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Physiological adaptations associated with host specialization: ecological implications for generalist and specialist saturniids (Callosamia spp.)

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# PHYSIOLOGICAL ADAPTATIONS ASSOCIATED WITH HOST SPECIALIZATION: ECOLOGICAL IMPLICATIONS FOR GENERALIST AND SPECIALIST SATURNIIDS (CALLOSAMIA SPP.)

By

Kelly Susanne Johnson

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# ABSTRACT

## PHYSIOLOGICAL ADAPTATIONS ASSOCIATED WITH HOST SPECIALIZATION: ECOLOGIAL IMPLICATIONS FOR GENERALIST AND SPECIALIST SATURNIIDS (*CALLOSAMIA* SPP.)

By

Kelly S. Johnson

The nutritional and chemical characteristics of plants are important determinants of herbivore fitness, thus an herbivore's metabolic and detoxicative capabilities can profoundly influence the evolution of host use patterns. I investigated physiological adaptations associated with host specialization in three Saturniids (*Callosamia promethea*, *C. angulifera* and *C. securifera*) which display a gradation of feeding specialization and distinct reciprocal inabilities to use each others' hosts. The importance of host chemistry and detoxification ability as determinants of host use were evaluated by isolating specific host chemicals responsible for differential performance of *Callosamia* on sweetbay and comparing their detoxification by the three species. We identified two compounds, magnolol and a biphenyl ether, that reduce growth and survival of *C. promethea* and *C. angulifera*. Mortality appears to be due to toxicity rather than antifeedancy. In all three species, compounds are metabolized *in vitro* by midgut enzymes, but *C. securifera* does so more rapidly than the two unadapted species. Degradation of the biphenyl ether was both NADPH dependent and inhibited by piperonyl butoxide, suggesting the involvement of cytochrome P450 enzymes. Magnolol metabolism was NADPH dependent but not inhibited by piperonyl butoxide. Detoxification activity was not induced by pentamethylbenzene or a mixture of the sweetbay allelochemicals. Contrary to the hypothesis that polyphagous feeders have higher levels of general detoxification enzymes, cytochrome P450 activity is more closely associated with host chemistry than diet breath in *Callosamia*. The hypothesis that hostplant specialization is accompanied by increased nutritional efficency was tested by comparing nutritional indices of the tuliptree specialist, *C. angulifera* and *C. promethea*, which feeds on many hosts in addition to tuliptree. The specialist exhibited higher gross efficiency of utilization (ECI) and net efficiency of utilization (ECD) on tuliptree compared to the generalist. Growth and consumption of the two species did not differ. The phylogenetic relationship between the three species was clarified using allozyme electrophoresis. The presence of fixed alleles indicated little or no gene flow between the taxa. Based on Nei's genetic identities, the three *Callosamia* species are genetically equidistant, making identification of ancestral host use patterns difficult.

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# INTRODUCTION

All organisms display some degree of ecological specialization, particularly with regard to habitat or resource use. The degree of specialization exhibited by different species varies enormously, however, prompting the question of what factors lead to the evolution or maintainence of specialist or generalist characteristics.

Many herbivorous insects display specialized feeding habits, using only a fraction of the plants in a community as host plants. Plant chemistry has been demonstrated to have a profound influence on insect fitness and is thought to be a primary directive factor in the evolution of host use patterns in many herbivorous insect groups. The inability to successfully ingest and metabolize some plants can impose a signficant constraint on host use, just as a novel metabolic trait may allow an herbivore to expand onto a new host. Enzymatic detoxification is one mechanism for coping with potentially detrimental host allelochemicals.

In contrast to the amount of research on mechanisms of detoxification of man-made toxins by insects, there are relatively few examples of how naturally occurring allelochemicals are metabolized. Understanding the physiological mechanisms underlying the metabolism of host chemicals is the first step to identifying patterns of physiological constraints and tradeoffs that may influence the evolution of host use patterns.

Current models of ecological specialization are founded on the assumption that there are tradeoffs associated with generalized and specialized resource use. Based on the tradeoff principle, generalists may be able to utilize a greater variety of resources, but specialists are more efficient at utilizing a few. In herbivorous insects, host specialization may allow improved utilization of that host, either through greater efficiency of digestion or detoxification. Although the tradeoff principle is frequently envoked in discussions of the evolution of host patterns in insects, there is little empirical evidence of nutritional or detoxicative tradeoffs associated with host specialization.

Metabolic costs associated with detoxification have been hypothesized as a currency for specialization tradeoffs. It has been suggested that generalist herbivores have higher levels of general detoxification enzymes (PSMO's) which enable them to detoxify a broad array of allelochemicals encountered in their diets. Herbivores that specialize on one or a few hosts may have the advantage of having to maintain lower and less metabolically costly levels of detoxification enzymes. Thus far, however, metabolic costs of detoxification have not been clearly demonstrated, nor is it clear how metabolic costs might translate into differences in fitness.

The wild silkmoths (genus *Callosamia*, Saturniidae) offer an ideal model system to test predictions about ecological specialization, physiological tradeoffs and patterns of detoxification enzymes. The three closely related species display not only a gradation of host specialization, but some distinct reciprocal inabilities to utilize each others' host plants. *C. securifera* is a specialist on sweetbay magnolia, a host on which neither of its congeners is able to survive. *C. angulifera* is also a specialist, feeding on tuliptree. *C. promethea* is the most polyphagous species in the group, feeding on plants from over five different families in addition to tuliptree.

In this dissertation, a comparative approach was used to investigate the importance of host chemistry and physiological adaptation in the evolution of host use patterns in this genus of moths. In the first chapter, allozyme electrophoresis is used to clarify the phylogenetic relationship among the three *Callosamia* species so that an evolutionary trajectory of detoxicative and host use patterns could be proposed. The second chapter evaluates the importance of host chemistry in determining the differential abilities of *Callosamia* to feed on sweetbay by identifying allelochemicals toxic to unadapted herbivores. In the third chapter, the physiological adaptations that enable the sweetbay specialist to detoxify host chemicals are characterized and a comparative approach used to evaluate the importance of detoxification enzymes in a phylogenetic context. Finally, the fourth chapter addresses the hypothesis that host specialization is accompanied by increased efficiency of resource utilization.

# CHAPTER ONE

## Estimates of genetic differentiation between *Callosamia* species and *Hyalophora cecropia* (Saturniidae) by allozyme electrophoresis

### Abstract

The genus *Callosamia* is composed of three morphologically similar species, *C. promethea*, *C. angulifera* and *C. securifera*, which can be hybridized by handpairing but are apparently reproductively isolated in the wild by differences in mating times. Observations of the cross-attraction of the pheromones between species and the occasional disruption of normal flight and calling patterns by local weather conditions suggest reproductive isolation by allochronic mating behavior may not be complete. Moreover, despite the current taxonomic separation of *Callosamia* and *Hyalophora* as genera, inter-generic hybrids have been produced through hand pairing. We performed cellulose acetate electrophoresis of the three *Callosamia* species, *C. angulifera* X *C. promethea* hybrids, and *Hyalophora cecropia* to quantify the genetic differentiation between these taxa. Each of the three *Callosamia* species were distinguishable by fixed alleles at five of eighteen loci, indicating that there is little or no gene flow between the taxa. Pairwise comparisons of Nei's genetic identities calculated from all 18 loci indicate that the three *Callosamia* species are phylogenetically equidistant from each other. *Hyalophora cecropia* shared alleles with *Callosamia* at only 1 of the 18 loci.

## Introduction

The genus *Callosamia* is composed of three closely related species of native silkmoths with overlapping distributions across Eastern North America. Although Michener (1952) considered *Callosamia* a subgenus of *Hyalophora*, Ferguson (1972) viewed the three species as a discrete group and elevated the taxa to full generic status. *C. securifera* was first described as a "variety" of *C. angulifera*, and was relatively recently elevated to species status after the temporal reproductive isolation mechanism between *Callosamia* species was described (Brown 1972). *C. securifera* females emit pheromone from mid-morning to early afternoon, while *C. promethea* are active from late afternoon to dusk, and *C. angulifera* females do not begin calling until after dusk. There appears to be little qualitative difference in the pheromone, as *C. securifera* and *C. angulifera* males can be attracted to captive *C. promethea* females that are emitting pheromone (Haskins and Haskins 1952, Peigler 1980, K. S. Johnson, personal observation).

Successful hybrids between *Callosamia* species can be obtained by hand-pairing, but hybridization in the wild is believed to be uncommon (Brown 1972, Ferguson 1972) since intermediate specimens are rarely collected (Peigler 1980). The genitalia of *C. promethea* are considerably larger than the other two species and this size differential can act as a mechanical barrier to cross-mating even when moths are handpaired (Peigler 1977). Post-mating incompatabilities often result in reduced egghatch, weak larvae, disruption of pupal diapause, and weak or malformed adults (Peigler 1980). Interspecific hybrids involving *C. promethea* are usually sterile, although in *C. angulifera* and *C. securifera* crosses a small proportion of hybrids are fertile for three generations (Haskins and Haskins 1952, Peigler 1977, 1980).

In addition to the premating behavioral and mechanical barriers to interspecific hybridization in *Callosamia*, differences in hostplant use may also contribute to the isolation between species. With few exceptions, neither *C. promethea* nor *C. angulifera* larvae survive when fed sweetbay magnolia (*Magnolia virginiana*: Magnoliaceae), the host of the monophagous *C. securifera* (Johnson et al. in preparation). This is despite the fact that *C. angulifera* is a near-specialist on another magnoliaceous host, tuliptree (*Liriodendron tulipifera)*, and *C. promethea* is quite polyphagous, feeding on a variety of hosts in the Magnoliaceae, Lauraceae, Rosaceae, Oleaceae, Rubiaceae, Styracifluaceae and others (Ferguson 1972, Stone 1991). Understanding the phylogenetic relationships of the *Callosamia* group would provide a valuable framework for hypotheses concerning the evolution of host use and physiological adaptation to host allelochemicals in this group.

Allozyme electrophoresis can be useful for estimating genetic divergence and phylogenetic relationships of closely related insect taxa (Berlocher 1984). We conducted this preliminary electrophoretic survey of *C. promethea*, *C. angulifera*, *C. securifera*, *C. promethea* X *C. angulifera* hybrids and *Hyalophora cecropia* to 1) evaluate the effectiveness of the reproductive isolating mechanisms in *Callosamia* 2) to estimate the relative degree of genetic differentiation between the three species and 3) compare genetic distances to the closely related genus *Hyalophora*.

## Materials and Methods

### *Sample Collection*

Representatives of the three *Callosamia* species, *C. promethea* x *C. angulifera* hybrids and *Hyalophora cecropia* (as a closely-related outgroup) were included in this study. Individuals of *C. promethea*, *C. angulifera* and *C. securifera* were wild-collected or were offspring of females collected from various (two to seven) sites across their natural geographic distributions (Figure 1 and Table 1). The number of individuals from each site ranged from 1 to 6, and because females were needed for other studies during this period, most of the samples for electrophoresis were males. Seven hybrid specimens were obtained from a semi-natural mating of a captive female *C. promethea* (collected in Southern WI) with a wild *C. angulifera* (Cass Co., MI) male. This female began calling near dusk and attracted seven wild *C. angulifera* males; one was allowed to mate and viable hybrids (n=6) were obtained from this crossing. An additional hybrid specimen was the result of a male *C. promethea* paired with a female *C. angulifera* in the laboratory. *H. cecropia* samples were pupae collected from several sites in Ingham and Clinton Co.'s, MI in 1991. Voucher specimens of the three *Callosamia* species (minus the abdomens) have been deposited at the Entomology Department of Michigan State University.

### *Allozyme Electrophoresis*

Both adults and pupae were used in allozyme analyses after preliminary studies indicated that there were no appreciable differences in allozyme frequencies between the life stages. Individuals were killed by freezing at - 80° C

Table 1. Collection locations for *Callosamia* specimens.

| Taxon | Region | Site and number of individuals | |
|---|---|---|---|
| *C. promethea* | Wisconsin | 1 | Kenosha Co. (2) |
| | Michigan | 2 | Otsego Co. (2) |
| | | 3 | Barry Co. (4) |
| | | 4 | Clinton Co. (2) |
| | Maryland | 6 | Montgomery Co. (2) |
| *C. angulifera* | Michigan | 5 | Cass Co. (9)* |
| | Virginia | 7 | Greensville Co. (3) |
| *C. securifera* | North Carolina | 8 | Bladen Co. (1) |
| | Florida | 9 | Highlands Co. site 1 (6) |
| | | 10 | Highlands Co. site 2 (4) |
| | | 11 | Lake Co. (1) |

* Specimens collected from this site over two separate years

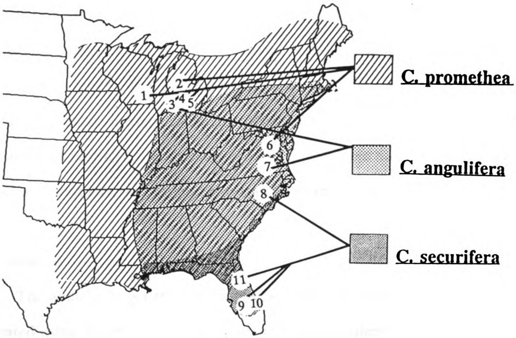


Figure 1. Geographic distribution of *Callosamia promethea*, *C. angulifera* and *C. securifera* and collection sites for specimens.

and stored until processing. The posterior half of the abdomen of adult moths and the posterior end (1 cm) of pupae were used for electroporesis. Tissue was placed in 1.5 ml Eppendorf tubes with 250 ul of extraction buffer (Tris-EDTA-B-mercaptoethanol, pH 7.0) and homogenized with a tissue grinder. The tubes were capped and centrifuged at 14,000 rpm for 8 minutes, then 0.25 $\mu$l of tissue supernatant was applied to cellulose acetate plates using the Super Z-12 application system (Helena Laboratories). Plates were placed in refrigerated rigs and run under the conditions indicated in Table 2, then stained using an agar overlay and covered to prevent backstaining. The zymeograms were scored by measuring the relative mobilities of alleles from the origin after arbitrarily assigning the most common allele a mobility value of "100". To insure consistency of scoring between runs, two individuals from each plate were run on the subsequent plate, and at least two species were always represented on a plate.

### *Data Analysis*

The amount of genetic divergence between the *Callosamia* species was estimated using Wright's F-statistics and Nei's genetic identity measures. F-statistics were calculated for the seven polymorphic loci according to the method of Weir and Cockerham (1984). For each locus, species-level differences in allele frequencies were compared by chi-square or F-ratio (loci with > 2 alleles). Nei's pairwise genetic identities and jackknifed standard errors were calculated across all loci (Hartl and Clark 1989).

Table 2. Allozymic loci resolved for *Callosamia promethea*, *C. angulifera*, *C. securifera* and *Hyalophora cecropia* and corresponding running conditions for each enzyme. Buffers and origin positions (an = anode, ce = center, ca = cathode) were selected to keep enzymes centered on the cellulose acetate plates.

| Locus | Enzyme name (E. C. number) | Running conditions | | | |
|---|---|---|---|---|---|
| | | buffer | origin | voltage | time |
| AAT-1 | Aspartate aminotransferase (2.6.1.1) | I | an | 275V | 40 |
| AAT-2 | | | | | |
| AC | Aconitase (4.2.1.3) | A | an | * | 40 |
| ACP | Acid phosphatase (3.1.3.2) | C | ce | 275V | 40 |
| ALD | Aldolase (4.1.2.13) | I | ce | 275V | 40 |
| FUM | Fumarase (4.2.1.2) | C | ce | 275V | 40 |
| GPI | Glucose phosphate isomerase (5.3.1.9) | I | | 273V | 40 |
| G6PDH | | | | | |
| HBDH | Hydroxybutyrate dehydrogenase | D | an | 300V | 40 |
| IDH | Isocitrate dehydrogenase (1.1.1.42) | A | an | * | 40 |
| LDH | Lactate dehydrogenase (1.1.1.27) | B | an | * | 40 |
| MDH | Malate dehydrogenase (1.1.1.37) | C | ce | 275V | 40 |
| MPI | Mannose-6-phosphate isomerase (5.3.1.8) | I | an | 275V | 40 |
| P3GDH | 3-phosphoglycerate dehydrogenase (1.1.1.95) | C | an | 275V | 40 |
| PEP-LA | Peptidase (leucyl-alanyl) (3.4.11-13..) | C | | 275V | 40 |
| PGM | Phosphoglucomutase (2.7.5.1) | I | an | 275V | 40 |
| SORDH | L-iditol dehydrogenase (1.1.1.14) | I | an | 275V | 40 |
| TPI | Triose phosphate isomerase (5.3.1.1) | I | an | 275V | 40 |

* Voltage is adjusted to maintain current between 9-12 mA per plate

## Results

Eighteen loci were resolved across the taxa surveyed (listed in Table 2). Six loci did not vary among *Callosamia* taxa (ALD, GPI, G6PDH, PEP-LA, SORDH, TPI). Fixed differences were observed between *Callosamia* species pairs at five loci (AC, ACP, FUM, MDH, and P3GDH) and the remaining seven loci were polymorphic in at least one species. Nine loci were polymorphic in *Hyalophora cecropia* and six were invariable; there was only one shared allele between *Callosamia* and *Hyalophora* (AAT-1). The allele frequencies and relative mobility of allozymes are summarized in Table 3.

*C. promethea*, *C. angulifera* and *C. securifera* were all distinguishable by fixed alleles at at three or more loci, as expected for genetically distinct species. There were four fixed differences between *C. securifera* and *C. angulifera*; three between *C. angulifera* and *C. promethea*; and three between *C. promethea* and *C. securifera*. Estimates of Wright's *F*-statistics are listed in Table 4.

The genetic identities (Table 5) calculated from invariant, polymorphic and fixed alleles indicate that *C. promethea*, *C. angulifera* and *C. securifera* are equally differentiated from each other. Because *Hyalophora cecropia* shared only one allele at a single locus with *Callosamia*, genetic identity between the two genera was not calculated. Overall heterozygosity was not calculated for any taxa, since the small sample sizes make interpretations difficult (Nei 1978).

Table 3. Allele frequencies for 18 loci resolved in *C. securifera*, *C. angulifera*, *C. promethea*, hybrids and *Hyalophora cecropia*. Sample sizes (no. of individuals) are listed in parentheses.

| Allozyme locus | | *C.sec* | *C.ang* | *C.pro* | *C.a.* x *C.p.* | *C.p.* x *C.a.* | *H. cecropia* |
|---|---|---|---|---|---|---|---|
| AAT-1 | | (12) | (12) | (11) | (3) | (1) | (5) |
| | 20 | 0.17 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 70 | 0.12 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 100 | 0.71 | 0.46 | 1.00 | 1.00 | 0.00 | 1.00 |
| | 140 | 0.00 | 0.50 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 200 | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 | 0.00 |
| AAT-2 | | (12) | (12) | (12) | (3) | (1) | (5) |
| | 67 | 0.08 | 0.08 | 0.00 | 0.33 | 0.00 | 0.00 |
| | 75 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.40 |
| | 100 | 0.92 | 0.92 | 1.00 | 0.67 | 1.00 | 0.00 |
| | 125 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.60 |
| HBDH | | (12) | (12) | (12) | (7) | (1) | (8) |
| | -250 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.125 |
| | -200 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.75 |
| | -100 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.125 |
| | 50 | 0.17 | 0.00 | 0.04 | 0.14 | 0.00 | 0.00 |
| | 100 | 0.83 | 1.00 | 0.96 | 0.86 | 1.00 | 0.00 |
| IDH | | (8) | (8) | (7) | (0) | (0) | (0) |
| | 100 | 0.69 | 0.69 | 0.93 | - | - | - |
| | 130 | 0.31 | 0.31 | 0.07 | - | - | - |
| LDH | | (12) | (12) | (12) | (7) | (1) | (8) |
| | 67 | 0.00 | 0.00 | 0.58 | 0.00 | 0.00 | 0.00 |
| | 100 | 1.00 | 1.00 | 0.42 | 1.00 | 1.00 | 0.00 |
| | 120 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.69 |
| | 150 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.31 |
| MPI | | (4) | (4) | (4) | (0) | (0) | (0) |
| | 50 | 0.00 | 0.125 | 0.00 | - | - | - |
| | 75 | 0.25 | 0.25 | 0.50 | - | - | - |
| | 100 | 0.75 | 0.50 | 0.50 | - | - | - |
| | 125 | 0.00 | 0.125 | 0.00 | - | - | - |
| PGM | | (7) | (8) | (8) | (0) | (0) | (0) |
| | 75 | 0.50 | 0.50 | 0.12 | - | - | - |
| | 100 | 0.50 | 0.50 | 0.88 | - | - | - |
| AC | | (4) | (4) | (4) | (7) | (0) | (8) |
| | 80 | 0.00 | 0.00 | 0.00 | 0.00 | - | 1.00 |
| | 100 | 1.00 | 0.00 | 1.00 | 0.50 | - | 0.00 |
| | 150 | 0.00 | 1.00 | 0.00 | 0.50 | - | 0.00 |
| ACP | | (8) | (8) | (8) | (7) | (1) | (8) |
| | 40 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 |
| | 60 | 0.00 | 1.00 | 0.00 | 0.00 | 1.00 | 0.00 |

Table 3 (cont'd).

| | | | | | | |
|---|---|---|---|---|---|---|
| 100 | 1.00 | 0.00 | 1.00 | 1.00 | 0.00 | 0.00 |
| FUM | (12) | (12) | (12) | (7) | (1) | (8) |
| 62 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.56 |
| 67 | 1.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 88 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.44 |
| 100 | 0.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 |
| MDH | (12) | (12) | (12) | (7) | (1) | (8) |
| 70 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.56 |
| 75 | 1.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 90 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.44 |
| 100 | 0.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 |
| P3GDH | (12) | (12) | (12) | (7) | (1) | (8) |
| 30 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.50 |
| 50 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.50 |
| 67 | 0.00 | 0.00 | 1.00 | 0.50 | 0.50 | 0.00 |
| 100 | 1.00 | 1.00 | 0.00 | 0.50 | 0.50 | 0.00 |
| GPI | (8) | (8) | (8) | (7) | (1) | (8) |
| 80 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.38 |
| 90 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.62 |
| 100 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 |
| G6PDH | (12) | (12) | (12) | (7) | (1) | (8) |
| 60 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.25 |
| 75 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.75 |
| 100 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 |
| PEP-LA | (12) | (12) | (12) | (4) | (0) | (3) |
| 75 | 1.00 | 1.00 | 1.00 | 1.00 | - | 0.00 |
| 100 | 0.00 | 0.00 | 0.00 | 0.00 | - | 1.00 |
| TPI | (12) | (12) | (12) | (7) | (1) | (8) |
| 60 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.50 |
| 80 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.50 |
| 100 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 |
| ALD | (12) | (12) | (12) | (0) | (0) | (0) |
| 100 | 1.00 | 1.00 | 1.00 | - | - | - |
| SORDH | (12) | (12) | (12) | (0) | (0) | (0) |
| 100 | 1.00 | 1.00 | 1.00 | - | - | - |

Table 4. Tests for species-level differences in allele frequencies at seven polymorphic loci in the genus *Callosamia*. Chi-square ( > 2 allele loci) and F - ratios (2 allele loci) for $F_{IS}$, $F_{ST}$, and $F_{IT}$ values.

| | Statistic | | lambda | Approx. chi sq | df | Approx. Z | Significant at P < 0.05 |
|---|---|---|---|---|---|---|---|
| AAT-1 | Fis | 0.3668 | 0.0356 | 163.5 | 96 | 4.083 | |
| | Fst | 0.1888 | 0.3753 | 30.4 | 6 | 3.919 | * |
| | Fit | 0.4863 | 0.0179 | 201.3 | 102 | 5.494 | |
| MPI | Fis | 0.1848 | 0.3636 | 14.7 | 27 | -1.938 | |
| | Fst | -0.0375 | 0.9394 | 0.5 | 6 | -2.734 | ns |
| | Fit | 0.1542 | 0.3429 | 16.6 | 33 | -2.414 | |
| | | | | F - ratio | df 1 | df 2 | |
| AAT-2 | Fis | -0.0476 | 0.5455 | 0.9091 | 33 | 36 | |
| | Fst | 0.0040 | 0.9375 | 1.1000 | 2 | 33 | ns |
| | Fit | -.00435 | 0.5294 | 0.9143 | 35 | 36 | |
| HBDH | Fis | 0.3400 | 0.3495 | 2.0303 | 33 | 36 | |
| | Fst | 0.0596 | 0.8855 | 2.1343 | 2 | 33 | ns |
| | Fit | 0.3793 | 0.3224 | 2.1619 | 35 | 36 | |
| IDH | Fis | -0.3063 | 0.6751 | 0.5310 | 29 | 32 | |
| | Fst | 0.1532 | 0.7000 | 6.2143 | 2 | 29 | * |
| | Fit | -0.1062 | 0.5926 | 0.7097 | 31 | 32 | |
| LDH | Fis | 1.0000 | 0.0000 | 1.7014 | 33 | 36 | |
| | Fst | 0.5455 | 0.5172 | 0.5455 | 2 | 33 | * |
| | Fit | 1.0000 | 0.0000 | 1.7014 | 35 | 36 | |
| PGM | Fis | 0.3378 | 0.3616 | 2.0204 | 21 | 24 | |
| | Fst | 0.1457 | 0.7755 | 3.0404 | 2 | 21 | ns |
| | Fit | 0.4343 | 0.3049 | 2.3789 | 23 | 24 | |

Table 5. Matrix of pairwise genetic identities and jackknifed standard errors calculated between *Callosamia promethea*, *C. angulifera* and *C. securifera*

| | *C. promethea* | *C. angulifera* | *C. securifera* |
|---|---|---|---|
| *C. promethea* | 1.00 | | |
| *C. angulifera* | 0.76 ± 0.08 | 1.00 | |
| *C. securifera* | 0.77 ± 0.08 | 0.79 ± 0.08 | 1.00 |

## Discussion

Despite the ease with which hybrids of all three *Callosamia* species can be produced, the genomes of these taxa are quite distinct, as evidenced by the presence of fixed allelic differences. The fixed differences between *C. promethea* and *C. angulifera* held true even in samples collected from a location where both moths were observed to be common (Cass Co. MI). Our electrophoretic results support the generally accepted view that reproductive isolation between the three species in the wild is complete (Ferguson 1972, Peigler 1980).

The estimated genetic identities between the three species range from 0.76 - 0.79, and are well within values reported for other congeneric Lepidoptera (Hagen and Scriber 1991). The interspecific compatability, at least to the F1 generation, reflects a fairly typical flexibility in mating compatability in the Attacine Saturniids in general. Intergeneric hybrids have been obtained between *C. promethea* and *Hyalophora cecropia*, and all three *Callosamia* species X *Samia cynthia* (Peigler 1978), despite the fact that these genera have been reported to have different chromosome numbers (Robinson 1971). There appears to be little chemical differentiation of mating pheromone, as well, since there are reports of cross-reactivity among the *Callosamia* species, and even between *C. promethea* and *Hyalophora cecropia* (Rau and Rau 1929). Although Wright's F-statistics were calculated, the small sample sizes reduced our confidence in any interpretations.

Because *Hyalophora* has so few shared alleles with *Callosamia*, it was not considered to provide a robust outgroup comparison in this study. The lack of a good outgroup, in combination with the equal genetic differentiation between the three *Callosamia* species, makes it impossible to determine which taxa might be ancestral. Although there has been some speculation that *C. angulifera* is the primitive member of the genus due to its nocturnal habit, Michener (1952) dismissed this idea, since the nocturnal/diurnal trait has apparently undergone frequent reversals in other Saturniids. The electrophoretic dissimilarity between *Callosamia* and *Hyalophora* may be an indication that these taxa are not as closely related as presumed. Although *Hyalophora* have occasionally been reported to feed on tuliptree, larval survival on it is generally quite low (Scarbrough et al. 1974, Manuwoto et al. 1985). *Callosamia* may be more closely allied with the Asiatic *Samia*, which also possess the ability to use tuliptree as a host (Stone 1991).

# CHAPTER TWO

## Phenylpropanoid phenolics in sweetbay magnolia as chemical determinants of host use in Saturniid silkmoths (*Callosamia*)

### Abstract

Host plant chemistry often plays an important role in determining the evolution of host use patterns in herbivorous insects. We tested the hypothesis that specialization on sweetbay magnolia within the wild silkmoth genus *Callosamia* is associated with physiological adaptations to host allelochemicals. Although all three closely related *Callosamia* species feed on magnoliaceous hosts, only the monophagous *C. securifera* survives on sweetbay (*Magnolia virginiana*). In laboratory assays with intact foliage, both *C. angulifera* and the polyphagous *C. promethea* fed readily on sweetbay but were unable to survive beyond the third instar. We identified two neolignan compounds, magnolol and a biphenyl ether, that reduce neonate growth and survival of the unadapted herbivores when painted on acceptable host leaves at concentrations similar to those in sweetbay. Both compounds significantly reduced neonate growth in *C. angulifera* and *C. promethea* but had no effect on the sweetbay specialist, *C. securifera*.

## Introduction

Plant chemistry was postulated to be a major determinant of the evolution of host plant patterns in phytophagous insects early in the development of plant-herbivore theory (Fraenkel 1959, Ehrlich and Raven 1964). Although the relative importance of plant chemistry in mediating insect-plant interactions remains controversial as the influence of natural enemies and other ecological restrictions on host use patterns have gained recognition (Lawton and McNeill 1979, Price et al. 1980;1986, Stamp 1984, Strong et al. 1984, Bernays and Graham 1988, Scriber and Lederhouse 1992, Schultz 1992), there is ample evidence that host characteristics can profoundly influence the evolution and diversification of phytophagous taxa (Benson et al. 1975, Mitter and Brooks 1983, Farrell and Mitter 1990).

Repeated demonstration of the importance of host chemistry for host selection, feeding behavior and growth of insects supports the idea that the evolution of insects onto novel or changing hosts may be constrained by behavioral and physiological adaptation to plant chemicals. Plant allelochemicals appear to have played a central role in the evolution of host patterns in *Papilio* butterflies (Feeny et al. 1983, Feeny 1987; 1991, Miller 1987, Scriber 1988), leaf beetles (Futuyma and McCafferty 1990), *Euphrydras* checkerspot butterflies (Bowers 1988), and *Heliconius* butterflies (Spencer 1988). In particular, the concept of toxic and deterrent compounds acting as chemical constraints or barriers to host use remains a prominent feature in discussions of phylogenetic patterns of host use (Miller 1987, Berenbaum et al. 1989, Feeny 1991, Scriber et al. 1991, Nitao et al. 1992).

An expansion or shift to a novel hostplant requires the metabolic capacity to utilize the particular nutritional and chemical characteristics of the new host as well as changes in oviposition and larval feeding behavior. Specific allelochemicals may act as chemical barriers if the sensory, detoxicative or other metabolic characteristics of the herbivore are inadequate for dealing with them effectively. Comparative measures of the effects of plant compounds on adapted herbivores and insects not associated with the plant in nature have proven especially valuable for identifying metabolic constraints on host use (Erickson and Feeny 1974, Blau et al. 1978, Berenbaum 1978; 1981, Miller and Feeny 1983, 1989, Lindroth et al. 1986, Lindroth 1988, Scriber et al. 1989, Nitao et al. 1992).

Although the wild silkmoth genus *Callosamia* (Saturniidae) as a group uses Magnoliaceous hosts, individual moth species differ dramatically in their ability to survive on sweetbay, *Magnolia virginiana*. The three closely related *Callosamia* species differ in host breadth: the specialist *C. securifera* feeds almost exclusively on sweetbay, the oligophagous *C. angulifera* uses primarily tuliptree (*Liriodendron tulipifera*: Magnoliaceae), and the polyphagous *C. promethea* feeds on deciduous trees in more than five plant families, including black cherry (Rosaceae), sassafras (Lauraceae), tuliptree (Magnoliaceae), ash and lilac (Oleaceae), buttonbush (Rubiaceae) and others (Ferguson 1972, Peigler 1976, Stone 1991). Although sweetbay occurs within the geographic distributions of the two *Callosamia* species with broader feeding habits, neither *C. promethea* (with the exception of some populations in Maryland, H. Pavulaan, personal communication) nor *C. angulifera* use it as a host. Laboratory observations of early instar feeding behavior led to the hypothesis that sweetbay foliage is toxic to unadapted herbivores. Using a

bioassay directed fractionation procedure, an effort was made to confirm and characterize the phytochemical basis for the differential performance of the three *Callosamia* species on sweetbay.

## Materials and Methods

### *Bioassays on fresh leaves*

The relative suitabilities of tuliptree, sassafras and sweetbay as hosts for each *Callosamia* species was evaluated in June 1989 using measures of neonate growth rates and survival on intact foliage. Newly eclosed larvae were weighed and reared individually in petri dishes on detached leaves kept fresh with water-filled plastic vials with rubber caps. The relative growth rate of each larva was calculated as RGR = [ln (final mass) - ln (initial mass)/ 2 days]. First instar survival was determined by the number of larvae that successfully completed the molt to the second stadium. Tuliptree and sassafras foliage used in the feeding trial was collected in Ingham Co. MI; sweetbay foliage was from potted trees (originally obtained from Herren Nursery, Florida Division of Forestry) maintained in a greenhouse at Michigan State University for several years. Insects used in the assays on host foliage were from five wild-collected *C. securifera* females (Highlands Co. FL), one *C. promethea* (WI) and one *C. angulifera* female (Lebanon Co. PA). While better representation of populations would have been desirable, these were the gravid females that could be obtained at the time. Larvae were distributed evenly across the three hosts (25 to 30 larvae per host) and reared at 24° C, 18:6 light:dark photoperiod. Because the *C. promethea* trial was conducted ten days earlier than the other two species, each species trial was analyzed separately. Significance of differences in growth rates on sassafras, tuliptree and sweetbay were tested by one way analysis of variance followed by Tukey's multiple range test. Differences in percent survival were not analyzed because the trial was not replicated.

*Isolation, separation and bioassay of extract from M. virginiana leaves.*

Fresh sweetbay leaves (mixture of young and old) were removed from trees, freeze-dried, and milled to a fine powder. The leaf powder (435 g) was sequentially extracted at room temperature with hexane (6 l), ethyl acetate (6 l) and methanol (2 l). Removal of solvent *in vacuo* yielded 36.9 g, 16.9 g, and 54.34 g of residue, respectively.

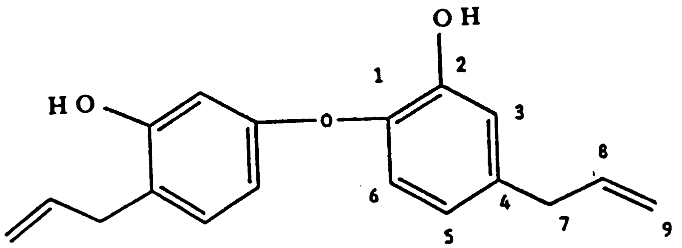
Because *Callosamia* larvae do not accept artificial diet, extracts were painted on fresh leaves of tulip tree, which is an acceptable host for both *C. promethea* and *C. angulifera.* Extracts were dissolved in an appropriate solvent (hexane or methanol) and applied to the top surface of detached leaves with a syringe. In order to approximate concentrations in fresh *M. virginiana* leaves, the volume of extract applied to each leaf was adjusted according to the fresh weight of that leaf, resulting in a 0.5 % concentration (g extract per g fresh leaf). Control leaves were treated with an equivalent volume of the appropriate solvent. Freshly treated leaves were added after 24 h or as needed.

Newly eclosed larvae were weighed and allowed to feed individually on treated leaves in plastic cups for 72 h at 24° C, 18:6 light-dark photoperiod, after which they were re-weighed and 48 h relative growth rate calculated as described above. A 1 cm layer of moistened plaster of Paris in the bottom of each cup served to maintain moisture and leaf turgor during the experiment. Larvae used in bioassays were obtained from field-collected or first generation laboratory females (*C. promethea* were from Cass Co. and Otsego Co. MI; *C. angulifera* were wild females from Greensville Co., VA and Florence Co., SC). Due to the difficulty of coordinating egg hatch of female moths to obtain similar-aged neonates, only two families of each species were represented in each assay. Ten to fifteen neonates were used in each treatment. Tukey's HSD multiple comparison test was used to detect differences ( $P < 0.05$) in growth rate of each species on the extracts.

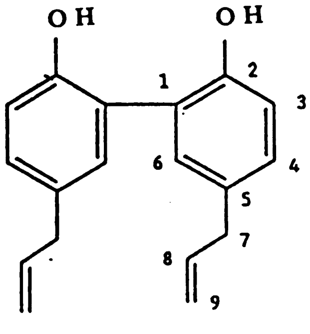
*Isolation and bioassay of individual compounds from active fraction*

The hexane fraction (12.78 g) of sweetbay extract was found to be the most active against neonates, so its constituents were further separated using centrifugal partition chromatography (Sanki Engineering, Kyoto) using the lower phase of hexane:acetonitrile:ethyl acetate:water (40:35:25:5) partition as the mobile phase at a flow rate of 3.2 - 3.8 mls/min at 900 rpm. Three major compounds were purified by flash column (Sigma silica 230-400 mesh, 60 A eluted with hexane:ether 4:1) and preparatory thin layer chromatography (silica gel, 2000um tapered Uniplate, Analtech, Newark, Delaware developed twice in hexane:ether 4:1). The final purification yielded three neolignan compounds: 4, 4'-diallyl-2,3'-dihydroxybiphenyl ether (147.8 mg), 5,5'-diallyl-2,2'-dihydroxybiphenyl (100mg) and 3,5'-diallyl-2'-hydroxy-4-methoxybiphenyl (207.6 mg). These compounds were identified from their NMR, MS, UV and IR spectra (Nitao et al. 1991). Structures for these compounds are shown in Figure 2 and hereafter they are referred to as the biphenyl ether ( 1 ), magnolol ( 2 ) and methoxyhonokiol ( 3 ), respectively.

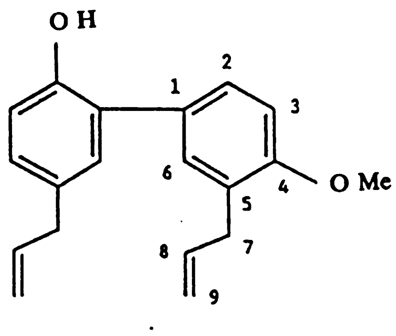
The biphenyl ether, magnolol and methoxyhonokiol were bioassayed at 0.03%, 0.125% and 0.5% (g compound per g fresh tulip tree leaf) against *C. promethea* neonates using the leaf-painting technique described previously. Natural concentrations of these compounds in sweetbay foliage were estimated to be 0.46, 0.31 and 0.64 % fw respectively from our extraction procedure, but dose response curves are more informative than single dose assays of plant secondary metabolites because concentrations of compounds in plants in the field may vary considerably. Assays were replicated at least three times using *C. promethea* collected from distantly separated geographic locations (Montgomery Co., MD; Lebanon Co., PA; several locations in WI and MI). Because *C. angulifera* were difficult to obtain, only magnolol was tested against this species. (*C. angulifera* larvae were from wild females collected in Cass Co. MI; Greensville Co., VA; and MS). In the *C. angulifera* assays, magnolol was assayed at five doses rather than three (0.1, 0.2, 0.3, 0.4, and 0.5% g compound per g fresh leaf). As an additional check of the leaf painting technique, the responses of *C. securifera* neonates (several families from Highlands Co. FL) to magnolol and the biphenyl ether were assayed at 0.1, 0.3 and 0.5%. Larval response to host chemicals was measured in terms of 48 h relative growth rate. Data from the replicate assays were pooled and a polynomial regression model used to test for linear and quadratic dose-dependent effects (SAS Institute, 1985).



biphenyl ether



magnolol



3,5'-diallyl-2'-hydroxy-
4-methoxybiphenyl

Figure 2. Structures of the major consituents (the biphenyl ether, magnolol and methoxyhonokiol) identified from the sweetbay fraction active against larvae of unadapted *Callosamia* silkmoth species.

## Results

### *Bioassays on intact hostplants*

Relative growth rate and first instar survival of both *C. promethea* and *C. angulifera* were considerably reduced on sweetbay compared to the two acceptable hosts (Figure 3), whereas *C. securifera* performed as well as or significantly better on sweetbay than on tuliptree and sassafras. First instar survival for *C. promethea* and *C. angulifera* was less than 30% on sweetbay but ranged from 90 - 100% on acceptable hosts. *C. securifera* survival was greater than 70% on sweetbay and ranged from 62 - 85% on sassafras and tuliptree. The patterns of short term larval growth rates corresponded closely with larval survival, with the exception of *C. securifera*'s response to tuliptree. On this host, growth was significantly lower than on sweetbay and sassafras although survival remained high. No conspicuous differences in larval feeding rate were observed, suggesting that the reduced growth and survival were not entirely due to strong antifeedant effects.

### *Separation and bioassay of crude extract.*

Of the three major fractions, the hexane extract produced the greatest reduction in larval growth rate (one-way ANOVA, *C. promethea* $F = 7.86$; df = 3; $P < 0.0001$, *C. angulifera* $F = 9.79$; df = 3; $P < 0.0001$). *C. promethea* and *C. angulifera* responded similarly to all three fractions with a significant reduction of growth on leaves treated with the hexane extract compared to controls (Figure 4. *C. promethea* (mean ± SE) 0.26 ± 0.04 $\text{mg} \cdot \text{mg}^{-1} \cdot \text{d}^{-1}$; *C. angulifera* 0.34 ± 0.03 $\text{mg} \cdot \text{mg}^{-1} \cdot \text{d}^{-1}$). *C. angulifera* growth on leaves painted with the ethyl acetate fraction was also lowered compared to control (0.45 ± 0.03 $\text{mg} \cdot \text{mg}^{-1} \cdot \text{d}^{-1}$).

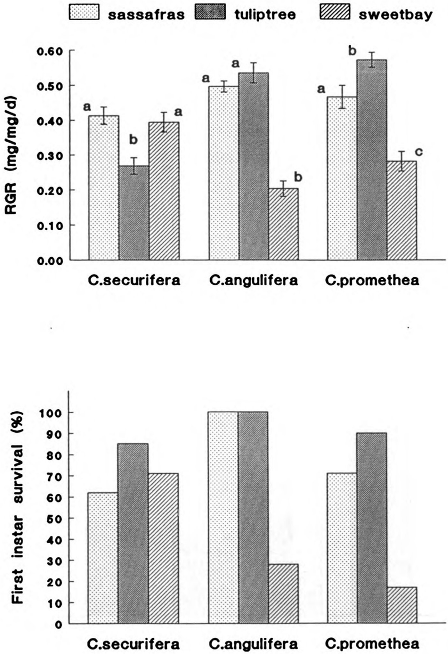


Figure 3. First instar relative growth rates (48 h) and survival of the three *Callosamia* species on sassafras, tuliptree and sweetbay.

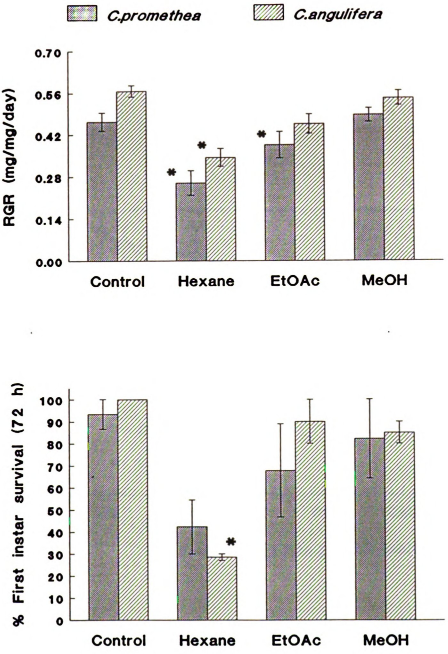


Figure 4. First instar growth (48 h) and survival of the two unadapted silkmoth species on tuliptree leaves painted with sweetbay extract (equivalent to 0.5 % g/g fw). Mean ± SE of 3-5 replicates with 5-10 larvae per replicate.

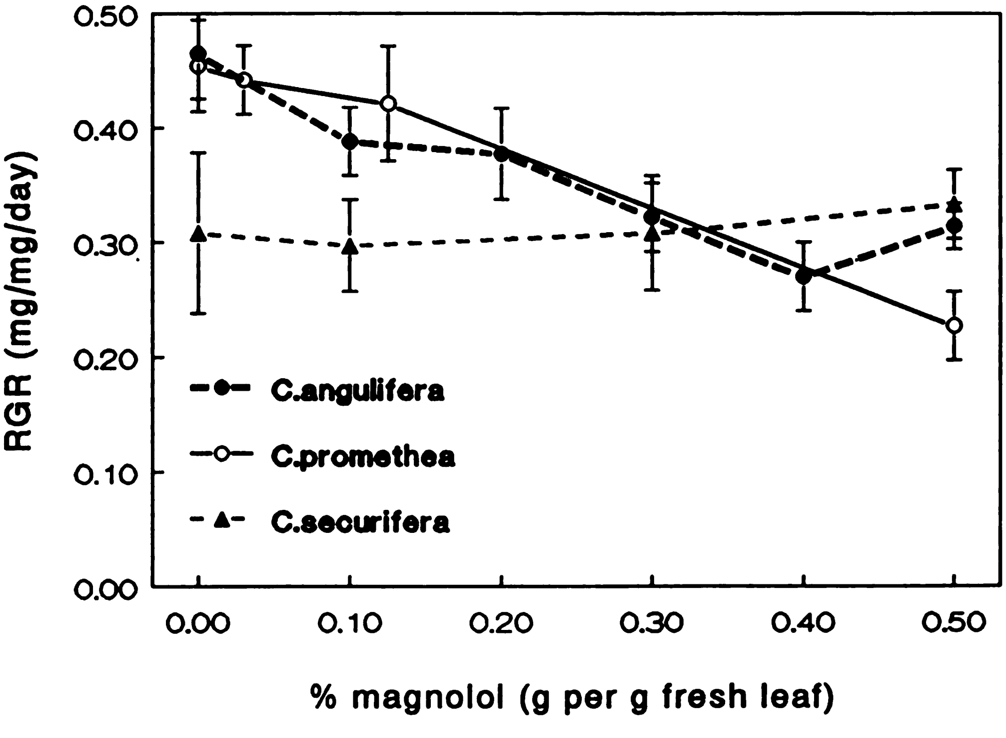


Figure 5. First instar growth of *Callosamia* species on tuliptree leaves painted with magnolol. Mean ± SE of 3-5 replicates of 5-10 larvae. *P* value indicates significance of linear dose-dependent effects.

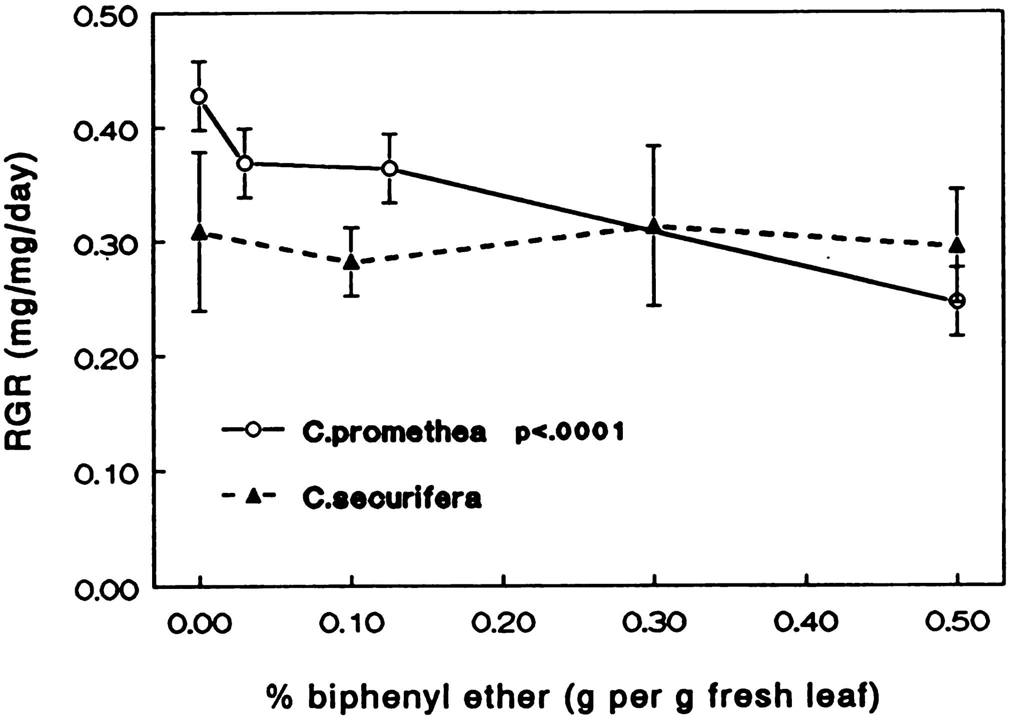


Figure 6. First instar growth of *Callosamia* species on tuliptree leaves painted with the biphenyl ether. Mean ± SE of 3-5 replicates of 5-10 larvae. *P* value indicates significance of linear dose-dependent effects.

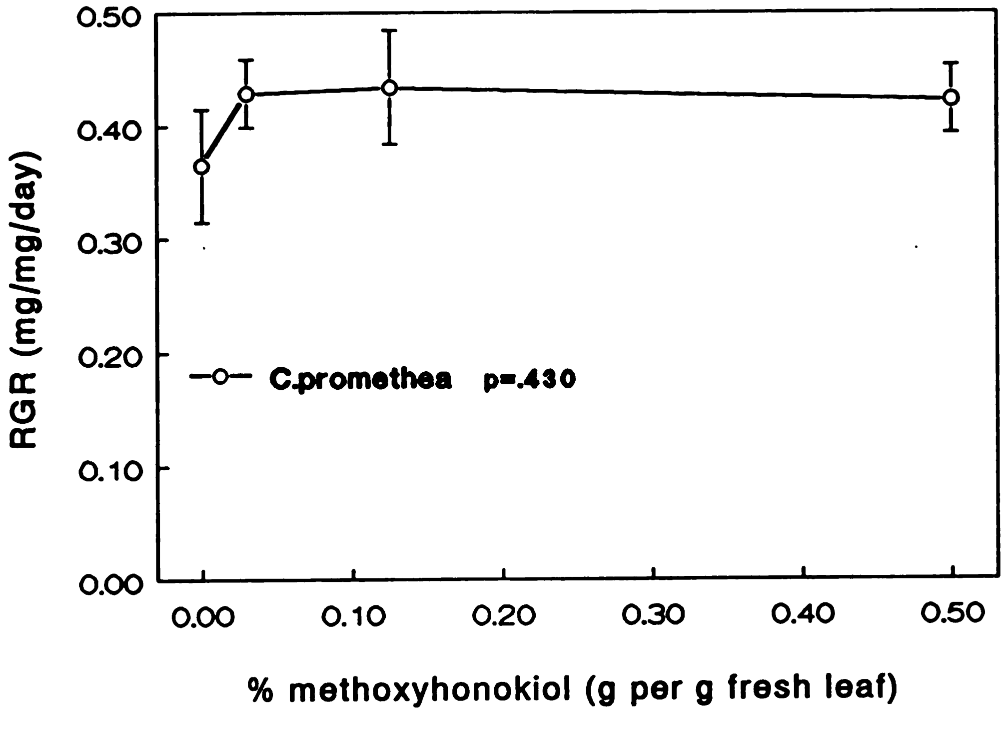


Figure 7. First instar growth of *Callosamia* species on tuliptree leaves painted with methoxyhonokiol. Mean ± SE of 3-5 replicates of 5-10 larvae. *P* value indicates significance of linear dose-dependent effects.

Percent survival of both species was lowest on leaves treated with the hexane fraction (*C. promethea* 42.3 ± 12.3 $mg \cdot mg^{-1} \cdot d^{-1}$; *C. angulifera* 28.5 ± 1.5 $mg \cdot mg^{-1} \cdot d^{-1}$).

*Isolation and bioassay of individual compounds from the active hexane fraction*

Magnolol and the biphenyl ether were active against larvae of the unadapted species at concentrations approximating those found in fresh leaf material (which were 0.31 and 0.46 % fw for magnolol and the biphenyl ether respectively). Relative growth rates of *C. promethea* larvae were significantly decreased by both magnolol (Figure 5. Linear response: $F = 14.31$; $df = 1,75$; $P = 0.0003$) and the biphenyl ether (Figure 6. Linear response: $F = 18.89$; $df = 1, 74$; $P = 0.0001$) but not by methoxyhonokiol (Figure 7. Linear response: $F = 0.64$; $df = 1, 30$; $P = 0.430$). *C. angulifera* neonate growth was also significantly decreased by magnolol (linear response: $F = 18.01$; $df = 1, 149$; $P = 0.0001$) in a dose-dependent fashion. As expected, *C. securifera* larvae were unaffected by magnolol and the biphenyl ether across all doses.

## Discussion

Our results demonstrate that foliar chemistry strongly influences the performance of *Callosamia* species on sweetbay. The poor growth and survival of *C. promethea* and *C. angulifera* on sweetbay can be explained by the activity of two neolignan compounds, magnolol and the biphenyl ether isolated from the foliage. When assayed individually at doses similar to those found in fresh leaf material, both compounds reduced larval growth to levels similar to those observed on intact foliage. Neither compound has been reported from tuliptree, a magnoliaceous host used by *C. promethea* and *C. angulifera*, nor are they detectable in hexane extract of fresh tuliptree foliage by thin layer chromatography (K. S. Johnson, unpublished). Potentially, the presence of either or both of these compounds could block host shifts by herbivores that lack specific metabolic mechanisms to cope with them, even within lineages that are adapted to utilize Magnoliaceous hosts, such as *Callosamia*. In this taxon, the use of sweetbay apparently requires specialized metabolic adaptations to allelochemicals other than those that accompany the ability to feed on tuliptree or those associated with a highly polyphagous feeding habit.

While host chemistry may constrain host use through behavioral or metabolic effects on some herbivores (Bernays and Chapman 1987, Feeny 1991), we saw no evidence of strong antifeedancy in our assays. Larvae appeared to feed equally on treated and control leaves; however, distinguishing toxic from behavioral responses (antifeedancy) in insect bioassays is often difficult (Blau et al. 1987). However, many lignans have been reported to have biological activity (McRae and Towers 1984, Bernard et al. 1989, Faure et al. 1990), and both neolignans isolated in this study appear to have a broad spectrum of antimicrobial (Clark et al. 1981) and insecticidal activity (Nitao et al. 1991, Nitao et al. 1992).

In our assays, the biphenyl ether was somewhat phytotoxic to the experimental leaves, causing browning at higher doses. Thus, there may have been additional changes in leaf chemistry that enhanced or confounded the effect of the biphenyl ether itself on *Callosamia* larvae. However, we would expect any artifactual effects to affect *C. securifera* as well; the fact that they were not affected by the sweetbay compounds further supports our hypothesis that the compounds themselves are toxic to insects that are not adapted to cope with them in their diet. In a separate study, leaves treated with the biphenyl ether (exhibiting similar browning) and magnolol at comparable doses had no effect on growth of tiger swallowtail (*Papilio glaucus*, Papilionidae) larvae from populations adapted to feed on sweetbay (Nitao et al. unpublished), which we interpret as further evidence that leaf browning in itself does not have significant toxic effects.

In these bioassays of *C. promethea* from different geographic locations (WI, MI, PA, MD), we found no evidence of the ability to utilize sweetbay as a host, with the exception of a population near Rockville, Maryland, where cocoons were found locally on sweetbay. However, neonates from these populations appeared to be as susceptible to deleterious effects of magnolol and the biphenyl ether as other *C. promethea* populations (0.5 % biphenyl ether reduced RGR from 0.41 mg/mg/d on control leaves to 0.19 mg/mg/d; 0.5 % magnolol reduced RGR from 0.37 on control leaves to 0.21 mg/mg/d).

It is possible that sweetbay plants in Maryland contain lower concentrations of neolignans than trees in other geographic regions. We chose to assay the isolated compounds across a range of doses because nothing is known about the quantitative variation of these compounds in plants in the field. Secondary plant metabolites exhibit considerable spatial, temporal, intra- and inter-plant variation in response to genetic and environmental factors. Sweetbay magnolia grows in a variety of habitats across its range and can be considerably plastic in growth habit; there may be geographic differences in foliar chemistry as well.

The differential responses of *Callosamia* species to magnolol and the biphenyl ether suggest that adaptation of *C. securifera* to sweetbay has involved specialized biochemical and/or physiological traits that afford larvae a greater tolerance of allelochemicals in this host. However, caution must be exercised when attempting to attribute the evolution of host use patterns to single causal determinants, such as current host chemistry (Barbosa 1988, Rausher 1988, Thompson 1988). Without accurate knowledge of the phylogenetic relationship of the three *Callosamia* taxa, interpretation of current ecological responses to allelochemicals in an evolutionary context is difficult. We have little information about ancestral and derived traits in *Callosamia*; estimates of genetic distance from allozyme electrophoresis (K. S. Johnson et al., in preparation), indicate that the three species are essentially equidistant from each other. This contrasts with our initial assumption that *C. securifera* was more closely related to *C. angulifera*, based on its historical placement as a subspecies of the latter by many taxonomists until the temporal reproductive isolating mechanism was described (Brown 1972, Ferguson 1972). Gene flow between the three species is prevented by diurnal separation of mating times: *C. securifera* females call in late morning, *C. promethea* in late afternoon, and *C. angulifera* mate after dark. Although the physiological mechanisms controlling mating time are unknown, it would not be difficult to imagine that a relatively simple change may be involved, and that it may have produced more or less simultaneous isolation of the three species.

Regardless of whether the differential ability of the three *Callosamia* species to cope with sweetbay allelochemicals played a role in speciation (e.g., selection against hybrids) or developed after reproductive isolation, an understanding of the specific biochemical/physiological adaptation of *C. securifera* to magnolol and the biphenyl ether should provide a better basis for evaluating the role of these compounds in the evolution of host use patterns in *Callosamia*.

# CHAPTER THREE

## Comparative detoxification of host allelochemicals (*Magnolia virginiana*: Magnoliaceae) by generalist and specialist Saturniid silkmoths

### Abstract

Sweetbay magnolia (*Magnolia virginiana*) contains two biologically active neolignan compounds (magnolol and a biphenyl ether) that apparently prevent unadapted insects from feeding on this host. To better understand the evolution of host use and feeding specialization in herbivorous insects, we compared the detoxification capability of the sweetbay silkmoth (*Callosamia securifera*), a specialist on sweetbay, with that of two closely related species, *C. angulifera* and the polyphagous *C. promethea*. *In vitro* degradation of the biphenyl ether by *C. securifera* midgut homogenate was NADPH dependent and inhibited by piperonyl butoxide, suggesting the involvement of cytochrome P450 enzymes. Degradation of magnolol was NADPH dependent but not inhibited by piperonyl butoxide. The sweetbay specialist degrades both compounds more rapidly than either of the unadapted silkmoth species. We were unable to induce higher degradation activity in *C. promethea* with pentamethylbenzene or a mixture of magnolol and the biphenyl ether, nor did activity vary significantly when larvae were reared on different hostplants. Use of sweetbay in this group of Saturniids is correlated with the ability to rapidly degrade specific host allelochemicals. Cytochrome

P450 activity is more closely associated with host plant chemistry than herbivore diet breadth.

## Introduction

The ability of herbivores to tolerate or detoxify ingested plant allelochemicals is thought to be an important determinant of ecological and evolutionary patterns in host use. In herbivorous insects, detoxification of plant compounds occurs via a number of oxygenases, reductases, hydrolases and transferases that are capable of metabolizing plant compounds and presumably evolved for that purpose (Brattsten 1979, Dowd et al. 1983, Ahmad et al. 1986, Lindroth 1990). Biochemical detoxification of host allelochemicals has been a central feature of hypotheses concerning the evolution of specialist and generalist feeding habits as well as phylogenetic patterns of host utilization patterns within taxa. Early theories regarding the evolution of host use patterns proposed that physiological or biochemical characteristics of herbivores evolve in response to toxins in their hosts (Ehrlich and Raven 1964), and these characteristics may constrain them to a subset of plants in the environment or preadapt them to colonize novel hosts (Janzen 1980).

In particular, the microsomal cytochrome P-450 based mono-oxygenase (or polysubstrate monooxygenase, PSMO) system serves as an important general-purpose detoxification mechanism for metabolizing host allelochemicals (Ahmad 1979, Brattsten 1979). The broad substrate specificity and inducible nature of the PSMO system is consistent with its proposed central role in polyphagous feeding habits, since generalist herbivores can be expected to encounter a broad array of secondary metabolites in their diet (Krieger et al. 1971, Ahmad 1983). Early comparisons of PSMO activity in generalists and specialist insects supported this hypothesis, and led to the generalization that PSMO activity is correlated with diet breadth in herbivorous insects (Krieger et al. 1971, Ahmad 1983, Yu 1987).

However, subsequent studies have shown that PSMO and other detoxification enzyme activities are not always correlated with a broader host range. Examples of equal or higher activity in specialist herbivores have been demonstrated for PSMO-based detoxification activity (Berenbaum 1991, Bull et al. 191986, Neal 1987, Nitao 1989, Rose 1985; Lindroth 1991), glutathione transferases (Lee and Berenbaum 1992, Gunderson et al. 1986, Wadleigh and Yu 1988) and various antioxidant enzyme systems (Aucoin et al. 1991, Berenbaum 1991, Lee and Berenbaum 1992, Pardini et al. 1989). Differences between detoxification activities of specialists and generalists appear to be correlated with specific chemical characteristics of the hostplant, and perhaps involve differences in inducibility and flexibility rather than peak levels of activity (Berenbaum 1991).

The absence of a strong correlation between detoxification enzyme levels and diet breadth has important implications for the view that metabolic costs associated with detoxification can influence the evolution of host specialization and other feeding patterns. Specific hypotheses concerning the role of detoxification enzymes in the evolution of host use patterns, such as whether generalist detoxification enzymes are more inducible or have greater substrate flexibility (Berenbaum 1991), whether there are tradeoffs in metabolic efficiency or risk of bioactivation associated with detoxification, and whether specialists are more likely to be derived from generalists can be better addressed through comparative studies of specific physiological/biochemical adaptations in phylogenetically related herbivores. There are presently few comparisions of detoxification of ecologically relevant phytochemicals in phylogenetically related generalists and specialists.

The wild silkmoth genus *Callosamia* (Saturniidae) serves as a good model system for simultaneously examining the relationship between diet breadth, host chemistry and patterns of detoxification activity within a phylogenetic context. The genus is composed of three closely related species that display various degrees of host specialization that is mediated in part by host phytochemistry. *Callosamia promethea* is highly polyphagous, feeding on trees from over five different plant families, including black cherry (*Prunus serotina*), ash (*Fraxinus* spp.), sassafras (*Sassafras albidum*), spicebush (*Lindera benzoin*), tuliptree (*Liriodendron tulipifera*), sweetgum (*Liquidambar styraciflua*), lilac (*Syringa vulgaris*) and others. *Callosamia angulifera* is a semispecialist on a subset of hosts used by *C. promethea*, most commonly feeding on tuliptree. The third silkmoth species, *Callosamia securifera*, is a strict specialist on another magnoliaceous host, sweetbay magnolia (*Magnolia virginiana*). With rare exceptions, neither *C. promethea* nor *C. angulifera* feed on sweetbay magnolia in the wild, and are unable to survive on it in laboratory assays; at one location in Maryland, *C. promethea* cocoons were found on sweetbay (H. Pavulaan, personal communication). Sweetbay foliage contains two biologically active neolignan compounds, magnolol and a biphenyl ether, that are toxic to insects (Nitao et al. 1991, 1992). When painted on leaves of an acceptable host (tuliptree) at concentrations equivalent to those found in sweetbay, both compounds reduced

the growth and survival of early instar *C. promethea* and *C. angulifera*, as well, but had no effect on *C. securifera* (Johnson et al. unpublished). The use of sweetbay by *Callosamia* appears to require physiological and/or biochemical adaptation to these toxic allelochemicals.

The purpose of this study was to evaluate the adaptive significance of enzymatic detoxification systems in the evolution of host use patterns in the genus *Callosamia*. We first characterized the detoxification of magnolol and the biphenyl ether in the sweetbay feeder, *C. securifera*, then evaluated its adaptive significance by comparing detoxification activity in this specialist with that of the two closely related, but unadapted silkmoths, *C. promethea* and *C. angulifera*.

## Materials and methods

### Insect rearing

Larvae used in experiments were first or second generation from wild collected adults or cocoons. *C. securifera* larvae originated from adults collected in Lake Co. and Highlands Co., FL.; *C. promethea* originated from populations at various sites in southern WI and MI. *C. angulifera* larvae were from a wild female captured at a blacklight in southern MI. Larvae were fed fresh foliage collected locally (tuliptree, sassafras and black cherry trees in Ingham Co., MI), or from potted trees (sweetbay originally obtained from Herren Nursery, Florida Division of Forestry) maintained at Michigan State University for several years. Larvae were reared in groups of 5 - 10 in plastic shoeboxes (10 cm x 20 cm x 27 cm) on leaves kept fresh with water-filled plastic vials with rubber caps at 24° C on a 18:6 light:dark photoperiod.

## Isolation of allelochemicals

Magnolol and the biphenyl ether used in the *in vitro* metabolism experiments were extracted and purified from sweetbay magnolia leaves (see methods, Chapter 2). Freeze-dried leaves were milled to a powder and sequentially extracted with hexane, ethyl acetate, and methanol. Magnolol, methoxyhonokiol and the biphenyl ether were isolated from the hexane extract using centrifugal partition chromatography and further purified by flash column (Sigma silica 60 A eluted with hexane:ether 4:1) and preparatory thin layer chromatography (silica gel, 2000 um tapered Uniplate, Analtech, Newark, Delaware developed twice in hexane:ether 4:1). For the *in vitro* metabolism experiments, magnolol and the biphenyl ether were presented as a 2:3 mixture to approximate the natural proportions extracted from sweetbay foliage (Nitao et al. 1991).

## Mechanism of detoxification of sweetbay neolignans in three *Callosamia* species

### *NADPH dependency*

Because larvae of the three species were not available simultaneously, NADPH dependency of magnolol and the biphenyl ether metabolism by *C. securifera*, *C. promethea* and *C. angulifera* midgut tissue was measured in three separate experiments. Midguts from two to four day old third instar larvae were removed, sliced longitudinally and rinsed with chilled 0.1 M potassium phosphate buffer (pH 7.8; EDTA). Groups of five to ten midguts were homogenized with ten strokes in a hand-held Ten Broeck homogenizer in approximately 1.0 ml phosphate buffer per g larval wet mass, and the resulting homogenate assayed immediately. An aliquot of each homogenate was set aside for protein estimation using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond CA 94804).

Detoxification of magnolol and the biphenyl ether was estimated from the disappearance of the parent compound during *in vitro* incubation with midgut homogenate. Incubations were conducted in uncapped glass scintillation vials at a standardized buffer conditions (0.01 M potassium phosphate at pH 7.8) and tissue:substrate concentrations. Reactions were begun with the addition of a 10:15 μg mixture of magnolol:biphenyl ether in 20 μl methanol to an incubation mixture of potassium phosphate buffer with or without 0.3 mM NADPH (with the exception of the NADPH dependency experiment with *C. securifera*, in which 1.0 mM was used due to an error in calculations) and 1.0 - 3.0 mg protein in a total volume of 1.0 ml. After 30 minutes at 30° C in a shaking water bath, reactions were stopped by adding 250 μl 1.0 N hydrochloric acid and immediately chilling the vials. Two controls consisting of inactivated (boiled) homogenate were included, one with NADPH and one without. Each incubation was done in duplicate and the experiment was replicated two to six times for each species over a period of two months.

The amount of magnolol and biphenyl ether remaining in each vial after the incubation period was extracted and quantified by high-pressure liquid chromatography. After addition of a standard to measure extraction efficiency (10 μg of methoxyhonokiol), samples were extracted with two volumes of ethyl acetate, dried under an airflow, then redissolved in 500 μl methanol. Samples were filtered through a nylon filter (Alltech 0.45 U) prior to injection into a Beckman Series model 167 high-performance liquid chromatograph with ultraviolet detector. Magnolol, biphenyl ether and methoxyhonokiol were separated on a C-18 column (150 mm X 4.6 mm OD) eluted with 80% methanol at a flow rate of 1.5 ml/min and quantified by comparison with standard curves. Unrecovered compound was assumed to have been metabolized.

Adjusting data by recovery of the internal standard did not reduce the amount of variation or significance of treatments, so values were not corrected before statistical analysis. Significant differences in the amount of magnolol and biphenyl ether degraded by active tissue with and without NADPH were determined by *t*-test at $P < 0.05$.

### *Inhibition by piperonyl butoxide*

Inhibition by piperonyl butoxide, a methylenedioxyphenyl compound, is characteristic of PSMO-based detoxification reactions (Brattsten 1979). We tested the effect of piperonyl butoxide on *in vitro* magnolol and biphenyl ether degradation by *C. securifera* and *C. promethea* gut homogenate (*C. angulifera* was not included due to the unavailability of larvae). Midgut homogenate was prepared from mid-fourth instar larvae (5-7 per replicate) as described previously. 300 $\mu$l (*C. securifera*) or 100 $\mu$l (*C. promethea*) of homogenate was incubated with 10:15 ug magnolol:biphenyl ether and 0, $10^{-2}$, $10^{-3}$, or $10^{-4}$ M of piperonyl butoxide (Aldrich Chemical Company, technical grade 90%) added in 10 $\mu$l methanol and a total volume of 1.0 ml potassium phosphate buffer (pH 7.8) supplemented with 0.3 mM NADPH. A check consisting of tissue inactivated by 250 $\mu$l hydrochloric acid at the beginning of the reaction and $10^{-2}$ M piperonyl butoxide, NADPH, and substrate was included in each trial. The mixtures were incubated for 10 minutes at 30° C, then stopped by the addition of 250 $\mu$l 1.0 N hydrochloric acid and immediate chilling. Undegraded magnolol and biphenyl ether in the incubation vials were extracted and quantified as described above. Dose-dependent effects of piperonyl butoxide on magnolol and biphenyl ether degradation were analyzed separately using a polynomial regression model ANOVA model (SAS Institute 1985).

### *Induction of detoxification enzymes by sweetbay allelochemicals and pentamethylbenzene*

Two experiments were conducted to investigate whether detoxification of magnolol and the biphenyl ether in the polyphagous *C. promethea* is inducible. In the first experiment, 1-3 day old third instar larvae were fed sassafras leaves painted with a mixture of magnolol:biphenyl ether at a 2 : 3 ratio (0.2 : 0.3 % g/g fresh leaf), 0.2% pentamethylbenzene (Aldrich Chemical Company, Inc., Milwaukee, WI), or methanol for 24 h, then switched to untreated foliage for 24 h prior to *in vitro* assays. Larvae were housed individually in plastic petri dishes lined with moistened filter paper and fed leaves kept fresh in water-filled plastic vials. After dissection, larval midguts were randomly allocated to two groups for tissue preparation, so that the homogenization process was replicated twice for each treatment. The homogenization and *in vitro* assays followed procedures described previously.

The second experiment differed only in that compounds were applied to tuliptree, rather than sassafras, and larvae were allowed to feed for 48 h, rather than 24 h, before being switched to untreated leaves. Because of differences in protocol, the two experiments were analyzed separately for differences in rates of *in vitro* degradation of magnolol and the biphenyl ether using one-way ANOVA (Systat, Wilkinson 1990).

### Comparative detoxification of sweetbay neolignans by *C.securifera*, *C. promethea* and *C. angulifera*

Rates of *in vitro* degradation of magnolol and biphenyl ether were compared in third instar *C. securifera* reared on sweetbay, *C. angulifera* reared on tuliptree, and *C. promethea* reared on tuliptree, sassafras and black cherry. The larval hostplant could not be standardized because *C. securifera* survives poorly on tuliptree, and neither *C. promethea* nor *C. angulifera* can be reared on sweetbay. Assays of the three species could not be precisely synchronized because of seasonal differences in larval availability, so trials with *C. angulifera* were performed a month later (September 5-7 1992) than those of *C. securifera* and *C. promethea* (July 9-16 1992). Assays were performed in duplicate as described earlier, with two to six replicate trials conducted on different days for each species-hostplant combination. A mean rate of degradation for each species-host combination was calculated from the replicate trials, and differences between means were tested using a Tukey-Kramer test after one-way ANOVA detected significant differences (Systat, Wilkinson 1990).

## Results

### Mechanism of detoxification of sweetbay neolignans in *Callosamia*

#### *NADPH dependency and inhibition by piperonyl butoxide*

Addition of NADPH to incubation mixtures significantly enhanced degradation of the biphenyl ether in both *C. securifera* and *C. promethea* (Table 6). The effect of NADPH on biphenyl ether degradation by *C. angulifera* tissue could not be statistically analyzed due to unequal variances and small sample size, but the incubations with NADPH also had a higher mean rate of degradation of biphenyl ether. In contrast, magnolol degradation appears to be NADPH dependent only in *C. securifera*; addition of NADPH did not increase metabolism of this neolignan in *C. promethea* or *C. angulifera*.

Piperonyl butoxide inhibited *in vivo* metabolism of biphenyl ether by *C. securifera* and *C. promethea* homogenate in a dose-dependent fashion, but had no effect on magnolol metabolism in either species (Table 7). $10^{-2}$ M piperonyl butoxide reduced biphenyl ether metabolism nearly by half in *C. securifera* (Figure 8), and to about one-tenth the activity of the control in *C. promethea* (Figure 9). The degree of inhibition in *C. securifera* incubations is likely underestimated; the total disappearance of compounds in the uninhibited control indicates that the substrate was fully metabolized before the incubations were halted. Had the controls not been substrate-limited, the difference between control and piperonyl butoxide incubations may have been greater. The NADPH dependency and piperonyl butoxide inhibition of biphenyl ether metabolism are consistent with a cytochrome P450-based detoxification pathway in the sweetbay specialist (*C. securifera*) and the polyphagous *C. promethea*. Metabolism of magnolol in *C. securifera* is also apparently via cytochrome P450, but this mechanism is lacking or different in the other two species, in which degradation was neither NADPH dependent nor inhibited by piperonyl butoxide.

### *Induction of detoxification enzymes by sweetbay allelochemicals and pentamethylbenzene*

*C. promethea* larvae fed leaves treated with pentamethylbenzene or the neolignan mixture for 24 or 48 h did not metabolize the biphenyl ether or magnolol more rapidly than larvae fed control leaves treated with methanol (Table 8).

## **Comparative detoxification of sweetbay neolignans by *C.securifera*, *C. promethea* and *C. angulifera***

*C. securifera* midgut tissue degraded magnolol and the biphenyl ether two to three times more rapidly than the two species unadapted to sweetbay (Figure 10). Because the linearity of reactions over the 30 minute incubation period could not be determined, degradation rates are expressed in terms of ug/mg protein/total incubation period rather than the more conventional ug/mg/min. The mean rate of magnolol degradation was 5.9 ± 0.8 ug/mg/30 min in *C. securifera* compared to 2.3 ± 0.1 ug/mg/30 min in *C. angulifera* and 2.3 ± 0.5 ug/mg/30 min in *C. promethea* reared on tuliptree. Biphenyl ether degradation in *C. securifera* was 12.8 ± 4.8 ug/mg/30 min compared to 2.6 ± 0.1 ug/mg/30 min and 4.7 ± 1.2 ug/mg/30 min in *C. angulifera* and *C. promethea* respectively. Detoxification activity in the highly polyphagous *C. promethea* was not

Table 6. NADPH dependence of biphenyl ether and magnolol degradation *in vitro* by midgut homogenate of third instar *C. securifera*, *C. angulifera* and *C. promethea* larvae. Checks consisted of midgut homogenate inactivated (stopped) by addition of 1 N HCl at the beginning of the incubation.

| Treatment | N | µg/vial unrecovered | | |
|---|---|---|---|---|
| | | Biphenyl ether | Magnolol | Extraction efficiency |
| *C. securifera* | | | | |
| NADPH | 3 | 15.0 ± 0.0 * | 8.3 ± 0.5 * | 66.3 ± 6.4 |
| no NADPH | 3 | 5.8 ± 0.7 | 1.3 ± 0.7 | 81.7 ± 16.3 |
| NADPH stopped | 3 | 5.1 ± 1.4 | 1.6 ± 0.8 | 69.3 ± 9.1 |
| no NADPH stopped | 3 | 2.6 ± 1.6 | 0.6 ± 0.4 | 92.3 ± 12.4 |
| *C. promethea* | | | | |
| NADPH | 6 | 7.5 ± 1.5 * | 4.5 ± 1.0$^{ns}$ | 64.4 ± 2.4 |
| no NADPH | 6 | 5.6 ± 0.9 | 4.3 ± 1.0 | 64.3 ± 2.0 |
| NADPH stopped | 6 | 2.1 ± 0.4 | 2.7 ± 0.8 | 69.3 ± 3.0 |
| no NADPH stopped | 6 | 2.8 ± 0.5 | 2.6 ± 0.9 | 83.9 ± 4.7 |
| *C. angulifera* | | | | |
| NADPH | 2 | 4.2 ± 0.1 ‡ | 3.7 ± 0.02 ‡ | 52.3 ± 1.7 |
| no NADPH | 2 | 4.0 ± 0.1 | 2.7 ± 0.5 | 55.1 ± 1.1 |
| NADPH stopped | 2 | 2.2 ± 20.7 | 1.7 ± 0.005 | 58.5 ± 3.4 |
| no NADPH stopped | 2 | 0.8 ± 9.8 | 1.0 ± 0.3 | 57.7 ± 1.5 |

Mean and SE of multiple homogenization events (N) with duplicate incubations.

* Significance between NADPH and no NADPH treatments determined by t-test, $P < 0.05$.

‡ Not statistically tested due to small sample size and unequal variances.

Table 7. ANOVA of dose-dependent inhibition of biphenyl ether and magnolol metabolism by piperonyl butoxide in *C. securifera* and *C. promethea*.

*C. securifera*

| | BIPHENYL ETHER | | | | MAGNOLOL | | | |
|---|---|---|---|---|---|---|---|---|
| Source | SS | df | *F* | P | SS | df | *F* | P |
| Treatment | 167.397 | 3 | 5.99 | 0.019 | 24.124 | 3 | 2.35 | 0.124 |
| linear | 154.272 | 1 | 16.57 | 0.004 | 22.164 | 1 | 6.48 | 0.026 |
| quadratic | 11.940 | 1 | 1.28 | 0.290 | 1.954 | 1 | 0.57 | 0.464 |

*C. promethea*

| Source | SS | df | *F* | P | SS | df | *F* | P |
|---|---|---|---|---|---|---|---|---|
| Treatment | 177.616 | 3 | 8.84 | 0.031 | 15.772 | 3 | 4.41 | 0.0928 |
| linear | 162.369 | 1 | 24.24 | 0.008 | 0.655 | 1 | 0.00 | 0.4995 |
| quadratic | 10.465 | 1 | 1.56 | 0.280 | 15.070 | 1 | 12.65 | 0.0237 |

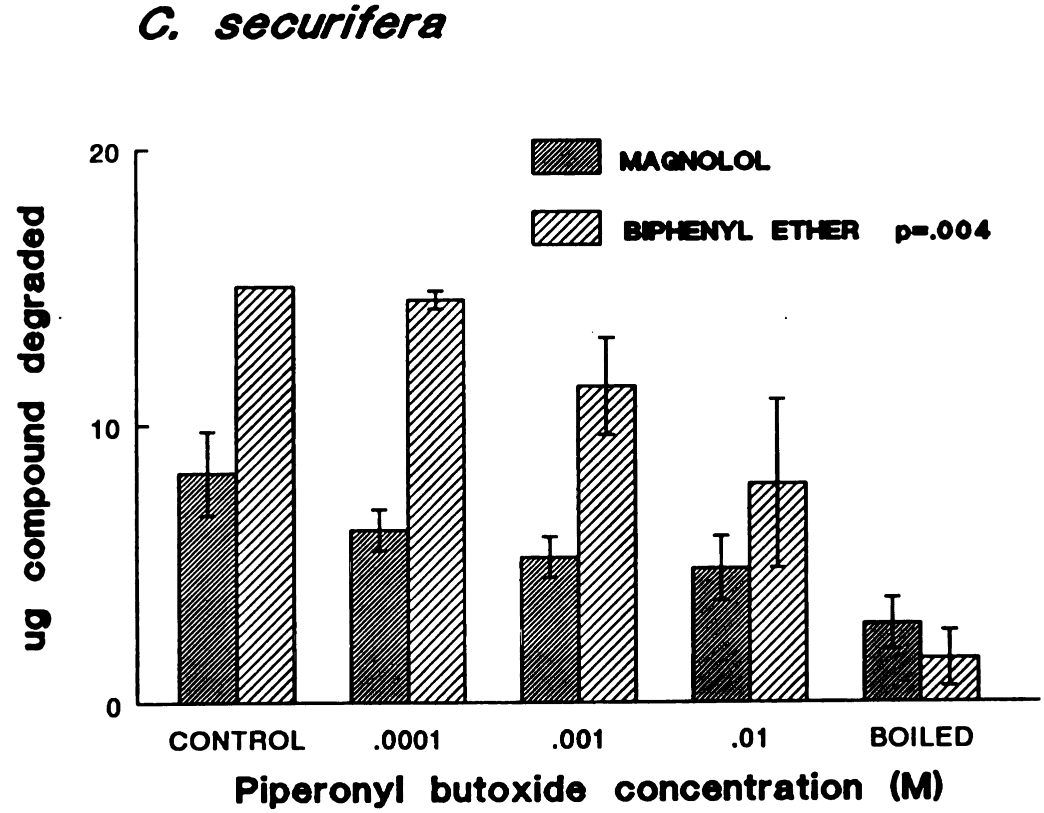


Figure 8. Piperonyl butoxide inhibition of magnolol and biphenyl ether degradation by midgut homogenate of *C. securifera*.

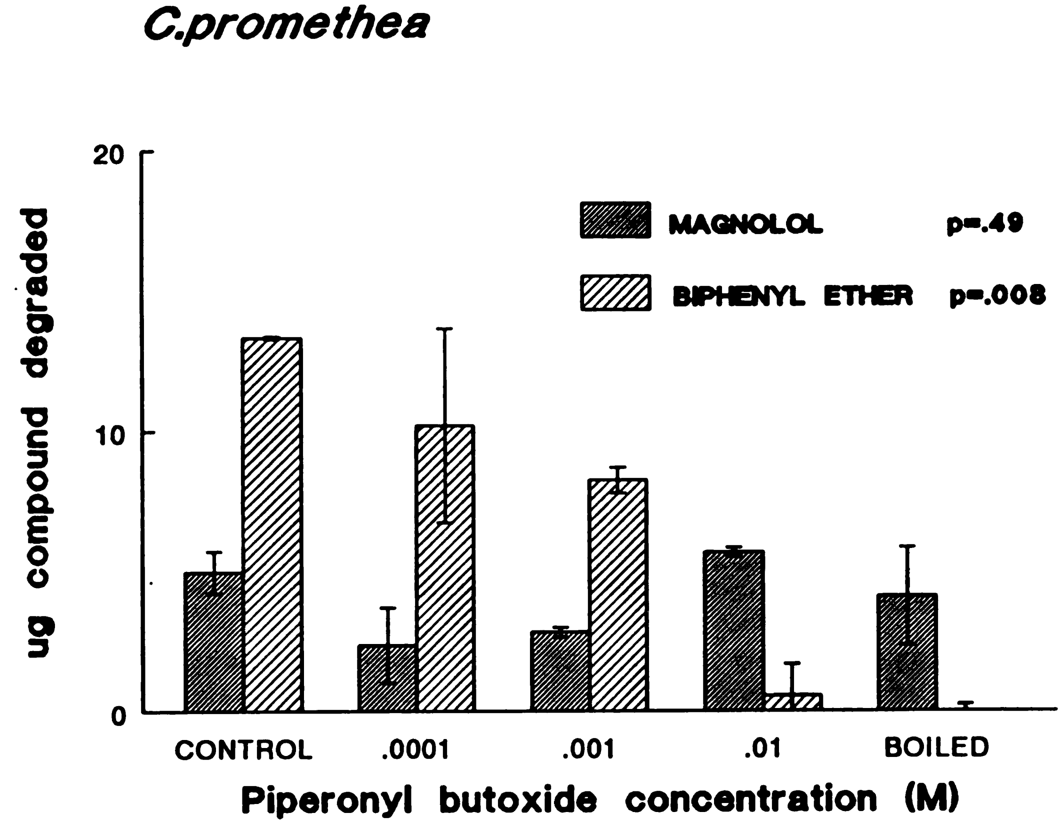


Figure 9. Piperonyl butoxide inhibition of magnolol and biphenyl ether degradation by midgut homogenate of *C. promethea*.

significantly different from that of the oligophagous *C. angulifera*, nor did hostplant signficantly alter the rate of metabolism of magnolol (2.39 ± 0.5, 1.7 ± 0.5, 1.6 ± 0.4 ug/mg/30 min) and biphenyl ether (4.7 ± 1.2, 2.3 ± 1.2, 2.9 ± 0.6 ug/mg/30 min) on tuliptree, sassafras and black cherry, respectively, in *C. promethea*.

## Discussion

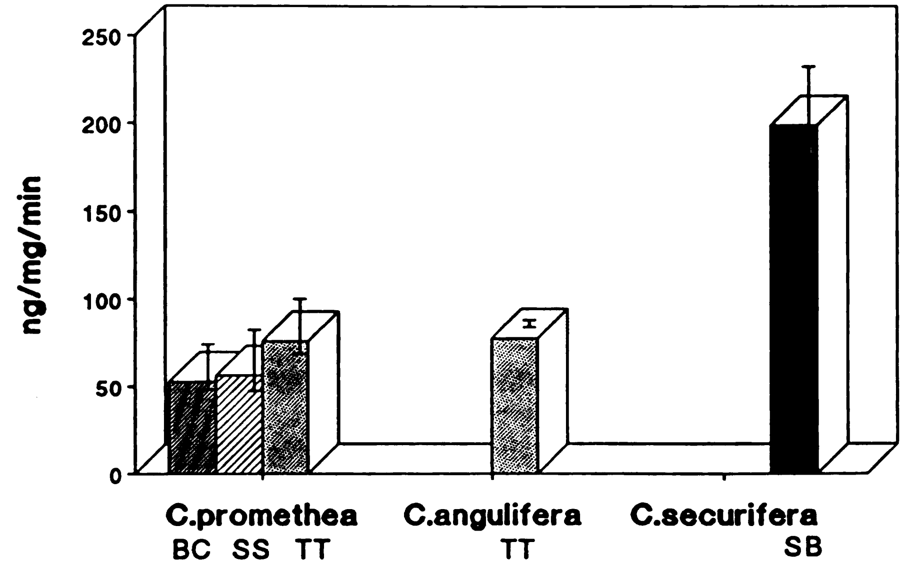
In *Callosamia* silkmoths, specialization on sweetbay magnolia has been accompanied by biochemical adaptations for metabolism of toxic neolignans encountered in this host. *C. securifera* midgut tissue is capable of rapidly detoxifying two toxins, magnolol and a biphenyl ether, that apparently act as phytochemical barriers to two closely related *Callosamia* species and other unadapted herbivores (Nitao 1992) that cannot feed on sweetbay.

Levels of detoxification activity, as measured by magnolol degradation and the P450-based detoxification of the biphenyl ether, are not correlated with diet breath (polyphagy) in *Callosamia*. The results of this study substantiate the growing body of evidence that detoxification activity is more closely correlated with specific chemistry of hosts than diet breadth *per se* in phytophagous insects (Nitao 1989, Berenbaum 1991).

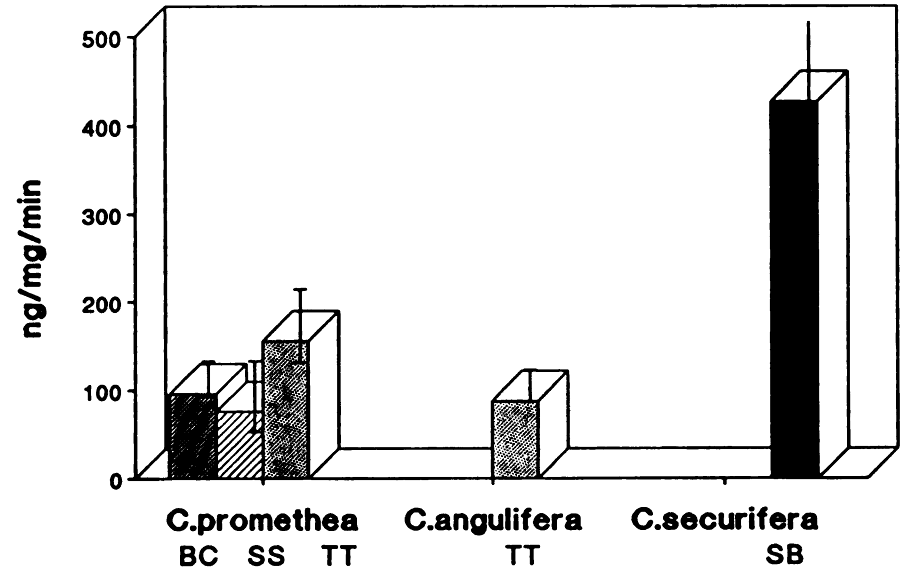
The comparative approach used in this study provides an evolutionary context in which to evaluate the correlation between detoxification enzymes and diet breath in *Callosamia*. In contrast to early findings that high PSMO activity is correlated with polyphagy using model substrates as indicators of detoxification activity, this study shows that PSMO activity, at least towards a compound of

Table 8. Degradation of sweetbay allelochemicals by midgut homogenate of *C. promethea* larvae exposed to host allelochemicals and a detoxification enzyme inducer for 24 h on sassafras (Experiment 1) and 48 h on tuliptree leaves (Experiment 2).

| Treatment | n | μg degraded/vial | |
|---|---|---|---|
| | | biphenyl ether | magnolol |
| **Experiment 1.** | | | |
| control | 2 | 5.3 ± 0.6 | 4.1 ± 0.4 |
| pentamethylbenzene | 2 | 4.4 ± 1.4 | 3.4 ± 0.4 |
| biphenyl ether:magnolol | 2 | 7.2 ± 0.7 | 4.2 ± 0.5 |
| **Experiment 2.** | | | |
| control | 2 | 7.3 ± 1.5 | 5.3 ± 0.4 |
| pentamethylbenzene | 2 | 6.7 ± 1.1 | 4.8 ± 0.4 |
| biphenyl ether:magnolol | 2 | 6.8 ± 1.1 | 4.5 ± 0.7 |



Magnolol degradation



Biphenyl ether degradation

Figure 10. Comparision of *in vitro* degradation of sweetbay allelochemicals (magnolol and the biphenyl ether) by third instar larvae of three species of *Callosamia*. The letters under the species names indicate the host larvae were reared on (BC = black cherry, SS = sassafras, TT = tuliptree, SB = sweetbay).

ecological interest, was highest in the specialist. Whether PSMO activity towardsbiphenyl ether is well-correlated with that towards other commonly used model substrates, such as aldrin, is not known. Detoxification activity towards different substrates is not always well correlated, making it even more difficult to make generalizations. Perhaps generalist detoxification enzyme systems have broader substrate specificity and/or greater inducibility than those of specialists, allowing polyphagous species greater flexibility to respond to the unpredictable profile of ingested allelochemicals (Berenbaum 1991).

Metabolism of the biphenyl ether is NADPH dependent and inhibited by piperonyl butoxide, suggesting a cytochrome P450-based detoxification mechanism in *C. securifera* and *C. promethea*. We were unable to assess NADPH dependency or the effect of piperonyl butoxide in *C. angulifera* due to the difficulty in obtaining larvae of this species. The exact detoxification pathway of the biphenyl ether is not known, although two metabolite peaks of greater polarity were evident in the HPLC chromatograms (see Appendix 2) in incubations of active tissue compared to inactivated tissue. Since biphenyl ether and magnolol were being metabolized simultaneously in the incubation mixture, specific metabolite peaks cannot be associated with their parent compound. Hydroxylation of biphenyl compounds is via PSMO's has been described in fall armyworms (Yu 1984). In a number of animals, biphenyl is metabolized by microsomal cytochrome P-450 to a variety of hydroxybiphenyls, which are then conjugated with glucuronic acid and/or sulfate (Powis et al. 1987, Pacific 1991).

The metabolic fate of magnolol is less apparent. In *C. securifera*, the response to NADPH and piperonyl butoxide suggest a cytochrome P450-based pathway, but this was not the case for *C. promethea*. The different characteristics of magnolol metabolism by *C. promethea* (not NADPH dependent and not inhibited by piperonyl butoxide) may indicate the absence of the specific P450 isozyme required for metabolizing magnolol. In rats, orally administered magnolol is extensively conjugated with glucuronides and other conjugates in the liver, and then isomerized or reduced, but the role of cytochrome P-450 in these processes is unclear (Ma et al. 1988). Specific P450 isozymes are required for xanthotoxin metabolism in umbellifer specialists (Cohen et al. 1989, Berenbaum et al. 1990); unadapted herbivores apparently lack the appropriate form for xanthotoxin metabolism. Alternatively, the *C. promethea* detoxification system may be inhibited by magnolol and the biphenyl ether. Naturally occurring enzyme inhibitors are not uncommon in plants (Berenbaum 1986, Berenbaum et al. 1990); moreover, both lignans and phenylpropanoids have been reported to inhibit cytochrome P450 enzymes (Bernard et al. 1989). Decreased sensitivity to detoxification enzyme inhibitors in host foliage can be an important adaptation to host chemistry, as in the umbellifer specialist *P. polyxenes*, which is less susceptible to methylenedioxyphenyl PSMO inhibitors found in its hosts (Neal and Berenbaum 1989). There is also the possibility that signficant detoxification occurs in tissues other than the midgut, such as the fatbody.

The rates of allelochemical degradation by gut homogenate in this study were considerably higher than *in vitro* rates typically reported for homogenate or microsomal preparations. Degradation of xanthotoxin by gut slices in the black swallowtail (*P. polyxenes*) range from 0.9 - 1.6 nmol/min/mg (Bull et al. 1984); metabolism of the cardenolide uscharidin by monarch (*Danaus plexippus*) caterpillar gut homogenate ranges from 1.97 - 4.99 nmol/min/mg (Marty and Kreiger 1984). However, salicin is degraded at much higher rates (304.2 + 6.3 ng/min/mg) by *Papilio glaucus* B-glucosidases (Lindroth 1988). Because our estimates of detoxification activity were based on the amount of undegraded compound recovered, it is possible that the absolute values were inflated by the amount of parent compound not fully extracted from the incubation mixture. Average recovery of the internal standard (methoxyhonokiol) was fairly low (around 60%) but consistent across species, so the relative differences in detoxification activity measured are likely valid.

Because *C. promethea* and *C. angulifera* do not survive on sweetbay, we cannot rule out the possibility that the high detoxification activity in *C. securifera* is induced by the hostplant and is not necessarily a physiological characteristic of the species. On the one hand, the failure of the biphenyl ether:magnolol mixture to induce higher detoxification activity suggests that the this is not the case, although the possiblity of induction by other sweetbay allelochemicals cannot be ruled out. On the other hand, the lack of induction by pentamethylbenzene, a strong inducer of cytochrome P450 in *Spodoptera eridania* and other insects (Brattsten and Wilkinson 1973), is somewhat surprising. It is possible that higher levels of the particular forms of enzymes involved in magnolol and biphenyl ether degradation are not responsive to this particular inducer; alternatively, it is possible that higher activity was induced but returned to low levels during the 24 h period that larvae were placed on untreated leaves after exposure to the inducers. Detoxification enzyme levels can be quite labile; measurable elevation in activity often occurs within 30 minutes of exposure to inducers. Likewise, induced enzyme levels may be relatively short-lived, returning to normal levels within a similar time period (Brattsten and Wilkinson 1973, Yu 1986). If this were the case, enzymes induced by the biphenyl ether: magnolol mixture or pentamethylbenzene in this study may have returned to constituitive levels within 24 h after switching to untreated foliage.

The rapid rate of detoxification of magnolol and the biphenyl ether by midgut tissue of *C. securifera* appears to be an adaptation for feeding on sweetbay magnolia. Not only are these compounds toxic to *C. promethea* and *C. angulifera* neonates (Johnson et al. unpublished), but they act as chemical barriers to lauraceae-adapted swallowtail butterflies and the highly polyphagous fall webworm as well (Nitao et al. 1992). The lower rates of metabolism in *C. promethea* and *C. angulifera* caterpillars may explain why these species do not use sweetbay magnolia as a host. These species are, however, well-adapted to another magnoliaceous host, tuliptree (*Liriodendron tulipifera*) which does not contain detectable amounts of magnolol or the biphenyl ether (K. S. Johnson, unpublished).

The use of magnoliaceous hosts is not unique to the genus *Callosamia* among the New World Attacini (Saturniidae) but use of this host family by other silkmoths is more sporadic. *Samia cynthia* has been reported to feed on tuliptree, and there are occasional reports of polyphagous species such as *Hyalophora cecropia* and *Anthaerea polyphemus* using tuliptree as well (Stone 1991). Use of sweetbay magnolia, however, is restricted to *C. securifera* (and a few local populations of *C. promethea*, H. Pavulaan, personal communication). Thus, although *Callosamia* are adapted to feed on some magnoliaceous hosts, the use of sweetbay is constrained to taxa that have the detoxicative capacity to cope with biphenyl ether and magnolol.

Without a detailed phylogeny of the three *Callosamia* species, we can only speculate about whether the ability to detoxify sweetbay toxins is ancestral or derived. Ferguson (1972) considered the genus to be a very "compact and discrete" group that is well-differentiated from *Hyalophora* and *Eupackardia*. The close relationship between the three species is evidenced by the fact that interspecific hybrids can be obtained by handpairing (Piegler 1977, 1980). Under natural conditions, the species are apparently reproductively isolated by temporal differences in flight and mating times (Ferguson 1972). Reproductive isolation is apparently complete, as evidenced by the detection of fixed differences at several alleles by allozyme electrophoresis (Johnson et al., unpublished). Although *C. angulifera* has been regarded as the more primitive member of the genus due to its nocturnal habit, preliminary calculations of genetic identities based on allele freqencies indicated equal genetic distance between the three species (Johnson et al. unpublished). This, in combination with the morphological similarities and the potentially rapid reproductive isolation from differences in mating time, suggests that *C. promethea*, *C. angulifera* and *C. securifera* arose quickly and perhaps simultaneously from a common ancestor having the ability to feed on magnoliaceous hosts.

# CHAPTER FOUR

## Hostplant specialization and nutritional efficiency in Saturniid silkmoths *Callosamia promethea* and *C. angulifera*

### Abstract

It has been hypothesized that ecologically specialized organisms enjoy an advantage of greater efficiency of resource utilization compared to generalists. In herbivorous insects, polyphagous species are predicted to have lower growth rates or efficiency of utilization of hosts due to increased metabolic costs associated with a broad diet. We tested this prediction by comparing relative growth rate, gross efficiency of utilization (ECD) and net efficiency of utilization (ECD) of the highly polyphagous *Callosamia promethea* to that of the oligophagous *C. angulifera* on tuliptree, a host commonly used by both species. Relative growth rates and duration of instars were not significantly different. The use of analysis of variance to test for differences in ratio-based measures (RGR and RCR) resulted in errors due to statistical artifacts. When analyzed by ANCOVA (using initial body mass as a covariate), no significant differences in consumption or growth were found. In our comparisons, the tuliptree specialist *C. angulifera* consistently had a higher gross efficiency of utilization, and in one year a higher net efficiency of utilization as well.

## Introduction

Ecological specialization in some form is exhibited by virtually all organisms, particularly with regard to the use of food resources. Models explaining the evolution and maintenance of niche breadth are generally constructed on the fundamental assumption that there are physiological, behavioral or functional tradeoffs associated with using many different resources (Levins and MacArthur 1969). The tradeoff principle is so intuitive that the familiar adage, "a jack of all trades is master of none" is often used to express the idea that although generalists can take advantage of a greater variety and unpredictability of resources, specialists may be more efficient at using fewer types of resources.

Herbivorous insects exhibit a wide range of feeding specialization, from monophagy (use of a single hostplant) to varying degrees of polyphagy (wide range of plants usually belonging to different families) and the idea of tradeoffs associated with generalist and specialist strategies has played a central role in theories explaining patterns of host plant specialization (Futuyma and Moreno 1988). In particular, metabolic costs associated with processing dietary allelochemicals has been identified as a likely currency for tradeoffs associated with host specialization. Enzymatic detoxification of plant chemicals has been shown to be of considerable importance in determining host use patterns (Ahmad 1986, Lindroth et al. 1988, Brattsten 1977, Ivie et al. 1983, Nitao 1989), and the ability of polyphagous herbivores to utilize a wide range of host plants has been attributed to increased levels of detoxification systems (Krieger et al. 1972,

Ahmad 1983, Rose 1985). It has been hypothesized that the maintanence of high levels of detoxification enzyme systems is metabolically costly (Whittaker and Feeny 1971, Schoonhoven and Meerman 1978, Brattsten 1979, Scriber 1981), and that a herbivore that specializes on the phytochemical characteristics of one or a few hostplants may eliminate the necessity of maintaining an energetically costly array of biochemical and physiological detoxification mechanisms. The reduced metabolic load associated with dietary specialization might be evidenced as increased growth rates, efficiency of conversion of ingested food (ECI), or efficiency of conversion of digested food to biomass (ECD) (Whittaker and Feeny 1971, Scriber and Feeny 1979).

Metabolic costs associated with physiological processes other than the detoxification of allelochemicals can also affect efficiency of hostplant utilization and potentially influence the evolution of dietary specialization. There are measurable metabolic costs associated with the increased consumption and processing of low quality protein (Karowe and Martin 1989, Slansky and Wheeler 1991), as well as from dietary nutrient imbalances (Slansky and Scriber 1985), and compensation for diet deficient in water (Van't Hof and Martin 1989). Moreover, digestive proteases of herbivorous insects have been shown to vary in specificity and efficiency in which they act on dietary protein (Broadway 1989). Thus, host specialization may involve tradeoffs in efficiency of use of particular protein or nutritional profiles that are as significant as those associated with detoxification of allelochemicals.

Attempts to empirically demonstrate tradeoffs in nutritional efficiency in specialist and generalist herbivores have generally been unsuccessful. Schroeder (1976, 1977) found no difference in the growth rate of milkweed specialists compared to growth rates of generalist and specialist lepidopterans on their natural hosts. Similarly, Smiley (1978) and Scriber and Feeny (1979) found that generalists do not grow more slowly on their hosts than specialists. More direct comparisons of nutritional efficiencies (ECD, ECI) have failed to show differences between generalists and specialists (Scriber and Feeny 1979, Futuyma and Wasserman 1981, Scriber 1983). Comparisons within Papilionidae and Saturniidae by Scriber and Feeny (1979) suggested that specialists had higher efficiencies of conversion of ingested food, but these trends were not statistically signficant.

It is important to recognize that differences in utilization efficiencies may be a result of, rather than a cause of, hostplant specialization (Futuyma and Wasserman 1981, Futuyma 1991). Comparisons of unrelated specialists and generalists on different hosts may not be particularly revealing, since long associations with different habitats or resources can result in adaptations in many traits (Futuyma and Moreno 1988). To date, there are few studies that compare utilization parameters of closely related specialists and generalists simultaneously on a common host (Scriber and Feeny 1979, Futuyma and Wasserman 1981, Futuyma et al. 1984). Scriber and Feeny (1979) demonstrated that *Papilio troilus*, a Lauraceae specialist, had higher growth rates and ECI than the

polyphagous *P. glaucus* on two Lauraceous hosts, sassafras and spicebush. Futuyma and Wasserman (1981) found no differences in net efficiency of utilization of black cherry between the eastern tent caterpillar (*Malacosoma americanum*), a Rosaceae specialist, with the more polyphagous forest tent caterpillar (*M. disstria*). Within species comparisons have also failed to demonstrate differences in performance associated with host specialization (Futuyma et al. 1984, Rausher 1984). Additional comparisons of related taxa on common hosts are needed to determine whether there are general patterns of host use efficiency and specialization.

This paper describes a comparison of the nutritional efficency of the polyphagous promethea silkmoth (*Callosamia promethea*) to that of the more specialized tuliptree silkmoth (*C. angulifera*) on tuliptree (*Liriodendron tulipifera*, Magnoliaceae), a host commonly used by both species. These two silkmoths have many similar morphological and ecological characteristics, but differ considerably in host breadth. *C. angulifera* is a specialist on tuliptree, with populations occasionally reported to use spicebush or sassafras (Lauraceae). In contrast, *C. promethea* is highly polyphagous and feeds on hosts from many plant families, including Rosaceae (black cherry), Oleaceae (ash, lilac), Lauraceae (sassafras, spicebush), Styracifluaceae (sweetgum) and others in addition to tuliptree. Thus, this system presented an opportunity to test the prediction that specialization of *C. angulifera* on tuliptree should be accompanied by an increase in the efficiency of utilization of this host compared to *C.*

*promethea*. Also, because we were also able to obtain hybrids from a semi-natural mating of *C. angulifera* (female) x *promethea* (male) during the course of the study, an additional trial was conducted to compare the nutritional efficiency of these hybrid larvae to pure *C. promethea*.

## Materials and methods

### *Experimental insects*

Nutritional indices of third instar *C. promethea* and *C. angulifera* reared simultaneously on tuliptree were calculated in two experiments, one conducted in early July 1989 and another in late July 1991. In the first experiment (1989), *C. promethea* and *C. angulifera* were derived from wild stock collected in Pennsylvania. In the 1991 experiment, *C. angulifera* originated from two wild-collected females (Cass Co. MI and Emporia, SC) and one *C. promethea* female (Clinton Co., MI). In a third experiment, hybrid *C. promethea* x *C. angulifera* larvae originating from a mating of a captive *C. promethea* female (collected in southern WI) with a wild male *C. angulifera* (Cass Co., MI) were compared to pure *C. promethea* (Wisconsin) in early July 1991.

### *Calculation of nutritional indices*

Nutritional indices were calculated for third instar larvae reared individually in plastic petri dishes at 24° C on an 18:6 light:dark photoperiod and fed leaves kept fresh in water-filled vials with rubber caps. Fresh tuliptree leaves were collected daily from potted saplings maintained at Michigan State University.

Initial and final wet weights of larvae were measured during the premolt period between the second and third and third and fourth stadia. Dry weights of larvae were calculated using a wet:dry conversion value obtained from subsets of five pre-molting larvae taken at the beginning and end of each experiment. The dry weight of fresh leaves consumed was calculated by subtracting the dry mass of uneaten leaf material from an initial dry mass calculated using a wet:dry conversion factor calculated from weekly subsamples of 20 leaves. Larvae were given only slightly more leaf material than they could consume in 24 h to minimize error in estimates of leaf dry mass (Schmidt and Reese 1986). Uneaten leaf material and frass was collected and oven dried at 40° C for two weeks.

For each larva, relative growth rate (RGR), relative consumption rate (RCR), assimilation efficiency (AD), gross growth efficiency (ECI) and net growth efficiency (ECD) were calculated on a dry weight basis following Waldbauer (1968):

Relative growth rate (RGR) = larval mass gained/average larval mass x days

Relative consumption rate (RCR) = mass of food ingested/average larval mass x days

Assimilation efficiency (AD) = mass of food ingested-mass of frass/mass of food ingested

Gross growth efficiency (ECI) = larval mass gained/mass of food ingested

Net growth efficiency (ECD) = larval mass gained/mass of food ingested - mass of frass

### *Statistical analysis*

Due to the likelihood of seasonal and year to year differences in tuliptree quality, the three experiments were analyzed separately . One-way analysis of variance was used to compare RGR, RCR, AD, ECI and ECD of the two species. There has been much recent discussion of the potential misuse of ratios to adjust physiological data for variation in body size, particularly when the variable of interest varies allometrically (i.e. is nonlinear or not isometric) with body size or when the measure used for scaling is not independent of treatment effects (Packard and Boardman 1987). Nutritional index parameters such as RGR and RCR that use an average body mass in the denominator often violate both of these assumptions. The use of analysis of covariance to adjust for effects of body size has been suggested as a superior method of analyzing conventional ratio-based measures such as these (Raubenheimer and Simpson 1992). Thus, in this study, we performed an additional and separate analysis of variance on total growth and total consumption using inital larval mass as a covariate.

## Results

In two of the three experiments, we detected a significantly higher net growth efficiency (ECI) in *C. angulifera*, and in the early season trial (1989), net growth efficiency (ECI) was also higher in the specialist. There were no signficant differences in the nutritional parameters of hybrid *C.*

Table 9 . Comparisons of nutritional indices of third instar *C. promethea* (generalist), *C. angulifera* (specialist) and *C. angulifera* X *promethea* hybrid larvae reared on tuliptree.

| | days | RGR | RCR | ECI | ECD | AD | N |
|---|---|---|---|---|---|---|---|
| early July 1989 | | | | | | | |
| *C. promethea* | 6.1 ± 0.6 | 0.22 ± 0.01 | 1.68 ± 0.08‡ | 13.62 ± 0.76$^{**}$ | 45.79 ± 19.6$^{*}$ | 0.36 ± 0.04 | 11 |
| *C. angulifera* | 6.8 ± 0.8 | 0.22 ± 0.01 | 1.17 ± 0.07 | 18.99 ± 0.70 | 83.76 ± 18.0 | 0.26 ± 0.03 | 13 |
| late July 1991 | | | | | | | |
| *C. promethea* | 10.7 ± 2.3 | 0.13 ± 0.02 | 1.53 ± 0.23 | 8.35 ± 0.24$^{**}$ | 55.84 ± 8.44 | 0.20 ± 0.04 | 3 |
| *C. angulifera* | 8.2 ± 0.4 | 0.17 ± 0.003 | 1.33 ± 0.02 | 13.07 ± 0.15 | 48.52 ± 0.77 | 0.27 ± 0.004 | 10 |
| early July 1991 | | | | | | | |
| *C. promethea* | 5.13 ± 0.12 | 0.25 ± 0.007 | 1.79 ± 0.08 | 14.09 ± 0.48 | 50.23 ± 4.08 | 0.29 ± 0.02 | 8 |
| *C. ang X promethea* | 5.06 ± 0.10 | 0.27 ± 0.007 | 1.80 ± 0.08 | 15.74 ± 0.54 | 56.76 ± 4.43 | 0.30 ± 0.02 | 17 |

$^{*}$ $P < 0.05$ ANOVA

$^{**}$ $P \leq 0.01$ ANOVA

‡ Not significant by ANCOVA

*promethea* x *C. angulifera* and pure *C. promethea* larvae. The mean values of RGR, RCR, ECI, ECD and AD are summarized in Table 9.

Proper interpretation of higher ECI and ECD as evidence of increased efficiency of digestion or of a lower metabolic load requires that both consumption rate and instar duration be examined as well. Relative growth rates of *C. promethea* and *C. angulifera* were not significantly different in either trial. Although one-way analysis of variance of relative consumption rates (RCR) detected a significant difference between species in 1989, a careful examination of the data suggests that the effect may be an artifact of using ratio-based scaling to adjust for differences in body size. The conventional measure of RCR was calculated by dividing total consumption by the mean larval mass to adjust for differences in larval size. However, the use of ratios to scale for body size effects is restricted to situations in which the variable of interest varies isometrically with body size (is a linear relationship that passes through the origin when plotted on x,y axes) (Packard and Boardman 1987). Figure 11 shows that the relationship between total consumption (numerator) and average larval mass (denominator) is not isometric, nor are the slopes of the regressions equal, two violations of restrictions on the use of scaling by ratios. In cases where slopes are different, the analysis of ratio variables does not detect interactive effects between the dependent value and covariate, which can lead to the incorrect conclusion that the dependent variable differs between treatments (Type I error). Furthermore, there is not a strong covariance between consumption and mean larval mass, a situation in which use of ratios can introduce a variety of problems such as decreased power or artifactual treatment effects (Packard and Boardman 1987;1988, Raubenheimer and Simpson 1992).

In contrast, ANCOVA provides a measure of the relationship between the variable of interest and covariate, and is not as prone to artifactual effects even when a non-significant covariate is retained in the analysis. A reanalysis by ANCOVA showed that initial larval mass was a signficant covariate for consumption in 1989 ($P = 0.013$), and when this effect was taken into account, there were no significant species differences in consumption in this or either of the other two experiments.

Comparison of results from the ANOVAs of the ratio-based measures of consumption (RGR, RCR) with ANCOVA's of total consumption and total growth using initial larval weight as a covariate (in all cases slopes between the covariate and dependent variable were homogeneous) shows that the two methods of statistical analysis lead to different conclusions about treatment effects (Table 10). The ANCOVA $P$ values are consistently higher than those obtained by ANOVA of ratio-based measures, although in our experiments, the differences in P values changed our interpretation of differences in only one case, the consumption rates in 1989.

Table 10. Comparison of the ANOVA of ratio-based measures of consumption and growth (RCR and RGR) with ANCOVA of the total consumption and total growth using initial larval weight as a covariate.

| | Analysis | SS | df | MS | F-ratio | *P* |
|---|---|---|---|---|---|---|
| *1989* comparison of *C. promethea* versus *C. angulifera* | | | | | | |
| growth | ANOVA | 0.000302 | 1 | 0.000302 | 0.377 | 0.544 |
| | ANCOVA | 0.000280 | 1 | 0.000280 | 1.875 | 0.183 |
| consumption | ANOVA | 1.363192 | 1 | 1.363192 | 7.534 | 0.011 |
| | ANCOVA | 0.012209 | 1 | 0.012209 | 0.853 | 0.364 |
| *1991* comparison of *C. promethea* versus *C. angulifera* | | | | | | |
| growth | ANOVA | 0.004367 | 1 | 0.004367 | 2.204 | 0.166 |
| | ANCOVA | 0.002780 | 1 | 0.002780 | 1.816 | 0.207 |
| consumption | ANOVA | 0.091402 | 1 | 0.091402 | 0.544 | 0.476 |
| | ANCOVA | 0.000080 | 1 | 0.000080 | 0.008 | 0.930 |
| *1991* comparison of *C. promethea* versus *C. promethea x angulifera* hybrids | | | | | | |
| growth | ANOVA | 0.001593 | 1 | 0.001593 | 1.508 | 0.228 |
| | ANCOVA | 0.000473 | 1 | 0.000473 | 2.428 | 0.129 |
| consumption | ANOVA | 0.130748 | 1 | 0.130748 | 1.337 | 0.256 |
| | ANCOVA | 0.000092 | 1 | 0.000092 | 0.008 | 0.928 |

## Discussion

Nutritional indices have frequently been used to assess metabolic costs associated with the utilization of different diets. In particular, a concomitant decrease in growth rate and ECD has been interpreted as evidence for increased metabolic costs associated with a diet (Erickson and Feeny 1974, Schoonhoven and Meerman 1978, Scriber 1981, Cresswell et al. 1992, Appel and Martin 1992).

However, as several workers have recently pointed out, ECD is not a direct measure of metabolic expenditure, and caution should be exercised in using it to infer metabolic costs. ECD is the proportion of total assimilate that is allocated to biomass; so a decrease may come about either from increased allocation to metabolic processes or from a decreased total assimilate pool effected, for example, by a reduction in consumption rate. Thus, ECD's can be difficult to interpret if there is a simultaneous change in consumption. In these experiments, ANCOVA showed that consumption and growth did not differ between species in any of the trials, increasing confidence that the observed differences in ECD can be interpreted as increases in metabolic expenditure, rather than reductions in total assimilate.

The gross efficiency of ingested food (ECI) was higher in *C. angulifera* compared to *C. promethea*, in both trials, suggesting that host specialization may involve improvement of the efficiency of digestion of tuliptree than greater efficiency of net utilization of the digested material.

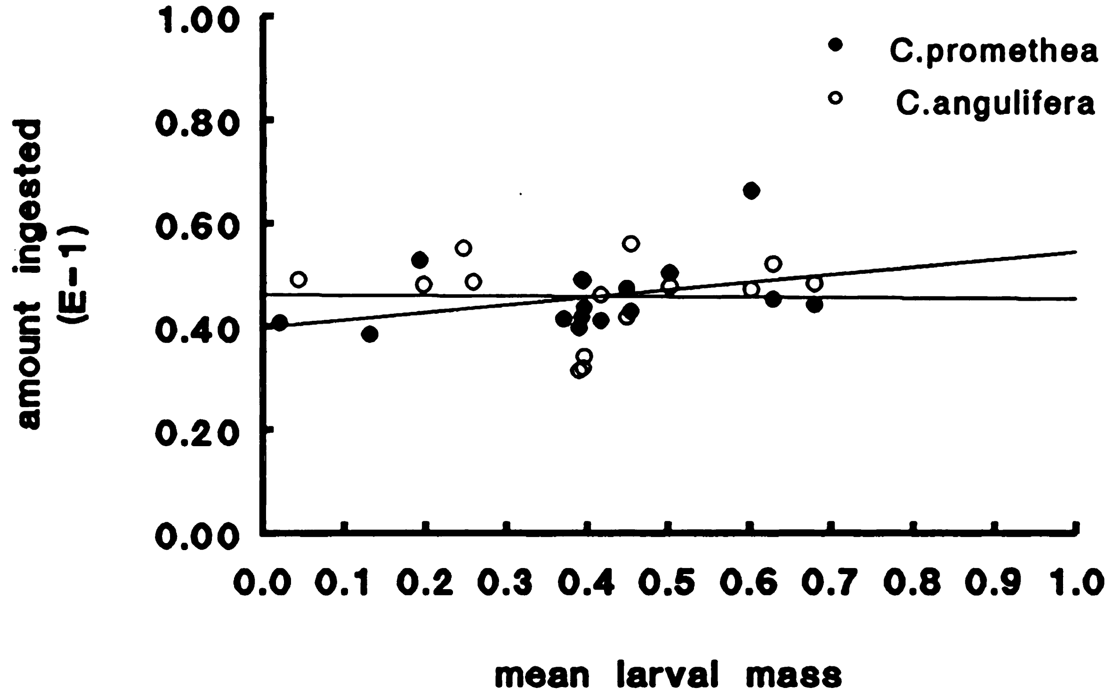


Figure 11. Illustration of the allometric relationship between consumption and mean larval mass for third instar *Callosamia* larvae in the 1989 experiment. Each point represents one larva.

Greater efficiency of digestion may be via differences in the efficacy of proteases (Broadway 1989), or other differences in digestive physiology that enhance absorption or utilization of nutrients.

Although we were able to demonstrate higher net growth efficiency in the tuliptree specialist in 1989, the absence of a significant difference in 1991 suggest that if *C. angulifera* has a greater net growth (metabolic) efficency, it is relatively subtle compared to the influence of differences in host quality, source of insect populations, and/or the degree of experimental error involved in gravimetric approximations. The between-experiment variation observed in our study underscores the importance of simultaneous comparisons, adequate population sampling and measures across a range of ecologically relevant host conditions for proper evaluation of the ecological and/or evolutionary significance of differences in net utilization efficiency.

Seasonal variation in host quality likely contributed to the different results obtained in the 1989 (early July) and 1991 (late July) trials, since the foliar water content varied from early July to August (Figure 12). Dietary water can significantly affect larval growth and the efficiency of conversion of digested food (Scriber 1977; 1979, Reese and Beck 1978, Martin and Van't Hof 1988, Schmidt and Reese 1988, Timmins et al. 1988), particularly by extending the duration of feeding stages (Van't Hof and Martin 1989). Variation in other host characteristics such as nitrogen or allelochemicals may have occurred during the season as well (Scriber 1984). Though our experiments fell within the normal phenological period experienced by *Callosamia* larvae in Michigan (the flight period generally ranges from mid- June to late July), variation in host quality within this period could have contributed to differences in utilization parameters.

Intraspecific variation in utilization parameters in *Callosamia* may have contributed to the variation seen between trials in our study as well. Local adaptation of herbivore populations to hosts is not uncommon in insects (Rausher 1982, Hare and Kennedy 1986, Scriber 1986, Horton 1988, Nitao et al. 1991), so it is possible that relative differences in nutritional parameters of *C. promethea* and *C. angulifera* in our study were due to the small number of families represented in each trial and/or the the particular populations compared. Our findings are consistent with an earlier comparisons of performance of these two species on tuliptree, which revealed no difference in growth or consumption rates (Scriber and Feeny 1979). Although *C. promethea* exhibits distinct local preferences for host use across its geographic range (Ferguson 1972), its generalist capabilities do not appear to be due to a mosaic composition of local specialists. At least in midwestern United States, offspring of adults from wild-collected cocoons do not perform better on the hostplants from which their parents originated (Scriber et al. 1991).

Metabolic costs associated with detoxification enzymes may be minor enough to be immeasurable by standard gravimetric procedures, which can be susceptible to large degrees of experimental error (Schmidt and Reese 1986) and do not always correspond with caloric (Slansky 1985) or respiratory (Van Loon 1991) estimates of metabolic expenditure. The

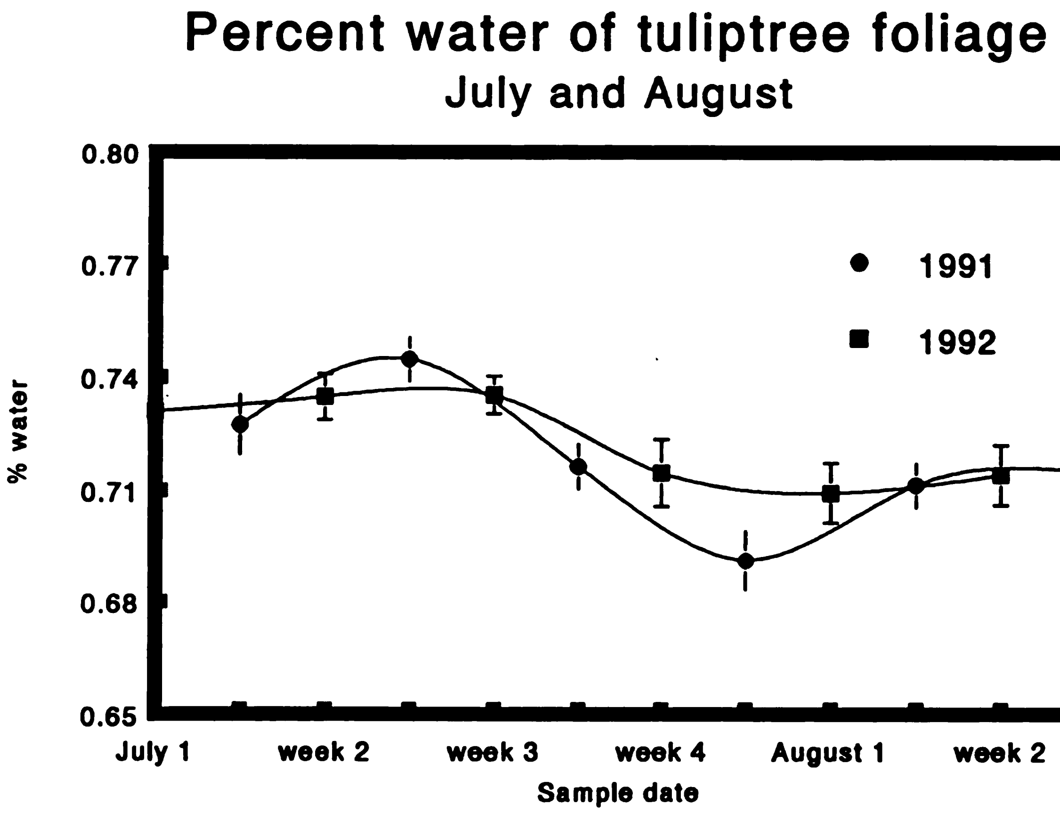


Figure 12. Variation in water content of tuliptree leaves during July and August (1991 and 1992). Each point represents the mean ± SE of 15 - 20 leaves.

equivocality of attempts to assess the energetic cost of allelochemical processing (Neal 1987, Cresswell et al. 1992, Appel and Martin 1992) suggests that metabolic costs may be highly situation-dependent. Some allelochemicals may be more costly to process, since detoxification is often via multiple steps involving different enzyme systems. In addition to the actual metabolic expenditure of reactions with each toxin molecule (Brattsten 1979), the rate of binding between enzyme and substrate as well as total detoxification enzyme turnover can be expected to contribute to the total metabolic load. Allelochemicals that cause tissue damage, such as gut lesions (Steinly and Berenbaum 1985) or interfere with digestive processes may exert additional indirect costs.

If tradeoffs in effiency between specialist and generalist strategies exist, it is becoming increasingly apparent that they are not likely to be energetic tradeoffs. The diets of insect herbivores are rarely energy-limiting (Slansky and Feeny 1977); rather, performance is more limited by nitrogen or water (Scriber and Slansky 1981, Mattson 1980, Slansky and Wheeler 1991, Martin and Van't Hof 1988). The more consistent differences in ECI between *C. angulifera* and *C. promethea* suggest that differences in efficiency in these species are more closely associated with digestive processes than metabolic costs of allelochemical detoxification. Empirical analysis of other biochemical, physiological or morphological functions related to nitrogen utilization, dietary water use or non-energetic constraints on detoxification are needed to determine what additional physiological tradeoffs may be associated with host specialization.

# SUMMARY AND CONCLUSIONS

Physiological adaptation to host chemistry is clearly an important determinant of current host use patterns within the genus *Callosamia*. Sweetbay magnolia contains at least two toxic neolignans, magnolol and a biphenyl ether, that reduce growth and survival of unadapted herbivores; the sweetbay specialist, however, is unaffected by similar concentrations. Although there is evidence that a few populations of *C. promethea* are able to use sweetbay as a host (in Maryland), the results of bioassays of larvae from a wide range of geographic locations indicates that this trait is not widespread. Offspring of the pupae collected from sweetbay in Maryland did not exhibit increased tolerance for magnolol or the biphenyl ether in bioassays, suggesting that the chemistry of the host, rather than the physiology of *C. promethea*, may be different at this location.

Physiological adaptation to sweetbay involves an increased ability to enzymatically detoxify host neolignans. Metabolism of the biphenyl ether appears to be via the cytochrome P450 enzyme system in both *C. securifera* and *C. promethea*. The mechanism of magnolol detoxification is less clear, but may also involve cytochrome P450 enzymes.

Comparison of the detoxification rates of magnolol and the biphenyl ether by *C. securifera*, *C. promethea* and *C. angulifera* indicates that the use of sweetbay is correlated with the ability to rapidly degrade these compounds. This,

in combination with confirmation from the bioassays that both compounds signficantly affect larval performance, provides strong evidence adaptation to host chemistry has played a primary role of chemistry in the evolution of host use patterns in this group of herbivores. However, demonstration of a key role of host chemistry does not necessarily lessen the importance of other ecological factors in initiating an early host shift or in contributing to differential selection between hosts.

In *Callosamia*, high detoxification activity is not correlated with diet breadth. Assuming that detoxification of the biphenyl ether is representative of general cytochrome P450 activity, the higher activity in the specialist *Callosamia* contradicts the prediction that P450 activity is correlated with diet breadth. The *Callosamia* system provided a powerful test of this hypothesis, since confounding effects of comparing herbivores from different phylogenies could be minimized. Our results support the growing body of evidence that levels of detoxification enzyme activity are more closely correlated with specific host chemistry than with diet breadth.

In addition to the evidence that specialist *Callosamia* have higher levels of cytochrome P450-based detoxification activity, there was only weak support for the hypothesis that the polyphagous feeding habit is accompanied by greater metabolic costs associated with detoxification. In the comparison of utilization efficiencies of the polyphagous (*C. promethea*) with the specialist (*C. angulifera*) on a shared host, in only one of two trials did the generalist have a lower net efficiency of utilization (ECD) expected if metabolic processes, including detoxification, were demanding greater energetic expenditure. However, the

generalist consistently had a lower gross efficiency of utilization (ECI), suggesting that physiological tradeoffs in efficiency of digestion or nutrient absorption may be more pronounced than tradeoffs associated with metabolic costs of detoxification.

Reconstruction of the evolution of host specialization and associated physiological adaptations in detoxication or nutritional efficiency in *Callosamia* silkmoths requires a sound phylogeny on which to overlay current species characteristics. Unfortunately, the similarity of genetic identities between *C. promethea*, *C. angulifera* and *C. securifera* and the lack of an outgroup for comparison provided little basis for determining ancestral versus derived traits in the group. Continued use of comparative approaches to evaluate physiological adaptations to hosts in an evolutionary context should provide the means for resolving whether there are in fact nutritional or detoxicative tradeoffs associated with specialization, and whether they are influential in the evolution of host use patterns.

# APPENDICES

## APPENDIX 1

### Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 1993-3

Title of thesis or dissertation (or other research projects):

Physiological adaptations associated with host specialization: Ecological implications for generalist and specialist Saturniids ( Callosamia spp.)

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name (s) (typed)
Kelly S. Johnson

Date 30 July 1993

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America. Bull. Entomol. Soc. Amer. 24:141-42.

Deposit as follows:

| | |
|---|---|
| Original: | Include as Appendix 1 in ribbon copy of thesis or dissertation. |
| Copies: | Included as Appendix 1 in copies of thesis or dissertation.<br>Museum(s) files.<br>Research project files. |

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

## APPENDIX 1.1

### Voucher Specimen Data

Page 1 of 3 Pages

| Species or other taxon | Label data for specimens collected or used and deposited | Number of: Eggs | Larvae | Nymphs | Pupae | Adults ♀ | Adults ♂ | Other | Museum where deposited |
|---|---|---|---|---|---|---|---|---|---|
| *Callosamia* *promethea* (Drury) | VA: Greenville<br>November 1988<br>T. R. Peerie | | | | | 1 | | | |
| *Callosamia* *promethea* (Drury) | MI: Barry Co.<br>February 1991<br>K. S. Johnson | | | | | 3 | 4 | | |
| *Callosamia* *promethea* (Drury) | MD: Montgomery Co.<br>Lab reared 1990<br>H. Pavulaan | | | | | 4 | 3 | | |
| *Callosamia* *promethea* (Drury) | MI: Otsego Co.<br>March 1990<br>K. S. Johnson | | | | | | 1 | | |

(Use additional sheets if necessary)

Investigator's Name(s) (typed)
K. S. Johnson

Date 30 July 1993

Voucher No. 1993-3
Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator Date

## APPENDIX 1.2

Voucher Specimen Data

Page 2 of 3 Pages

| Species or other taxon | Label data for specimens collected or used and deposited | Number of: Eggs | Larvae | Nymphs | Pupae | Adults ♀ | Adults ♂ | Other | Museum where deposited |
|---|---|---|---|---|---|---|---|---|---|
| *Callosamia angulifera* (Walker) | PA: Lebanon Co.<br>Lab reared 1989<br>J. Frey | | | | | 1 | 2 | | |
| *Callosamia angulifera* (Walker) | MI: Cass Co.<br>July 1991<br>K. S. Johnson | | | | | 1 | 5 | | |
| *Callosamia angulifera* (Walker) | SC: Florence<br>Lab reared 1990<br>J. Frey | | | | | | 1 | | |
| *Callosamia securifera* (Maassen) | FL: Highlands Co.<br>Lab reared March 1989<br>K. S. Johnson | | | | | 4 | 2 | | |
| *Callosamia securifera* (Maassen) | FL: Highlands Co.<br>Lab reared March 1992<br>K. S. Johnson | | | | | 2 | 2 | | |

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

K. S. Johnson

Date 30 July 1993

Voucher No. 1993-3

Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator Date

## APPENDIX 1.3

Voucher Specimen Data

Page 3 of 3 Pages

| Species or other taxon | Label data for specimens collected or used and deposited | Number of: Eggs | Larvae | Nymphs | Pupae | Adults ♀ | Adults ♂ | Other | Museum where deposited |
|---|---|---|---|---|---|---|---|---|---|
| Callosamia securifera (Maassen) | FL: Lake<br>Lab reared 1990<br>L. Simon | | | | | | 1 | | |
| Callosamia securifera (Maassen) | FL: Marion<br>December 1988<br>K. S. Johnson | | | | | | 1 | | |
| C. angulifera x promethea (male) | Lab mating<br>July 1991<br>K. S. Johnson | | | | | 1 | 3 | | |
| C. promethea x angulifera (male) | MI: Barry Co. x Cass Co.<br>July 1991 | | | | | | 5 | | |

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

K. S. Johnson

Date 30 July 1993

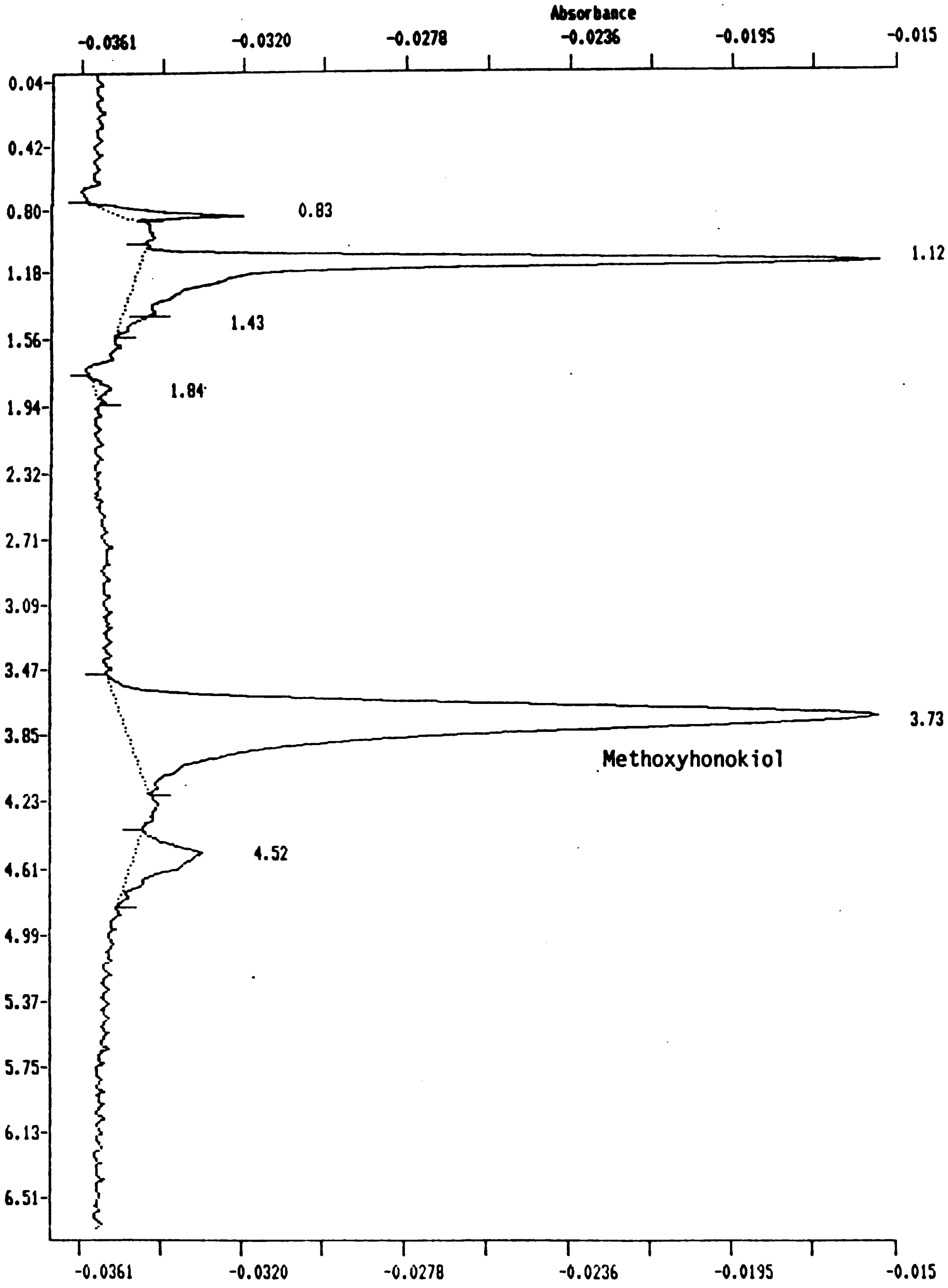
Voucher No. 1993-3

Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator Date

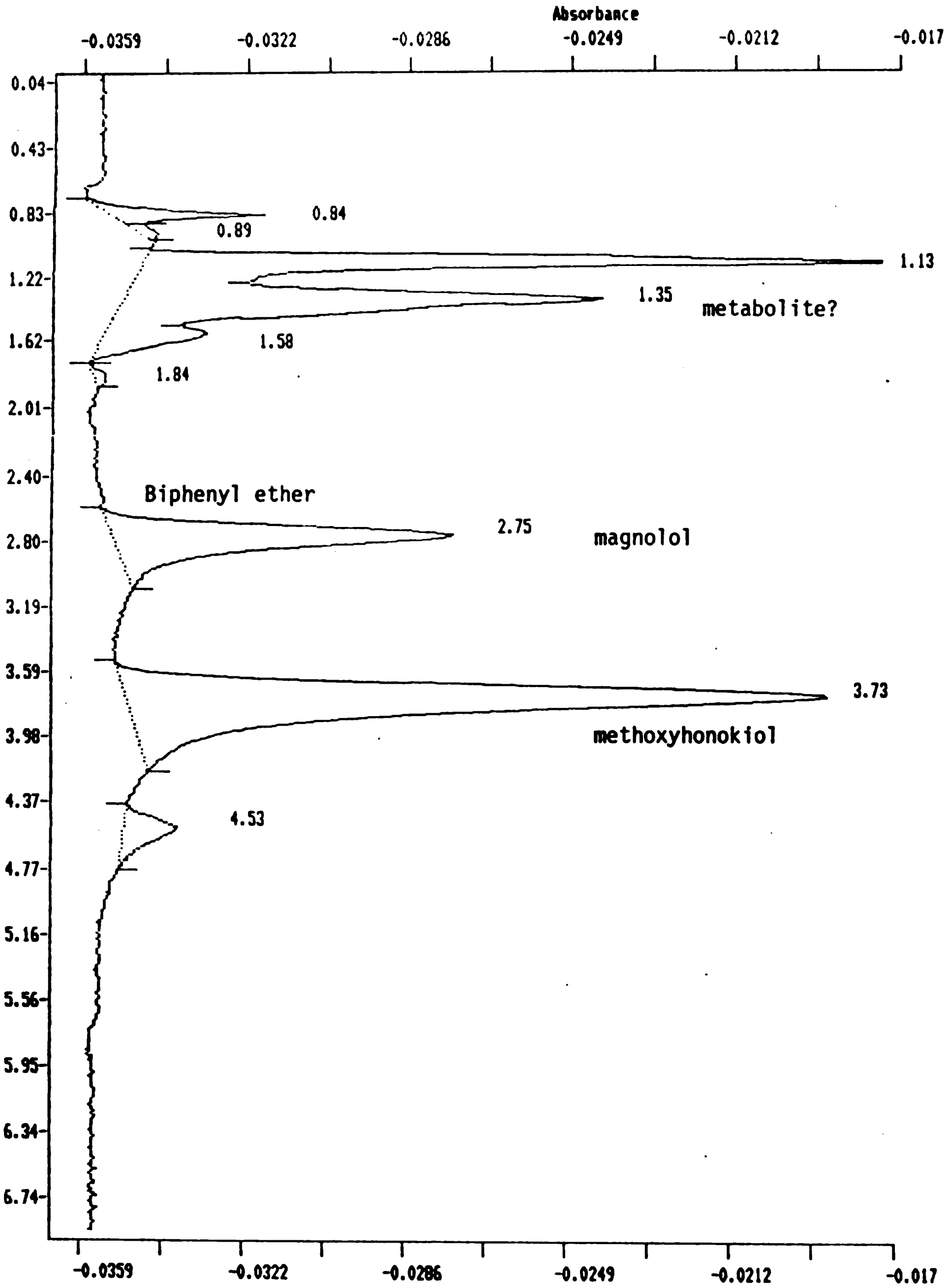
## Appendix 2.1

HPLC chromatogram of incubation extract: *C. securifera* midgut tissue + NADPH (no biphenyl ether or magnolol). Methoxyhonokiol was added as a standard after the incubation was halted.



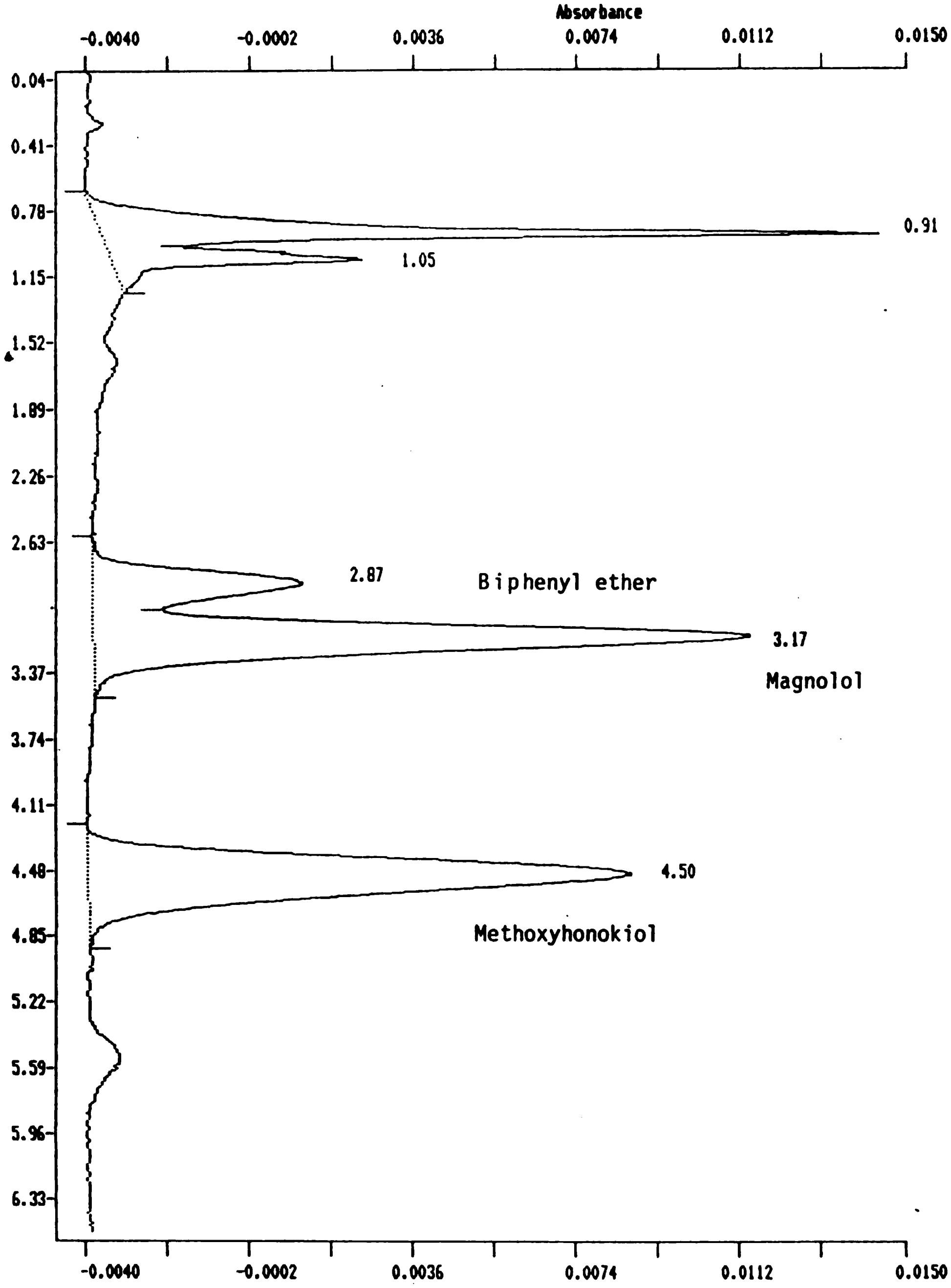
## Appendix 2.2

HPLC chromatogram of incubation extract: *C. securifera* midgut tissue + NADPH + biphenyl ether: magnolol mixture. Methoxyhonokiol was added as a standard after the incubation was halted.



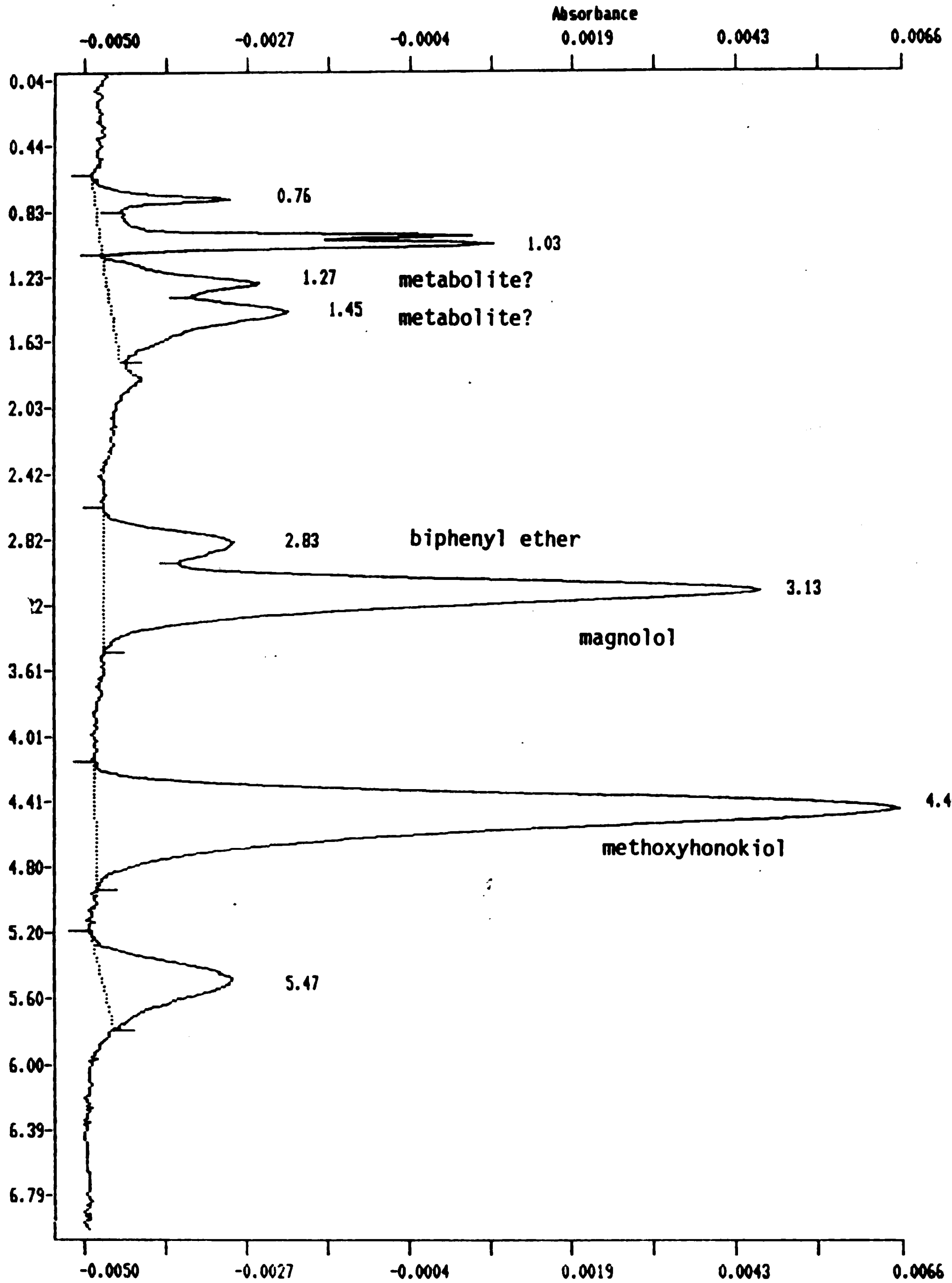
## Appendix 2.3

HPLC chromatogram of incubation extract: *C. promethea* midgut tissue + NADPH + biphenyl ether: magnolol mixture stopped at zero time with acid. Methoxyhonkiol was added as a standard after the incubation was halted. Larvae were reared on tuliptree.



## Appendix 2.4

HPLC chromatogram of incubation extract: *C. promethea* + NADPH + biphenyl ether: magnolol mixture. Methoxyhonokiol was added as a standard after the incubation was halted. Larvae were reared on tuliptree.



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