price 1987; Williams 1999; Fordyce and Shapiro 2003). Trade-offs can exist within a tri-trophic context, for example the benefit of avoiding exposure to natural enemies through diapause can be tempered by seasonal variation in host plant quality (Lill 2001). Also

important in a tri-trophic context, and widespread in insects, is plant-derived chemical defense. However, the effect of diapause on sequestered plant toxins has not been explored. Variation in sequestered chemical defense may be associated with diapause if sequestrates are metabolized, are energetically costly, or if their presence poses the risk of autotoxicity during diapause. The present study investigates trade-offs associated with diapause for the pipevine swallowtail butterfly, *Battus philenor* (L.) (Papilionidae), including adult mass, adult fat content, and the quantity of host plant-derived chemical defense.

The range of *B. philenor* extends from the southeastern and central United States to Arizona and south into Central America, with a disjunct population in California's Central Valley (Racheli and Pariset 1992). *B. philenor* is a specialist herbivore on plants in the genus *Aristolochia* (Aristolochiaceae), commonly called pipevines, which contain toxic alkaloids called aristolochic acids (Chen and Zhu 1987). Larvae sequester aristolochic acids, rendering both larvae and adults chemically defended against many natural enemies (Brower 1958; Jeffords 1979; Fordyce 2001; Sime et al. 2000). Facultative diapause occurs in the pupal stage and to some extent can be influenced by environmental factors. Sims and Shapiro (1983a) found that *B. philenor* caterpillars reared at higher temperatures were less likely to diapause compared to those reared at cooler temperatures and that females were more likely to diapause compared to males.

In this study, we examined the trade-offs associated with diapause for *B. philenor*. Specifically, we addressed the effect of diapause on chemical defense and the effect of diapause on stored energy reserves and adult mass. We explored geographic variation in the propensity to diapause, the effect of host plant species on diapause and associated trade-offs, and the adaptive significance of diapause based upon late season host plant availability and quality.

Materials and methods

Study populations and rearing

Host plant quality can be an important factor affecting the initiation of diapause in insect herbivores. To examine the effect of host plant on diapause, we reared *B. philenor* caterpillars on one of two host plant species, *A. californica* (Torrey) or *A. erecta* (L.). In California, only one host plant is available to *B. philenor*, the endemic *A. californica*, a climbing liana characteristic of riparian habitats of the Sacramento Valley and the surrounding foothills of the Coast Range and Sierra Nevada. The California population of *B. philenor* exhibits at least two broods, with the early season brood being the larger, followed by a partial second brood likely composed of first brood offspring that do not enter pupal diapause (hereafter referred to as direct development) (Fig. 1). In central

Texas, *A. erecta*, a semi-erect procumbent vine characteristic of arid, oak-juniper savanna, is the primary host plant, although other species of *Aristolochia* are recorded from this region and may also serve as larval host plants (Pfeifer 1966, 1970). Previous experience indicated that the Texas population is less prone to enter pupal diapause compared to the California population (C.C. Nice, personal observations). In central Texas, *B. philenor* is multiple brooded with overlapping generations and may be migratory (Scott 1986). Thus, two distinct life histories are possible for *B. philenor*. Individuals can exhibit a univoltine life history where pupal diapause can extend from early summer to the following spring, as is frequently observed in California. Alternatively, individuals can exhibit a bi- or multivoltine life history where direct development of pupae leads to two or more generations observed in a single year, as is observed for some California individuals and nearly all Texas individuals. We reared caterpillars from both populations on each host plant. This design permitted us to examine both the effect of host plant species and population of origin on the propensity to diapause.

B. philenor eggs were collected from naturally laid clutches in California in the Vaca Mountains of the Coast Range (Solano Co.) and in Texas at Freeman Ranch (Hays Co.), a field station operated by Texas State University. In total, a minimum of 800 eggs, representing more than 100 clutches, was collected from each population. Individuals from both populations were reared in California on *A. californica* and in Texas on *A. erecta*. Captive populations were reared on fresh plant material ad libitum until pupation in laboratory cages under ambient photoperiod at 24–27°C. Due to the time involved with caterpillar maintenance and a limited supply of host plant material, only a sub-sample of these caterpillars was permitted to develop to pupation (30 Texas individuals and 45 California individuals on *A. erecta*, and 60 Texas individuals and 90 California individuals on *A. californica*). Pupal weight and date of emergence

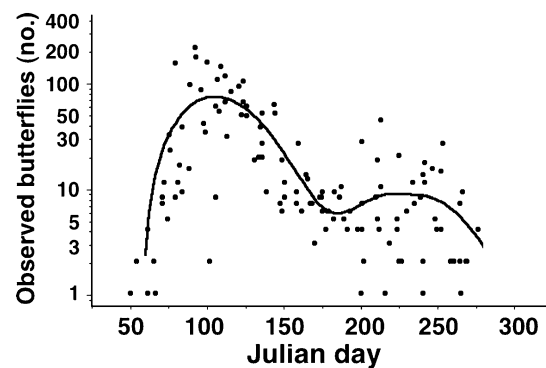


Fig. 1 Number of individual pipevine swallowtail (*Battus philenor*) adults observed by Julian day for 1999–2005 (combined). Data obtained using a fixed-course walk along a road at Gates Canyon (Solano Co., Calif., USA) during sunny, low wind conditions approximately every 2 weeks throughout the year. Fitted curve for illustrative purposes (cubic spline, generalized cross validation implemented in R; R Development Core Team 2005)

were recorded for each individual. Individuals were recorded as having entered diapause if they emerged the following spring. Non-diapausing individuals emerged within 4 weeks of pupation. Within 24 h after eclosion, after butterflies had expelled meconium and their wings had hardened, sex was recorded, and each individual was placed in a glassine envelope and stored at -20°C until chemical analysis.

Chemical analysis and fat content

Butterflies were freeze-dried and weighed to the nearest milligram. Each butterfly was placed in a 15-ml centrifuge tube, defatted twice via homogenization in 5 ml of hexane followed by 20 min of sonication. No aristolochic acids are lost to the hexane fraction (Fordyce et al. 2005). The fat content of the hexane fraction was determined by standard gravimetric technique. Aristolochic acids were extracted from the remaining butterfly tissue twice with 5 ml of 100% ethanol and sonicated for 30 min at 50°C . The ethanol extract was dried under reduced pressure leaving a yellow residue. This residue was re-suspended in 0.5 ml of 100% methanol and passed through a $0.45\text{-}\mu\text{m}$ filter into an autosampler vial for HPLC analysis. HPLC analyses were performed using a Waters Alliance HPLC system and Empower Pro Software with 2,996 diode array detector monitoring at 248 and 320 nm. Each injection was 10 μl eluted isocratically with methanol, water, and 1% acetic acid (52:47:1) at a flow rate of 1 ml/min on a Symmetry C-18 reverse phase column ($3.5\ \mu\text{m}$, $4.6\times 75\ \text{mm}$) (Waters). Aristolochic acid content was determined using retention time and absorption spectra of known standards as a reference. Aristolochic acid content of butterflies was measured as total amount (μg) and concentration (μg aristolochic acid per mg butterfly dry weight). Effects of diapause and sex on dry weight, fat content, and aristolochic acid concentration was examined using MANOVA.

Phenology of host plant quality

Aristolochia californica normally stops growing by early June. However, following disturbance, such as mowing or fire, new foliage will flush as late as August (J.A. Fordyce and A.M. Shapiro, personal observations). In early August 2000, a disturbance caused by road construction through a patch of *A. californica* provided an opportunity to compare the quality of old and newly flushed leaves for *B. philenor* larval growth. Naturally laid *B. philenor* eggs were collected from a mowed site along the American River Parkway (Sacramento Co.). Neonate larvae were confined to either older leaves or newly flushed leaves and permitted to feed for 48 h. Previous investigations have demonstrated that this time period is sufficient to measure the effects of leaf quality on caterpillar growth (Fordyce 2003, 2006; Fordyce and Shapiro 2003; Fordyce and Nice 2004). Only previously undamaged leaves were used for the experiment. Both treatments were replicated 12 times. After 48 h of feeding,

larvae were weighed to the microgram as a measure of leaf quality for larval growth.

Results

Rearing

The tendency to enter diapause differed significantly between the two study populations. Diapause was observed for 40% of California individuals and 13% of Texas individuals reared on *A. erecta* ($df=1$, $\chi^2=4.955$, $P=0.026$). For individuals reared on *A. californica*, 63% of the California individuals diapaused and 14% of Texas individuals diapaused ($df=1$, $\chi^2=22.328$, $P<0.001$). California larvae reared on *A. californica* were more likely to diapause compared to those reared on *A. erecta* in this study ($df=1$, $\chi^2=5.700$, $P=0.016$). The variation in diapause for the California population between these experiments may reflect unknown differences in rearing conditions, such as photoperiod (light periods in Texas were between 30 and 45 min shorter than those experienced in California during the rearing period of this study), or may reflect differences in the quality of the two host plants. Pupal weight did not predict whether California individuals diapaused when reared on *A. californica* ($df=1$, $\chi^2=0.878$, $P=0.3488$) or *A. erecta* ($df=1$, $\chi^2=0.13557$, $P=0.7127$). California females were more likely to diapause (31 of 41) compared to males (26 of 49) ($df=1$, $\chi^2=3.965$, $P=0.046$) when reared on *A. californica*. In contrast, there was no difference observed in diapause between sexes for individuals reared in Texas on *A. erecta* ($df=1$, $\chi^2=0.026$, $P=0.872$).

Chemistry and fat content

Because so few Texas individuals entered diapause, analysis of fat content and chemical defense as related to diapause is restricted to the California population. Similar to results of previous studies (Fordyce et al. 2005; Sime et al. 2000), aristolochic acid I was the primary aristolochic acid present in the butterfly ($39.1\pm 1.87\ \mu\text{g}$; mean \pm SE) and aristolochic acid II was present in lower amounts (7.06 ± 0.56). The relative amounts of these two compounds were correlated (Pearson product-moment correlation: $r=0.68$, $P<0.001$) Individuals that had diapaused had a significantly higher concentration of sequestered aristolochic acids and significantly less fat reserves compared to individuals that had undergone direct development on *A. californica* (Wilks' $\lambda=0.299$, $F_{9,204.6}=14.572$, $P<0.001$) and *A. erecta* (Wilks' $\lambda=0.368$, $F_{9,90.2}=5.096$, $P<0.001$) (Table 1; Fig. 2). Qualitatively similar results for diapause were observed when aristolochic acid content was analyzed as total amount sequestered on *A. californica* (diapause $F_{1,86}=4.627$, $P=0.033$) and *A. erecta* (diapause $F_{1,39}=6.970$, $P=0.012$) and when fat content was measured as a percent of body mass (not shown). For individuals reared on *A. californica*,

diapaused individuals weighed less (dry weight) than directly developing individuals. However, this effect was not detected for individuals reared on *A. erecta*. Females were consistently larger and contained greater amounts of aristolochic acids compared to males. None of the analyses detected an interaction between sex and diapause, suggesting that diapause similarly affects chemical defense and fat content for both sexes.

Phenology of host plant quality

One larva in each of the treatments died, leaving 11 replicates for each treatment. Older leaves were a substantially lower quality food compared to newly flushed leaves. Larval weight after 48 h of feeding on newly flushed leaves was 1.47 ± 0.18 mg (mean \pm SE), whereas larvae feeding on older foliage weighed 0.83 ± 0.05 mg (*t*-test: *df*=20, *t*=3.283, *P*=0.002). The average hatching weight of California larvae is 0.93 ± 0.10 mg (*n*=50) (Fordyce and Nice 2004) indicating that larvae feeding on older foliage had no net gain in mass over the course of the experiment.

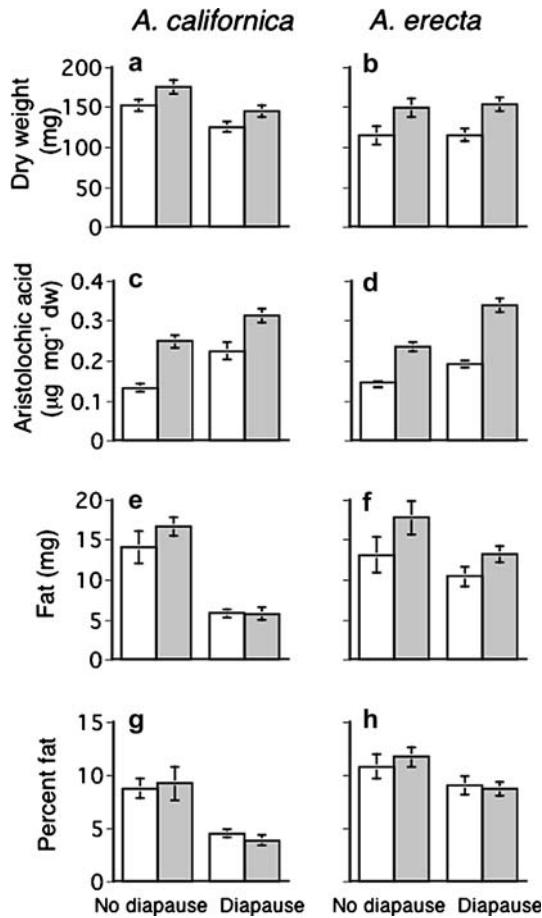


Fig. 2 Variation of pipevine swallowtail body conditions (a, b dry weight; c, d aristolochic acid; e, f fat; g, h percent fat) between males (open bars) and females (shaded bars) with direct development (no diapause) and diapause for California individuals reared on *A. californica* and *A. erecta* host plants. See text and Table 1 for statistical details. Error bars are ± 1 SE

Discussion

A trade-off between adult energy reserves and chemical defense was found for California *B. philenor*. On average, individuals that had undergone direct development had a significantly higher fat content compared to those that diapaused, regardless of larval host plant. For individuals reared on *A. californica* there was also a negative effect on total dry weight associated with diapause. Most butterfly species, including *B. philenor*, are nectar feeders as adults and are dependent on resources, such as fat, obtained as larvae for reproductive success (Karlsson 1995; Boggs 1997). Although direct development resulted in increased energy reserves that can translate into higher reproductive success, it came at the cost of reduced chemical defenses obtained as a larva. *B. philenor* undergoing diapause had on average 60% higher concentration of sequestered aristolochic acids and 40% higher total aristolochic acids compared to individuals that had undergone direct development. The physiological mechanisms by which aristolochic acids are sequestered by *Aristolochia* feeders are poorly understood, and thus the mechanisms responsible for the increased aristolochic acid content observed for diapausing individuals remain unknown. Previous work on other species of *Battus* have indicated that a substantial amount of the aristolochic acids in the larval diet are metabolized or excreted and that adults may contain as little as 2% of the total aristolochic acids consumed by larvae (Urzúa and Priestap 1985; Urzúa et al. 1987). Diapause might reduce the amount of aristolochic acids that are metabolized, or the rate that they are metabolized, resulting in greater sequestration of these toxins. The biological relevance of concentration versus total amount of aristolochic acid as related to chemical defense of the butterfly will likely depend on how a predator samples the butterfly, specifically whether the butterflies are entirely consumed or not (Fordyce et al. 2005). Regardless, both measures of aristolochic acid indicated that adults emerging from diapaused pupae contain greater amounts of aristolochic acids obtained as larvae. It is not known how important the differences in aristolochic acids we observed between diapausing and non-diapausing butterflies are for defense. However, experimental manipulation of larval aristolochic acid content has shown that these compounds have a direct effect on larval defense and are likely responsible for the toxicity, or unpalatability, of larvae and adults. Larvae containing more aristolochic acids are better defended against predators compared to larvae containing less aristolochic acids (Fordyce 2001). Codella and Lederhouse (1989) observed variation in palatability of *B. philenor* in feeding trials with captive blue jays, suggesting that variation in palatability, presumably due to variation in aristolochic acid content, can have defensive consequences for adults. Studies on the cardenolides of *Danaus gilippus* and *D. plexippus* have suggested that not only do sequestered toxins affect defense, but also the dynamics

Table 1 Univariate ANOVA tables for dry weight (mg), aristolochic acid concentration (log $\mu\text{g}/\text{mg}$ dw), and total fat content (mg) for California pipevine swallowtail (*Battus philenor*) individuals reared on *A. californica* and *A. erecta* host plants

Source	Dry weight			Aristolochic acid			Total fat content		
	MS	F^a	P	MS	F^a	P	MS	F^a	P
<i>A. californica</i>									
Diapause	15,444.01	19.286	< 0.001	0.41	20.927	< 0.001	1,386.62	42.063	< 0.001
Sex	8,909.08	11.125	0.001	0.95	48.442	< 0.001	1.35	0.041	0.8399
D \times S	47.86	0.056	0.809	0.06	3.298	0.073	1.46	0.044	0.8336
<i>A. erecta</i>									
Diapause	59.71	0.054	0.818	0.25	7.132	0.011	145.66	4.202	0.047
Sex	13,973.45	12.557	0.001	0.66	19.23	< 0.001	149.95	4.326	0.044
D \times S	46.40	0.042	0.839	0.02	0.49	0.487	9.74	0.281	0.599

^a *A. californica*: $F_{1,86}$; *A. erecta*: $F_{1,39}$

of mimicry complexes (Moranz and Brower 1998; Ritland 1994; Alonso-Mejía and Brower 1994). The increased aristolochic acid content observed in adult *B. philenor* might also have important consequences for their offspring. Female *B. philenor* transfer aristolochic acids that they obtain as larvae to their eggs, both on the surface of the egg (Sime et al. 2000) and within the egg, resulting in a decrease of aristolochic acid content in females over the course of the season (Fordyce et al. 2005). For California *B. philenor*, diapause results in increased amounts of chemicals associated with defense at the cost of decreased energy reserves.

Host plant quality will undoubtedly influence the magnitude of the diapause-related trade-offs. There was no apparent effect of host plant on differences in aristolochic acid content associated with diapause. Diapaused individuals consistently had a higher concentration and total amount of aristolochic acids. However, butterflies reared on *A. californica* had substantially less fat following diapause compared to individuals reared on *A. erecta*. Previous experiments have indicated that larvae develop at a substantially accelerated rate on *A. erecta* compared to *A. californica*, suggesting that it is a host plant species of superior quality (Fordyce and Nice 2004). The superior quality of *A. erecta* compared to *A. californica* may ameliorate some of the costs associated with diapause for individuals developing on the Texas host plant. However, we were unable to access the effect of diapause for the Texas population because so few individuals entered pupal diapause.

The variation in the propensity to diapause observed between the two populations studied here may also be influenced by qualitative differences of the two host plants, and by seasonal variation in host plant availability and quality. Individuals from central Texas were less likely to diapause compared to individuals from California, regardless of larval host plant. The higher rate of diapause observed for the California population in both of the rearing experiments might reflect selection pressure resulting from the seasonal decline in *A. californica* quality for larval development. Females in California generally avoid laying eggs on older foliage (J.A. Fordyce, personal observations), and thus suitable oviposition sites for females from this population are scarce by the

time the second brood emerges, thereby favoring diapause over direct development. Previous work on these two populations has shown significant differences in thermal tolerance of larvae, indicating between-population differences in other ecologically relevant traits (Nice and Fordyce 2006). Although it is possible that some of the between-population variation in the propensity to diapause is a consequence of parental conditions, laboratory colonies maintained over three generations similarly showed little pupal diapause for the Texas population, suggesting a genetic component of this variation (J.A. Fordyce, unpublished data).

Rausher (1986) observed that the first brood of a *B. philenor* population in eastern Texas using *A. serpentaria* and *A. reticulata* diapause at a rate of 41–55%, comparable to that of the California population in this study. He suggested that this diapause polymorphism could be maintained via frequency-dependent selection resulting from competition for suitable oviposition sites in the second brood. Whereas the California population has only one potential host plant, numerous species of *Aristolochia* occur in the region of our central Texas study population (Pfeifer 1966, 1970). Direct development might be favored in this population if suitable host plants are available for subsequent broods, if competition among females for suitable oviposition sites is low, or if individuals can successfully disperse to adjacent areas with suitable host plants.

Numerous studies have examined insect diapause in the context of trade-offs. The geographic variation in the propensity to diapause observed for *B. philenor* suggests that the costs and benefits associated with diapause will similarly vary throughout its range. One important benefit of diapause for the California population is that individuals temporally avoid periods when suitable host plants for larval development are scarce. The costs of diapause for *B. philenor* in the currency of stored energy reserves is consistent with that observed for many insects that undergo diapause (Danks 1987). Most individuals in Texas avoid this cost by developing directly. Future study will be required to assess if Texas populations have similar chemical and fat responses to diapause. Pupal diapause can also impose a cost if the diapausing stage is at increased risk of discovery or increased susceptibility

to parasites and predators, and such risks may be present for *B. philenor* pupae (Sims and Shapiro 1983b). However, upon emerging from diapause, *B. philenor* adults contain greater amounts of sequestered chemical defenses compared to individuals that developed directly. This first examination of the consequences of diapause on sequestered chemical defense indicates that this life history tactic may have important consequences for adult toxicity.

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