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HOST FINDING AND RECOGNITION BY *PAPILIO POLYXENES*  
(LEPIDOPTERA: PAPILIONIDAE): THE EFFECTS OF THREE HOST CUES  
AND OF HOST-PLANT EXPERIENCE ON OVIPOSITION BEHAVIOR

A Dissertation

Presented to the Faculty of the Graduate School  
of Cornell University

in Partial Fulfillment of the Requirements for the Degree of  
Doctor of Philosophy

by

Cheryl Ann Heinz

January 2002

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## BIOGRAPHICAL SKETCH

Cheryl Ann Heinz was born on 27 December 1970 in Rockford, Illinois. Adopted at birth, her early years in Westchester, Illinois, led to even more complex family changes. By the time she was eight years old, she was surrounded by two loving sets of parents, Craig and Nancy Burda and Ronald and Donna Heinz, as well as a new younger brother, Darren Burda.

A product of an excellent local public school system, Cheryl attended Mary Jane Kennedy School from kindergarden through fourth grade. Fifth grade brought a reorganization of the school system, and a transfer to the new Westchester Elementary School. Sixth through eighth grades were at the Westchester Middle School. During this time, Cheryl participated continuously in the band program, eventually winning the Outstanding 8<sup>th</sup> Grade Band Student award for her clarinet playing. She also participated occasionally in the chorus program, playing a bum named Sophie in the school presentation of Annie. She was already honing some computer skills, winning an award for writing a program in BASIC that would teach someone how to use high resolution (for 1984) graphics.

Cheryl attended Proviso West High School in Hillside, Illinois for one year, still playing the clarinet, as well as competing on the math and varsity track teams in the West Suburban League. Encouraged by her math coach and SDL Geometry teacher, she applied to attend a new, residential high school for Illinois students gifted in math and science that was to begin the following fall, with its first class of sophomores. In the fall of 1986, Cheryl became a member of the Charter Class of the

Illinois Mathematics and Science Academy, in Aurora, Illinois. In this new learning community, students were immersed in intellectual as well as creative pursuits.

While a student at IMSA, as it is affectionately known, Cheryl participated variously in the varsity volleyball, track, and bowling teams, a short-lived and loosely-defined jazz band that eventually evolved into a concert band, orchestra, Student Pugwash, Psychology Club, and Future Problem Solving Bowl (FPSB). In 1989, her senior year, a group of friends formed an FPSB team that won at the state level in Illinois, including an eighth place individual finish for Cheryl. The team took a post-graduation trip with the inspired Bernie Hollister to Ann Arbor, Michigan, for the "International" FPSB Competition, but failed to 'place' due to neglecting world peace in the final solution.

From IMSA, Cheryl went to the University of Illinois at Urbana-Champaign. She began working in the laboratory of Nancy Burley in the fall of her freshman year. Apparently impressed by a senior project on the extinction patterns of birds across the K/T boundary completed under the supervision of Ronald Pine at IMSA, Burley gave Cheryl an independent project to work on during her first summer in Champaign-Urbana. In addition, Cheryl spent several late evenings in the field tracking field voles with Lowell Getz, who advised her to not major in Ecology, Ethology, and Evolution (EEE). After spending one semester majoring in General Biology, Cheryl transferred to the EEE program. However, in the spring of her freshman year, she interviewed for one of 30 positions in the Honors Biology major. Cheryl was invited into the three-year program, which, she was later informed by a member of the campus curriculum committee, was considered to be one of the most difficult majors on campus due to very strict and advanced curriculum requirements.



Nonetheless, Cheryl persevered (only once contemplating a transfer to the English literature program during the final exam of an engineering–major level physics course), and graduated with high distinction in the spring of 1993.

After her early work with birds and mammals, Cheryl was looking for a different type of research project for her junior year and discussed the matter with her major advisor, Stuart Berlocher. He provided a list of names, and Cheryl did some research to see what sorts of projects each person was doing. She was intrigued by the work of May Berenbaum, not realizing that one could combine evolution, ecology, and chemistry. She spoke with May, which led to nearly two years of work in her lab, assisting graduate students in their research, helping with the catching of *Papilio polyxenes* and *Depressaria pastinacella*, and doing general gopher work in the campus libraries. Cheryl also completed a research project on the effects of anti–depressant compounds on the feeding behavior of *Trichoplusia ni*, resulting in her graduation with high distinction. May also greatly influenced Cheryl’s decision to attend Cornell for a graduate degree, as several of her other choices of potential major advisors at other schools had radiated from the same laboratory that she had been in while at Cornell, that of Paul Feeny.

While at Cornell, Cheryl was active for several years in the Graduate and Professional Student Assembly and the financial committees thereof. She also acted as a teaching assistant in a wide array of courses, culminating the in the Outstanding Teaching Assistant Award from the Department of Entomology in 2001.

One of the secrets of Cheryl’s past is a number of years of service at a McDonalds near the University of Illinois campus. Although the store itself no longer

exists, it was while working there that she met Robert Miroballi, another student working to make ends meet. Over time, an acquaintance became a friend, and the friendship slowly grew into something greater, culminating in marriage on 18 July 1997.

For Robert,  
my best friend, soulmate,  
and best of all, husband

and

For my parents,  
who have always been there,  
even when they didn't understand quite what I was getting into

## ACKNOWLEDGMENTS

I am deeply indebted first and foremost to my major advisor, Paul Feeny. He has consistently and graciously provided advice, time, space, effort, and funding, as well as social gatherings and sails on Cayuga Lake. This project would not have been possible without his stalwart advice and extensive knowledge of the systems involved. I am also deeply indebted to the other members of my committee: Cole Gilbert, J. Alan Renwick, and Thomas Seeley. All provided their own expertise and thoughtful advice, and have helped me to develop this project much more fully than I could have without them.

The Feeny lab group has been a constant source of support, feedback, and advice, as well as diversions, despite its changing nature. During my time in the lab, the group has variously included Maureen Carter, Meena Harbibal, Janie Sue Brooks, Karen Sime, Brian Traw, Shannon Murphy, and Michael Marsello. I am also indebted to Daniel Cousins for his careful attention to the plants used in my experiments, and for the idea of putting the cage for free-flight experiments in a greenhouse module.

Throughout my years at Cornell, I have shared an office variously with Jill Gregg, Pierre Gingerich, Erik Lilleskov, Karen Sime, Pamela Templer, and Janie Brooks. All have provided further support, feedback, advice, as well as conversation.

For advice, feedback, and discussions of my research, I thank Rick Harrison, Ron Hoy, Dan Papaj, Jack Schultz, Michael Singer, John Thompson. For assistance in recording the reflectances of the model leaf surfaces, I thank Ellis Lowe.

Three undergraduate assistants allowed me to complete this work in the time allotted. David Boyce, Marc Lachs, and Crystal Tung all demonstrated the dedication and work ethics of Cornell undergraduates to me.

I am indebted to the staff of the Entomology and especially Ecology and Evolutionary Biology Departments. From support in the construction of my NSF Doctoral Dissertation Improvement Grant to assistance in the regulations involving the hiring of students to the smiles and hellos in the hallways, the staff are exemplary.

The National Science Foundation provided support for much of this research in the form of a Doctoral Dissertation Improvement Grant (NSF IBN 99-02160). Additional NSF support was in the form of grants to Paul Feeny (IBN 91-19674, IBN 94-20319, and IBN 99-86250). Grants from the Andrew W. Mellon Student Research Fund at Cornell University, the Ecology and Evolutionary Biology Graduate Student Research Fund, the Grace H. Griswold Fund, the W. Arthur Rawlins and Alma D. Rawlins Graduate Student Endowment, and the Graduate School of Cornell University supported this research in various ways. Additionally, I was supported by teaching assistantships from Introductory Biology, Entomology, and Ecology and Evolutionary Biology during fall and spring semesters, and the Departments of Entomology and Ecology and Evolutionary Biology as well as the Graduate School during summers.

Many thanks to May Berenbaum for helping me to decide to come to Cornell. I am also indebted to my email support group of Elizabeth Doyle and Erika Tracy for encouragement, amusement, and reminders that life will go on. My cats have provided warmed legs and reminders to get up to feed them. Unfortunately, Kit, the cat that

moved to Ithaca with me did not see me through to the end, but Skippy and Gypsy have done their best in her absence.

My parents have always given me all of the emotional support I needed, as well as occasional other forms of support as needed. Although they have probably thought much about biology since their high school classes, all have been firm believers in my ability to complete anything I have started, including this degree. I could never find better cheerleaders, and I thank them for all of their wholehearted support.

My husband, Robert Miroballi, has provided all of the support and encouragement I have ever asked for, and still loves me in spite of it. It would be hard to find a truer friend or love, and I thank him dearly.

## TABLE OF CONTENTS

BIOGRAPHICAL SKETCH	iii
ACKNOWLEDGEMENTS	viii
TABLE OF CONTENTS	x
LIST OF TABLES	xii
LIST OF FIGURES	xv
CHAPTER ONE: INTRODUCTION	1
CHAPTER TWO: RESPONSES TO CONTACT CHEMISTRY	25
CHAPTER THREE: RESPONSES TO VOLATILE CHEMISTRY	48
CHAPTER FOUR: RESPONSES TO LEAF SHAPE. WITH AND WITHOUT CHEMICAL CUES	84
CHAPTER FIVE: RESPONSES TO CONTACT AND VOLATILE CHEMISTRY COMBINED	155
CHAPTER SIX: RESPONSES TO ALL THREE CUES TOGETHER. AND RESPONSES TO WHOLE PLANTS	191
CHAPTER SEVEN: EPILOGUE	231
APPENDIX ONE: LEAF SURFACE AREA REGRESSIONS	239

## LIST OF TABLES

Table 1.1	List of species of Lepidoptera that have been tested for an oviposition response to volatile chemical cues	6
Table 2.1	First responses of females before and after host-plant experience in Experiment 1.	31
Table 3.1	Categories of behaviors observed	58
Table 3.2	Leaf surface area regression parameters	60
Table 4.1	Categories of behaviors observed during trials	96
Table 4.2	Leaf surface area regressions	99
Table 4.3	First model plant approached and model plant most often approached within a trial during Experiment 2	117
Table 4.4	First model plant landed on and model plant most often landed on within a trial during Experiment 2	118
Table 4.5	Within-trial effects of experience and model plant on post-approach behaviors in leaf shape and contact chemical cues experiment	122
Table 4.6	Within-trial effects of experience and model plant on post-landing behaviors in leaf shape and contact chemical cues experiment	123
Table 4.7	First model plant approached and model plant most often approached within a trial during Experiment 3	133



Table 4.8	First model plant landed on and model plant most often landed on within a trial during Experiment 3	133
Table 4.9	Within-trial effects of experience and model plant on post-approach behaviors in leaf shape and volatile chemical cues experiment	136
Table 4.10	Within-trial effects of experience and model plant on post-landing behaviors in leaf shape and volatile chemical cues experiment	138
Table 5.1	Categories of behaviors observed during trials	162
Table 5.2	First model plant approached and model plant most often approached within a trial	174
Table 5.3	First model plant landed on and model plant most often landed on within a trial	174
Table 5.4	Within-trial experience effects of experience and model plant on post-approach behaviors	178
Table 5.5	Within-trial experience effects of experience and model plant on post-approach behaviors	179
Table 6.1	Categories of behaviors observed during trials	201
Table 6.2	Within-trial experience effects of experience and model host-plant cues on post-approach behaviors in Experiment 1	209
Table 6.3	Within-trial experience effects of experience and model plant on post-landing behaviors in Experiment 1	210

Table 6.4	Within-trial experience effects of host-plant experience and host-plant species on post-approach behaviors in Experiment 2 (whole plants)	221
Table 6.5	Within-trial experience effects of host-plant experience and host-plant species on post-landing behaviors in Experiment 2	222
Table A1.1	Leaf surface area regression parameters for each species	256

## LIST OF FIGURES

Figure 2.1	Cumulative first responses of naïve (A) and experienced (B) females to <i>D. carota</i> extracts in Experiment 1	32
Figure 2.2	Cumulative first responses of females to plant extracts for each of the four treatments in Experiment 2	35
Figure 2.3	The cumulative percent curling (A) or egg-laying (B) response of females to <i>D. carota</i> extract	38
Figure 3.1	The leaf shape used in this experiment	53
Figure 3.2	The arrangement of model plants, nectar plants, and extracts in the array	54
Figure 3.3	The timeline for an individual female	56
Figure 3.4	Median number of approaches and landings per female in naïve trials	62
Figure 3.5	Frequency of landings or other behaviors immediately following approaches to model plants	63
Figure 3.6	Number of approaches (A) and landings (B) by females in experienced trials	64
Figure 3.7	Median number of approaches and landings by experienced females on model plants with respect to the two host-plant volatile extracts	65
Figure 3.8	Median number of approaches and landings by experienced females on model plants with host-plant volatile extracts with respect to the host plant experienced	66

Figure 3.9	Number of females approaching (A) and landing (B) on model plants bearing each of the two host–plant extracts ( <i>D. carota</i> and <i>C. maculatum</i> ) with respect to the type of model plant most frequently landed upon within a single trial	68
Figure 3.10	Frequency of landings or other behaviors immediately following approaches to either of the two host plants, <i>D. carota</i> or <i>C. maculatum</i> , by experienced females, with respect to the the experience of the female	69
Figure 3.11	Distribution of landings by experienced females on <i>C. maculatum</i> model plants	70
Figure 3.12	Distribution of eggs laid on <i>C. maculatum</i> by females	71
Figure 4.1	The leaf shapes used in these experiments	88
Figure 4.2	Timeline for individual females in all three experiments	91
Figure 4.3	The arrangement of model and nectar plants in Experiment 1	93
Figure 4.4	The arrangement of model and nectar plants in Experiment 2	94
Figure 4.5	The arrangement of model and nectar plants in Experiment 3	95
Figure 4.6	Median number of approaches and landings per female in naïve leaf shape trials	101
Figure 4.7	Frequency of landings or other behaviors immediately following approaches to model plants with non–host or host–plant leaf shapes by naïve females	102
Figure 4.8	Number of approaches (A) and landings (B) by females in experienced trials	104

Figure 4.9	Median number of approaches and landings by females in experienced trials on model plants with host–plant leaf shapes with respect to the two host–plant leaf shapes	105
Figure 4.10	Median number of approaches and landings by experienced females on model plants with host–plant leaf shapes	106
Figure 4.11	Number of females approaching (A) and landing on (B) model plants bearing each of the two host–plant leaf shapes ( <i>P. sativa</i> and <i>D. carota</i> ) with respect to the type of model host plant most frequently landed upon	107
Figure 4.12	Frequency of landings or other behaviors immediately following approaches to model plants	108
Figure 4.13	Median number of approaches and landings per female in naïve trials	110
Figure 4.14	Frequency of landings or other behaviors immediately following approaches to (A) and landings on (B) model plants by naïve females	111
Figure 4.15	Number of approaches (A) and landings (B) by females in experienced trials on model plants with host plant cues (leaf shapes and contact chemicals) with respect to the two host–plant leaf shapes	112
Figure 4.16	Number of approaches (A) and landings (B) by females in experienced trials on model plants with host plant cues (leaf shapes and contact chemicals) with respect to the two host–plant contact extracts	114

<b>Figure 4.17</b>	<b>Median number of approaches and landings by experienced females on model plants with host–plant cues</b>	<b>115</b>
<b>Figure 4.18</b>	<b>Median number of approaches and landings by experienced females on model plants with host–plant cues (leaf shape and contact chemical extracts) with respect to the host plant experienced</b>	<b>116</b>
<b>Figure 4.19</b>	<b>Distribution of approaches (A) and landings (B) of individual females within independent trials on model plants with <i>D. carota</i> leaf shapes</b>	<b>119</b>
<b>Figure 4.20</b>	<b>Distribution of approaches (A) and landings (B) of individual females within independent trials on model plants with <i>D. carota</i> contact chemical extracts</b>	<b>120</b>
<b>Figure 4.21</b>	<b>Reflectance spectra of <i>D. carota</i> and <i>P. sativa</i> extracts on model leaf surfaces</b>	<b>124</b>
<b>Figure 4.22</b>	<b>Median number of approaches and landings per female in naïve trials with leaf shapes and volatile chemical cues</b>	<b>126</b>
<b>Figure 4.23</b>	<b>Frequency of landings or other behaviors immediately following approaches (A) and landings (B) to model plants with non–host or host–plant cues by naïve females</b>	<b>127</b>
<b>Figure 4.24</b>	<b>Number of approaches (A) and landings (B) by females in experienced trials on model plants with host plant cues with respect to the two host–plant leaf shapes</b>	<b>128</b>

Figure 4.25	Number of approaches (A) and landings (B) by females in experienced trials on model plants with host plant cues with respect to the two host–plant volatile extracts	129
Figure 4.26	Median number of approaches and landings by experienced females on model plants with host–plant cues	131
Figure 4.27	Median number of approaches and landings by experienced females on model plants with host–plant cues with respect to the host plant experienced	132
Figure 4.28	Distribution of approaches (A) and landings (B) of individual females on model plants with host–plant leaf shapes during independent trials	134
Figure 4.29	Distribution of approaches (A) and landings (B) of individual females on model plants with host–plant volatiles during independent trials	135
Figure 5.1	Timeline of females in this experiment	158
Figure 5.2	Arrangement of model and nectar plants in the experimental array	160
Figure 5.3	Median number of approaches and landings per female in naïve trials	166
Figure 5.4	Frequency of landings or other behaviors immediately following approaches (A) and landings (B) to model plants with non–host or host–plant cues by naïve females	167

Figure 5.5	Number of approaches (A) and landings (B) by females in experienced trials on model plants with host plant cues with respect to the two host–plant volatile chemical cues	169
Figure 5.6	Number of approaches (A) and landings (B) by females in experienced trials on model plants with host plant cues with respect to the two host–plant contact chemical extracts	170
Figure 5.7	Median number of approaches and landings by experienced females on model plants with host–plant cues	171
Figure 5.8	Median number of approaches and landings by experienced females on model plants with host–plant cues ( <i>C. maculatum</i> + <i>D. carota</i> ) with respect to the host plant experienced	172
Figure 5.9	Distribution of approaches (A) and landings (B) of individual females within single trials on model plants with host–plant volatiles	175
Figure 5.10	Distribution of approaches (A) and landings (B) by individual females within single trials on model plants with host–plant contact chemical extracts	176
Figure 5.11	Reflectance spectra of <i>D. carota</i> and <i>C. maculatum</i> extracts on model leaf surfaces	180
Figure 6.1	The arrangement of model and nectar plants in the first experiment, with all three plant cues	198
Figure 6.2	Timeline for females in Experiment 1	199
Figure 6.3	Median number of approaches and landings per female in naïve trials in Experiment 1	202



Figure 6.4	Frequency of landings or other behaviors immediately following approaches on model plants with non-host or host cues by naïve females	203
Figure 6.5	Number of approaches (A) or landings (B) by females in experienced trials on model plants with host-plant cues with respect to host-plant experience	205
Figure 6.6	Median number of approaches and landings by females in experienced trials on model plants with host-plant cues with respect to the two types of host-plant cues	206
Figure 6.7	Median number of approaches and landings by experienced females on model plants with host-plant cues with respect to the host plant experienced	207
Figure 6.8	Number of females approaching (A) and landing (B) most often on model plants bearing host-plant ( <i>C. maculatum</i> or <i>P. sativa</i> ) cues with respect to the type of model host plant first landed upon during a single trial	208
Figure 6.9	The arrangement of model and nectar plants in the second experiment	211
Figure 6.10	Timeline for females in Experiment 2	212
Figure 6.11	Median number of approaches and landings per female in naïve trials in Experiment 2	214
Figure 6.12	Frequency of behaviors following approaches (A) and landings (B) by naïve females in Experiment 2	215

Figure 6.13	Number of approaches (A) and landings (B) by females in experienced trials on host plants with respect to the host plant experienced and the plant species	216
Figure 6.14	Median number of approaches and landings by females in experienced trials on host plants, with respect to only the plant species	218
Figure 6.15	Median number of approaches and landings by experienced females on host plants with respect to the host plant experienced	219
Figure 6.16	Number of females approaching (A) and landing (B) most often on host plants with respect to the species of host plant first landed upon during a single trial	220
Figure A1.1	Regression of leaf length on total surface area for <i>D. carota</i>	241
Figure A1.2	Regression of leaf width on total surface area for <i>D. carota</i>	242
Figure A1.3	Regression of leaf weight on total surface area for <i>D. carota</i>	243
Figure A1.4	Regression of leaf length on total surface area for <i>C. maculatum</i>	244
Figure A1.5	Regression of leaf width on total surface area for <i>C. maculatum</i>	245
Figure A1.6	Regression of leaf weight on total surface area for <i>C. maculatum</i>	246
Figure A1.7	Regression of leaf length on total surface area for <i>P. sativa</i>	247
Figure A1.8	Regression of leaf width on total surface area for <i>P. sativa</i>	248

Figure A1.9	Regression of leaf weight on total surface area for <i>P. sativa</i>	249
Figure A1.10	Regression of leaf length on total surface area for <i>V. faba</i> , var. unknown	250
Figure A1.11	Regression of leaf width on total surface area for <i>V. faba</i> , var. unknown	251
Figure A1.12	Regression of leaf weight on total surface area for <i>V. faba</i> , var. unknown	252
Figure A1.13	Regression of leaf length on total surface area for <i>V. faba</i> , var. Broad Windsor	253
Figure A1.14	Regression of leaf width on total surface area for <i>V. faba</i> , var. Broad Windsor	254
Figure A1.15	Regression of leaf weight on total surface area for <i>V. faba</i> , var. Broad Windsor	255

## CHAPTER ONE

### INTRODUCTION

Herbivorous insects are rarely able to eat all kinds of available plant material. The majority of phytophagous insects are oligophagous, feeding on a variety of host plants that are botanically and/or chemically related (Strong, et al. 1984; Jermy, et al. 1990; Bernays and Chapman 1994). This provides these insects the flexibility to exploit a variety of plant species; however, even closely-related plants can differ in such phenotypic characters as growth form, leaf shape, and chemistry. How then can we account for the fidelity of oligophagous insects for a particular set of plants growing in complex vegetation? Clearly, host-finding responses to at least some plant traits must have a heritable component, and may be subject to experience-induced modification. Remarkably, little is known of the relative roles of "nature" and "nurture" in host-finding by herbivorous insects.

In the case of ovipositing butterflies, the series of host-finding behaviors is traditionally broken into six phases: search, approach, inspection, landing, abdomen curling, and oviposition (Damman and Feeny 1988; Renwick and Huang 1994). During each of these phases, a female judges the suitability of the plant and either accepts it and continues to the next phase, or rejects it and starts the search again. In making her judgement, a female uses three primary categories of host-plant traits as cues: visual, volatile chemical (olfactory), and contact chemical (gustatory). Visual cues, such as leaf shape, leaf color, and presence or absence of floral structures or leaf buds, are used during the entire sequence, and are learned by some species of searching butterflies (Traynier 1986; Rausher 1995). Volatile chemical cues can direct a female to the proper habitat at searching distances, giving more specific host

information during the remaining phases (Renwick and Huang 1994). Volatile chemical cues are learned by some moths, including *Tricoplusia ni* (Landolt and Molina 1996) and *Helicoverpa armigera* (Cunningham, et al. 1999). Contact chemical cues are the most restrictive; they require contact with the plant, and are thus available only after landing. These have been demonstrated to be the unconditional stimulus in the associative learning of leaf shapes by *Battus philenor* (Papaj 1986).

To account for specificity in host choice from one generation to the next, responses to at least one of the three host-finding cues must be heritable. Although oviposition preference within the *Papilio machaon* group of swallowtail butterflies is heritable (Thompson 1998), the heritability of responses to specific host-plant traits is unknown. Except for *B. philenor* females learning leaf shapes (Papaj and Rausher 1987a; Rausher 1995), it has been assumed that the responses to host plant cues are heritable. We do not know which responses to which cues have a heritable component; the assumption is that response to contact chemical cues is innate, but there is no direct, experimental proof.

The goal of the experiments in this dissertation has been to determine which of the three host plant cues elicit innate oviposition responses, with an underlying assumption that at least one of the three must evoke a fixed response to account for host specificity in an oligophagous species. The role of experience in modifying responses to host plant cues has also been examined, as even responses that have an innate basis may be altered by experience with a host plant. The focus of this research was the oligophagous species *Papilio polyxenes* (Lepidoptera: Papilionidae), the black swallowtail butterfly. This species has been shown to use contact chemical as well as volatile chemical cues to identify host plants in the family Apiaceae; several of the

stimulatory compounds as well as contact chemical receptors have been identified (Feeny, et al. 1988; Feeny, et al. 1989; Roessingh, et al. 1991; Baur, et al. 1993; Baur and Feeny 1994/1995; Carter, et al. 1998). Only anecdotal evidence exists for the use of visual cues in this species, but this evidence is nonetheless very suggestive of a role of visual stimuli in host location (Åhman, unpublished; pers. obs.). In addition, the host plants for this species are particularly diverse in terms of leaf shape, volatile chemistry, and contact chemistry.

### CUES FOR HOST FINDING

The use of all three types of host-plant cue by searching or ovipositing females has been well documented in several lepidopteran groups (Renwick and Chew 1994). The use of contact stimulant chemicals by butterflies has been particularly well studied in the Papilionidae (Nishida 1995) and the Pieridae (Renwick 1989). Volatile chemicals have been shown to be important for a number of lepidopteran groups (Renwick and Chew 1994; Haribal and Feeny 1998), and there is evidence for learning of host volatiles by the noctuid moths, *Trichoplusia ni* (Landolt and Molina 1996) and *Helicoverpa armigera* (Cunningham, et al. 1999). Visual cues are well known to be used in host finding by butterflies, and have been implicated in learning behavior in at least two families, Papilionidae and Pieridae (Stanton 1984; Renwick and Chew 1994; Rausher 1995).

#### *Contact chemical cues*

Contact chemicals are important, non-volatile host-recognition cues that act as oviposition stimulants for many phytophagous insects. These compounds are not available to a searching insect until it has landed upon the leaf, at which point gustatory receptors on the tarsi (Roessingh, et al. 1991; Simmonds, et al. 1994; Baur,

et al. 1998; Shimoda and Kiuchi 1998) or antennae (Haribal and Renwick 1998a; Hora and Roessingh 1999) come into contact with the leaf surface. Stimulants have been identified for several groups, including several phytophagous flies (Städler 1982; Simmonds, et al. 1994; Baur, et al. 1996; Degen, et al. 1999; Hurter, et al. 1999) a sawfly (Roininen, et al. 1999), and several species or groups of Lepidoptera. There has been some work with Noctuidae (Ramaswamy, et al. 1987), Yponomeutidae (Hora and Roessingh 1999) and Nymphalidae (Baur, et al. 1998; Haribal and Renwick 1998b), but most of the effort has focused on Pieridae (Traynier and Truscott 1991; Renwick, et al. 1992; Huang and Renwick 1993; Huang, et al. 1993a; Huang, et al. 1993b; Huang, et al. 1994/1995) and Papilionidae (reviewed in Nishida 1995). Deterrent non-volatile cues can also play an important role in the identification of hosts or non-hosts (Huang, et al. 1993a; Huang, et al. 1993b; Huang, et al. 1994/1995; Honda 1995).

In the Papilionidae, contact stimulants typically interact synergistically to produce a characteristic oviposition response (Nishida 1995; but see Haribal and Feeny 1998). Similar types of compounds, such as flavonoid glycosides and cyclitols, are required by butterflies using host plants from a botanically diverse group of families: Annonaceae, Apiaceae, Aristolochiaceae, and Rutaceae (Feeny 1995; Nishida 1995; Carter, et al. 1998; Haribal and Feeny 1998). It has been hypothesized that the similarity in the structure of these chemical cues is due to constraints on the evolution of the responses (by either behavioral or receptor-level mechanisms) (Feeny 1991). However, naïve females had never been tested for their responses to contact chemical cues, so whether the responses to reported stimulants were heritable rather than consequences of experience was not known.

### *Volatile chemical cues*

While contact chemical cues require contact with the leaf surface, volatile chemical cues allow for relatively specific information to reach an insect prior to contact with the plant. Volatile chemical cues have long been known to be important in insect–plant interactions (Visser 1986). Most receptors for volatile chemical cues are found in the antennae, although a few can be found on the palps of some Orthoptera and Lepidoptera (Bernays and Chapman 1994).

Across the Lepidoptera, several species have been shown to respond to volatile chemical cues for host finding or recognition, while only three have been reported not to respond at all (Table 1.1). Among the Papilionidae, female *Papilio demoleus* butterflies visited volatile-containing ether extracts of hosts more often than controls, and some butterflies even attempted to lay eggs in the absence of contact with the extracts (Saxena and Goyal 1978). Females of *P. polyxenes* increased activity in the presence of host volatiles, increasing landing and therefore oviposition rates on test leaves treated with both contact and volatile chemicals (Feeny, et al. 1989). No direct effect of volatiles on post–alighting oviposition behavior was found, agreeing with earlier findings by Ichinosé and Honda (1978) for *P. protenor*. However, female *Eurytides marcellus*, another papilionid, increased oviposition activity significantly in the presence of host volatiles, with some butterflies attempting to lay eggs when only volatile chemical cues were present (Haribal and Feeny 1998).

### *Visual cues*

Visual cues may be the only directional cues available to a butterfly as she begins searching; they may interact with volatile cues during orientation and inspection and with both volatile and contact chemical cues once landing has taken place (Damman



Table 1.1 List of species of Lepidoptera that have been tested for an oviposition response to volatile chemical cues. Responses are positive (“+”) or negative (“-”).

Family	Species	Response	References
Yponomeutidae	<i>Yponomeuta</i> <i>cagnagellus</i>	-	(Hora and Roessingh 1999)
Plutellidae	<i>Plutella xylostella</i>	+	(Justus and Mitchell 1996)
Tortricidae	<i>Cochylis hospes</i>	+	(Barker 1997)
	<i>Epiphyas</i> <i>postvittana</i>	+	(Suckling, et al. 1996)
Pyralidae	<i>Dioryctria amatella</i>	+	(Hanula, et al. 1985)
	<i>Ostrinia nubalis</i>	+	(Binder and Robbins 1997)
Geometridae	<i>Cideria albulata</i>	+	(Douwes 1968)
Papilionidae	<i>Eurytides marcellus</i>	+	(Haribal and Feeny 1998)
	<i>Papilio demoleus</i>	+	(Saxena and Goyal 1978)
	<i>Papilio polyxenes</i>	+	(Feeny, et al. 1989)
	<i>Papilio protenor</i>	-	(Ichinosé and Honda 1978)
Pieridae	<i>Pieris rapae</i>	(possible)	(Hern, et al. 1996)
Nymphalidae	<i>Agraulis vanillae</i>	+	(Copp and Davenport 1978)
Sphingidae	<i>Agrius convolvuli</i>	+	(Shimoda and Kiuchi 1998)
Noctuidae	<i>Helicoverpa zea</i>	+	(Hartlieb and Rembold 1996; Jallow, et al. 1999)
	<i>Heliothis virescens</i>	-	(Ramaswamy, et al. 1987)
	<i>Mamestra</i> <i>brassicae</i>	+	(Rojas and Wyatt 1999)
	<i>Trichoplusia ni</i>	+	(Landolt and Molina 1996)

and Feeny 1988; Renwick and Huang 1994). Visual cues can take several forms, including shapes, colors, and contrasts, and all have been shown to play a role in the ability of phytophagous insects to find their host plants (Gilbert 1975; Rausher 1978; Traynier 1979; Harris and Miller 1982; Wiklund 1984; Mackay and Jones 1989; Aluja and Prokopy 1993).

In two lepidopteran genera, *Colias* (Pieridae) and *Battus* (Papilionidae), females use visual cues to enhance searching efficiency (Stanton 1984; Rausher 1995). *Colias philodice eriphyle* and *Colias meadii* oviposit on legumes, and often, when searching for oviposition sites rather than nectar sites, land on non-legume plants with leaf shapes similar to legume host plants (Stanton 1984). However, the occurrence of these landing "errors" decreases during an oviposition bout, suggesting that there is short-term learning. Female *Battus philenor* in Texas use either of two host-plant species, the broad-leaved *Aristolochia reticulata* or the narrow-leaved *A. serpentaria*. Single females land preferentially on plants (hosts and non-hosts; more than 80% of landings occur on non-hosts for this species) of either broad or narrow leaves, and recent experience of the female determines leaf-shape preference (Papaj 1986; Papaj and Rausher 1987b; Rausher 1995). Although the percentage of landings on non-hosts is high, the short-term specialization on broad or narrow leaves is adaptive early in the season, when only the broad-leaved host is available, and later in the season, when only the narrow-leaved host can support caterpillar growth (Rausher 1995). Although searching efficiency can be increased by learning leaf shapes, this is not always the case. The nymphalid butterfly, *Euphydryas editha* is unable to increase its searching efficiency through experience, presumably due to environmental factors such as a short flight season and high environmental predictability (Parmesan, et al. 1995).

## LEARNING

The simplest definition of learning is “a change in behavior with experience” (Mackintosh 1983; Shettleworth 1984). This definition has its faults, mainly in including several categories of behavior that are not ideally considered learning, such as motor programs or maturational changes. Better is to add criteria, as suggested by Papaj and Prokopy (1989). Requiring the repeatability of the phenomenon, as measured by statistical probabilities, excludes the possibility of a chance correlation. Only accepting gradual changes in behavior as learning, as demonstrated by a learning curve, excludes motor programs, but, unfortunately, excludes single trial learning as well. Adding a requirement for reversibility, the ability to “unlearn” or replace one learned item with another, excludes maturational processes. Clearly, the strict application of all of these criteria will exclude many of the instances that would generally be thought of as learning; therefore, a more judicious use is required.

Learning can be predicted, or assumed to be adaptive, for a phytophagous insect when the availability of host plants is constant within a generation but varies from one generation to the next (Stephens 1993). A fitting example is that of *Battus philenor*, as described in the preceding section (Rausher 1995). In a habitat in east Texas, two hosts species are found: broad-leafed *Aristolochia reticulata* and the narrow-leafed *A. serpentaria*. *A. reticulata* is easier to find, as the narrow leaves of *A. serpentaria* provide this species with some level of crypsis. Early in the season, during the first flight of butterflies, females are more likely to be caught searching for the broad leaf shape of *A. reticulata*. Later in the same season, during the second generation of butterflies, *A. reticulata* leaves are too tough for larval feeding, and the females are more likely to be caught searching for the narrow-leafed, and still palatable, *A. serpentaria*. This variation from one generation to the next is not caused

by a genetic effect, but rather by learning, as females can associate the contact chemical cues of the host with the leaf shape of the plant landed upon (Papaj 1986; Papaj and Rausher 1987b).

This type of learning is known as associative learning, or conditioning (Mackintosh 1983). In this type of learning, an initially neutral stimulus, the conditioned stimulus (CS) is presented to the insect in conjunction with a stimulus, the unconditioned stimulus (US), that innately releases the desired behavior, the unconditioned response (UR). With repeated pairings, the CS alone will release the US, as first demonstrated in dogs by Pavlov (1927). A similar type of learning, a simple increase in a response with repeated presentation of a stimulus, is known as sensitization. In practice, sensitization and associative learning can be difficult to distinguish in studies of phytophagous insects due to the usual lack of knowledge concerning the exact stimuli involved in the presentation (Papaj and Prokopy 1989). Food aversion learning (Dethier 1988) is considered by some to be an additional type of learning (Papaj and Prokopy 1989), though it appears to simply be a special form of associative learning. In this case, a feeding insect learns to avoid a previously ingested food that resulted in a sort of malaise after ingestion. An additional non-associative form of learning is known as habituation, in which repeated presentation of a stimulus results in the waning of the response (Thorpe 1963). Finally, "induction of preference" seems to be a catchall category for effects of experience on feeding or oviposition preferences that cannot otherwise be explained by one of the earlier categories (Papaj and Prokopy 1989). For example, *Manduca sexta* caterpillars will initially accept *Lycopersicon esculentum*, *Nicotiana tabacum*, and *Solanum pseudocapsicum*. However, after a caterpillar has fed on *L. esculentum* or *S. pseudocapsicum*, it is much more likely to eat the same plant than any of the other two (Jermy, et al. 1968).

## STUDY SYSTEM

*Papilio polyxenes asterius*, the eastern black swallowtail butterfly, is a scarce to moderately abundant butterfly throughout much of North America east of the Rocky Mountains (Scudder 1889; Tyler 1975). In central New York, oviposition is limited to the Apiaceae, but larvae will also feed on several plants in the Rutaceae, believed to be the basal host plant for the genus (Dethier 1941; Berenbaum 1995). Adults live for an average of two weeks in the field, although some individuals can survive for up to five weeks (Lederhouse 1983). Larval mortality in the field is high; a female must lay at least 60 eggs, on average, to replace herself in the next generation (Feeny, et al. 1985). Larval mortality is due mostly to attack by invertebrate and vertebrate predators (Feeny, et al. 1985); adult mortality is due mostly to predation at overnight roosting sites (Rawlins and Lederhouse 1978).

In central New York, the most common host plant is wild carrot (*Daucus carota*); other local hosts include wild parsnip (*Pastinaca sativa*) and poison hemlock (*Conium maculatum*) (Scriber and Feeny 1979). Most are introduced biennial (*D. carota* is a winter annual) weeds of hayfields, roadsides, and other disturbed areas (Wiegand and Eames 1925). Larvae are also found on cultivated umbellifers, such as celery (*Apium graveolens*), fennel (*Foeniculum vulgare*), and parsley (*Petroselinum crispum*). Because larvae, in general, are unable to move from one host to another, especially in the earliest instars, the placement of the eggs by the ovipositing female ultimately determines the host plant and the fate of the larvae (Rausher 1979; Honda 1995). The larval host does not, however, have an effect on the oviposition choice of the resulting female (Wiklund 1974 ; Feeny and Rosenberry, unpublished). Lekking characterizes mating behavior; adult males are territorial, preferring areas of high altitude (Lederhouse 1982).

All three of the types of cue used for host-plant recognition are important for this species. Previous work has identified two compounds from carrot leaf extracts and one compound from parsnip leaf extracts that elicit oviposition behavior from experienced *P. polyxenes* females: luteolin 7-*O*-(6''-*O*-malonyl)- $\beta$ -D-glucoside and *trans*-chlorogenic acid from carrot and tyramine from parsnip (Feeny, et al. 1988; Carter, et al. 1998). The two compounds from carrot, combined in the correct proportions and presented as contact chemicals, evoked egg-laying behavior from over 75 percent of the females tested (Feeny, et al. 1988). Responses are enhanced significantly by the addition of volatile compounds from the carrot leaf, with females landing more often on model plants containing volatile extracts, leading to more eggs being laid on such models (Feeny, et al. 1989). Several compounds (sabinene hydrate, 4-terpineol, bornyl acetate, and (*Z*)-3-hexenyl acetate) showed high electroantennagram (EAG) activity, though their contribution to oviposition behavior is not yet known (Baur, et al. 1993; Baur and Feeny 1994/1995). Visual characteristics of host plants have never been tested. However, Åhman (unpublished) notes that females in the field appeared to be using flowers as a host finding cue. She noticed that released females landed only on species of host that were in bloom; Wiklund (1974) noted similar behavior by females of the closely-related *P. machaon*.

The systematics of the family Papilionidae is now firmly established (Miller 1987b; Sperling 1993), providing an evolutionary context for work with *P. polyxenes*. Swallowtails and their host plants have commonly been used as a model system for understanding host shifts (Dethier 1941; Miller 1987a; Thompson 1998); identifying the heritable components of host finding is essential for understanding the mechanisms behind these shifts.

Previous study of the effect of experience on *P. polyxenes* oviposition behavior was conducted by Åhman (unpublished). She compared results of a choice test between carrot and parsley for three different groups of females: females exposed to carrot plants for three days, females exposed to parsley plants for three days, and females with no previous experience with any plants. She found a slight, non-significant preference for carrot in all treatments and a slight enhancement of this preference with experience on carrot. The evidence was insufficient to conclude that learning was occurring; however, the evidence was inadequate to reject the possibility.

## EXPERIMENTS

In the chapters that follow, the host plants of *Papilio polyxenes* are broken down into three components: contact chemicals, volatile chemicals, and leaf shapes. Leaf shapes were chosen as the representative visual cue due to the importance of leaf shapes in the host-finding behavior of the pipevine swallowtail butterfly, *Battus philenor*. Each cue is tested individually in turn, followed by the three pairs of cues, the combination of all three cues together, and finally, whole, real plants. For each cue, two questions are asked: 1) Do females respond to the cues presented prior to adult experience with a host plant? and 2) Is there any evidence of learning in the females' responses to each cue or set of cues? The first question was meant to determine which cues released innate responses from mated females. The second question looked at the effect of experience on these responses. In other words, the relative roles of "nature" and "nurture" were to be explicitly examined.

The first type of cue tested, in the experiments in Chapter 2, was contact chemicals. Several oviposition stimulants have already been identified from two of the host plants of *P. polyxenes* (Feeny, et al. 1988; Carter, et al. 1998), and the same

bioassay could be co-opted for use for these questions. Females were presented with contact chemical-containing extracts applied to strips of filter paper, and the responses recorded. Two experiments were needed, one for each of the two questions, as age was seen to affect the results in the first experiment. A third experiment verified the non-deterrent nature of the non-host used as a control, *Vicia faba*, fava bean.

In Chapter 3, the responses to volatile chemistry were tested using a single experiment making use of a free-flight bioassay. Model plants were developed that could be used to present any combination of volatile chemical cues, contact chemical cues, and leaf shapes, to allow their use in subsequent experiments. Females were tested prior to host-plant experience to look for an innate response. They were then allowed to lay eggs on one of two host plants, giving them varied experiences to remove the compounding effects of age. After the host-plant experience, they were tested again, with extracts of each of the two hosts in the array. In this way, both questions could be answered using a single set of females. As naïve female butterflies were expensive in terms of time, effort, and money, this was an important facet of the experimental design.

Chapter 4 examines the role of leaf shapes in much the same way as Chapter 3 examined volatiles. The same model plants were used, but with no chemical cues present for one experiment. Instead, several different leaf shapes were tested with no other cues present. The second and third experiments combined the leaf shapes with contact chemistry and volatile chemistry, respectively. In this manner, the role of leaf shape alone and in conjunction with the chemistry of the hosts was examined, with the luxury of being able to mix and match cues in an effort to determine the relative roles



of each. The combination of volatile and contact chemicals was the subject of Chapter 5, with the same experimental design utilized once again.

Chapter 6 concludes the experimental chapters with two experiments. The first combines all three cue types, still making use of the model plants and the same experimental design. The final experiment is a similar bioassay, but finally using real plants instead of models. The assay with entire plants was left to the end as a check for the remaining experiments. First, the hosts were taken apart into their component parts, and finally, at the end, the plants were reconstructed — first with models, and ultimately, whole plants were used.

An Epilogue is found in Chapter 7, to bring together the results of the previous five chapters and draw conclusions that can only be made by looking at all of the experiments together.

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## CHAPTER TWO

### RESPONSES TO CONTACT CHEMISTRY

Phytophagous insects are highly dependent upon plant chemistry for host-plant identification (Bernays 2001). In particular, contact chemical cues are extremely important to Lepidoptera, in which the ovipositing female bears the responsibility for choosing the larval food plant, and such cues may even be required for oviposition (Renwick and Chew 1994; Honda 1995; Nishida 1995). The situation is particularly complex among the swallowtail butterflies (family Papilionidae), in which multiple stimulants act synergistically, whereas little to no activity occurs when presented singly (Renwick and Chew 1994; Honda 1995; Carter, et al. 1999).

An ovipositing female perceives contact chemical cues only after she lands on the plant surface. Upon landing, females “drum” their foretarsi on the surface of the leaf, so that a large number of chemoreceptors come in contact with chemicals present at the leaf surface (Ilse 1937; Roessingh, et al. 1991). If the correct compounds are present, a female will curl her abdomen, and, if sufficiently stimulated, it will lay an egg. For swallowtails, great effort has been placed into the identification of the contact stimulants (Nishida 1995; Haribal and Feeny 1998; Carter, et al. 1999). However, despite this attention, it is not known whether the responses to these compounds are truly innate, or whether they might be learned or otherwise altered by experience. An indirect test was made by Papaj (1986), who found that naïve female *Battus philenor* could learn to associate methanolic extracts of their *Aristolochia* host plants with leaf shape, but there has yet been no direct test of the contact chemical response with this or any other swallowtail butterfly.

The eastern black swallowtail butterfly, *Papilio polyxenes*, is a species for which contact stimulants have been partly identified (Feeny, et al. 1988). Three compounds have been identified as contact chemical oviposition stimulants: luteolin-7-O-(6''-O-malonyl)- $\beta$ -D-glucoside and *trans*-chlorogenic acid from wild carrot, *Daucus carota* (Feeny, et al. 1988), and tyramine from wild parsnip, *Pastinaca sativa* (Carter, et al. 1998). None of these compounds elicits significant activity when presented singly, but the malonylated glucoside and chlorogenic acid together elicit a significant level of response, as does the combination of chlorogenic acid, tyramine, and an as-yet unidentified neutral compound from *P. sativa*. As for the other swallowtails for which contact stimulants have been identified, all of these experiments have utilized females with prior adult experience (Feeny, et al. 1988; Carter, et al. 1998).

In central New York, oviposition by female *P. polyxenes* is limited to the Apiaceae, but the larvae will also feed on several plants in the Rutaceae, which is believed to be the basal host family for the genus (Dethier 1941; Berenbaum 1995). The most common host plant in central New York is wild carrot (*D. carota*); other local hosts include wild parsnip (*P. sativa*) and poison hemlock (*Conium maculatum*) (Scriber and Feeny 1979). Most are introduced biennial (carrot is a winter annual) weeds of hayfields, roadsides, and other disturbed areas (Wiegand and Eames 1925). Because larvae, especially in the earliest instars, are generally unable to move from one host to another, the placement of the eggs by the ovipositing female ultimately determines the host plant and the fate of the larvae (Rausher 1979). The larval host does not have an effect, however, on the oviposition choice of the resulting female (Wiklund 1974 for *Papilio machaon*; Feeny and Rosenberry, unpublished, for *P. polyxenes*).

Three experiments were conducted to determine whether the response to contact cues is innate. In the first experiment, naïve female *P. polyxenes* were tested for their response to extracts from one host, *D. carota*, and one non-host, *Vicia faba*, fava bean. After testing, females were allowed to lay eggs on *D. carota*, and then they were tested again after the host-plant experience. In addition, both naïve and experienced females were held for five different time periods to address the effect of deprivation on motivation. In the second experiment, an effect of age that confounded some results of the first experiment was removed by controlling for age and varying host-plant experience to better address the effect of experience on the response to contact chemical extracts. Finally, the third experiment verified that *V. faba* was non-deterrent.

## GENERAL METHODS

### *Extractions*

Leaves of carrot, *Daucus carota* (Apiaceae), and poison hemlock, *Conium maculatum* (Apiaceae) were collected either from wild-growing plants near Ithaca, New York and transported to the laboratory in a cooler with crushed ice, or from greenhouse plants grown from wild-collected seed from Ithaca, New York. Leaves of bean, *Vicia faba* (Fabaceae) were collected from greenhouse plants, grown from commercial seed (Agway). Leaves were weighed, and 100g batches were blended in 500ml boiling 95% ethanol. The slurry was filtered, and the ethanol was removed by evaporation under reduced pressure. The remaining aqueous solution was centrifuged, and the supernatant extracted three times each with diethyl ether, chloroform, and ethyl acetate. This aqueous extract contained virtually all of the stimulant activity of the parent ethanolic extract (Feeny, et al. 1988; Brooks, et al. 1996). The extract was

evaporated under reduced pressure to a concentration of 10 gram leaf equivalents (gle) per ml and frozen in 0.5ml aliquots.

### *Butterflies*

Butterflies were kept in an environmental growth chamber (Environmental Growth Chambers, Chagrin Falls, OH) with 16:8 LD, 27°C daytime temperature and 15.5°C nighttime temperature, at 75±5% relative humidity. Butterflies were fed and numbered as described in Carter and Feeny (1985), except that butterflies were fed a 20%, instead of 10%, solution of honey in water daily. Females were mated by hand pairing (Carter and Feeny 1985) at least 24 hours after emergence, on the first or second day after eclosion.

## EXPERIMENT 1: EXPERIENCE AND TIME

### *Specific Methods: Butterflies*

Female *Papilio polyxenes* were the first and second generation offspring of wild females caught in central Illinois (University of Illinois Phillips Tract Research Area, Urbana) in May of 1996 and in central New York (Town of Locke) in August of 1997. The butterflies emerged in a room without plant material, and they were not exposed to plant material before experimentation.

Each female was numbered successively upon emergence and then assigned to one of five treatments: 3, 6, 24, 27, or 30 hours. The number of hours for each treatment was the time between the initiation of mating (naïve females) or removal from the host plant (experienced females) and the time of the bioassay for response to contact stimulants described below. Females were fed before the bioassay to eliminate effects of hunger on their behavior.

Each female was tested twice, once before any plant experience (“naïve”), and once after plant experience (“experienced”). After the naïve test, a female was placed in a cage with a carrot plant and allowed to lay eggs ad libitum for 40 hours, at which time she was returned to the room without plant material to await the second bioassay, this time as an experienced female.

Ninety-eight females were tested for both naïve and experienced responses. Three additional butterflies were tested when naïve, but died of unknown causes before the subsequent test.

#### *Bioassay*

The bioassay for determining activity of the plant extracts for naïve *P. polyxenes* followed Feeny et al. (1988). Serial dilutions of *D. carota* extract were applied to confined areas (6.45 cm<sup>2</sup>) of narrow strips (2.54 cm wide) of Whatman (#1) filter paper in the following concentrations: 0.001 gle, 0.01 gle, and 0.1 gle. The same concentrations were used to make control strips with the *V. faba* extract. A negative control using only distilled water was also made. Strips were hung from a wooden frame and misted with distilled water. Females were placed individually, with the wings loosely held, on each of the control strips for five seconds, and the response (accept or reject, as measured by the curling of the abdomen) was recorded. The order of presentation of strips was distilled water, 0.001 gle, 0.01 gle, and 0.1 gle *V. faba* extract. The procedure was repeated with the experimental strips. A *D. carota* plant was used as a positive control; any female that did not respond to any strip of filter paper was presented with the plant. Any female accepting distilled water was discounted.



### *Analysis*

Data were analyzed using the SAS software (version 6.12, SAS Institute, Inc). The data from the females of different geographic origins were lumped together after testing for goodness of fit with a Kolmogorov–Smirnov test (Conover 1980; KS = 0.12 for naïve females, KS = 0.09 for experienced females). First responses to extracts were used for analysis: all females responded to all higher concentrations after the initial response. A comparison of naïve and experienced responses was made using McNemar’s test for matched pairs in the FREQ procedure of SAS (Conover 1980; Stokes, et al. 1995). The effect of time, in the guise of treatment, was tested using the Kruskal–Wallace test in the NPAR1WAY procedure of SAS (Conover 1980; Stokes, et al. 1995).

### *Results*

Naïve females, without any host–plant experience, responded positively to contact with the *D. carota* extracts. Of 101 females tested, 49 gave a positive response to the *D. carota* extract while none responded to the *V. faba* extract. No females responded to distilled water at any time.

Experienced, older females were significantly more likely to respond to the *D. carota* extract than were the younger, naïve females (Table 2.1; McNemar’s test:  $T_1 = 25.0$ ,  $P = 0.001$ ). In addition, the older, experienced females first responded at lower concentrations than they did when younger and naïve (Table 2.1; Wilcoxon–Mann–Whitney test:  $\chi^2_4 = 14.016$ ,  $P = 0.007$ ). Both naïve and experienced females were more likely to respond with increasing treatment time (time since mating for naïve and time off plant for experienced; Figure 2.1; Kruskal–Wallace test: Naïve:  $\chi^2_4 = 20.922$ ,  $P = 0.0003$ ; Experienced:  $\chi^2_4 = 11.249$ ,  $P = 0.0239$ ). For both naïve and experienced

Table 2.1: First responses of females before and after host-plant experience in Experiment 1. For the McNemar's test, positive responses included any positive response to an extract-treated filter paper strip (0.001gle, 0.01gle, or 0.1gle) while negative responses included responses to the whole plant, as well as a lack of response.

Experienced Response	Naïve Response				
	0.001gle	0.01gle	0.1gle	Whole Plant	No response
0.001 gle	1	0	0	0	0
0.01 gle	0	6	4	0	1
0.1 gle	2	6	24	1	2
Whole Plant	0	2	1	0	0
No response	0	10	20	2	16

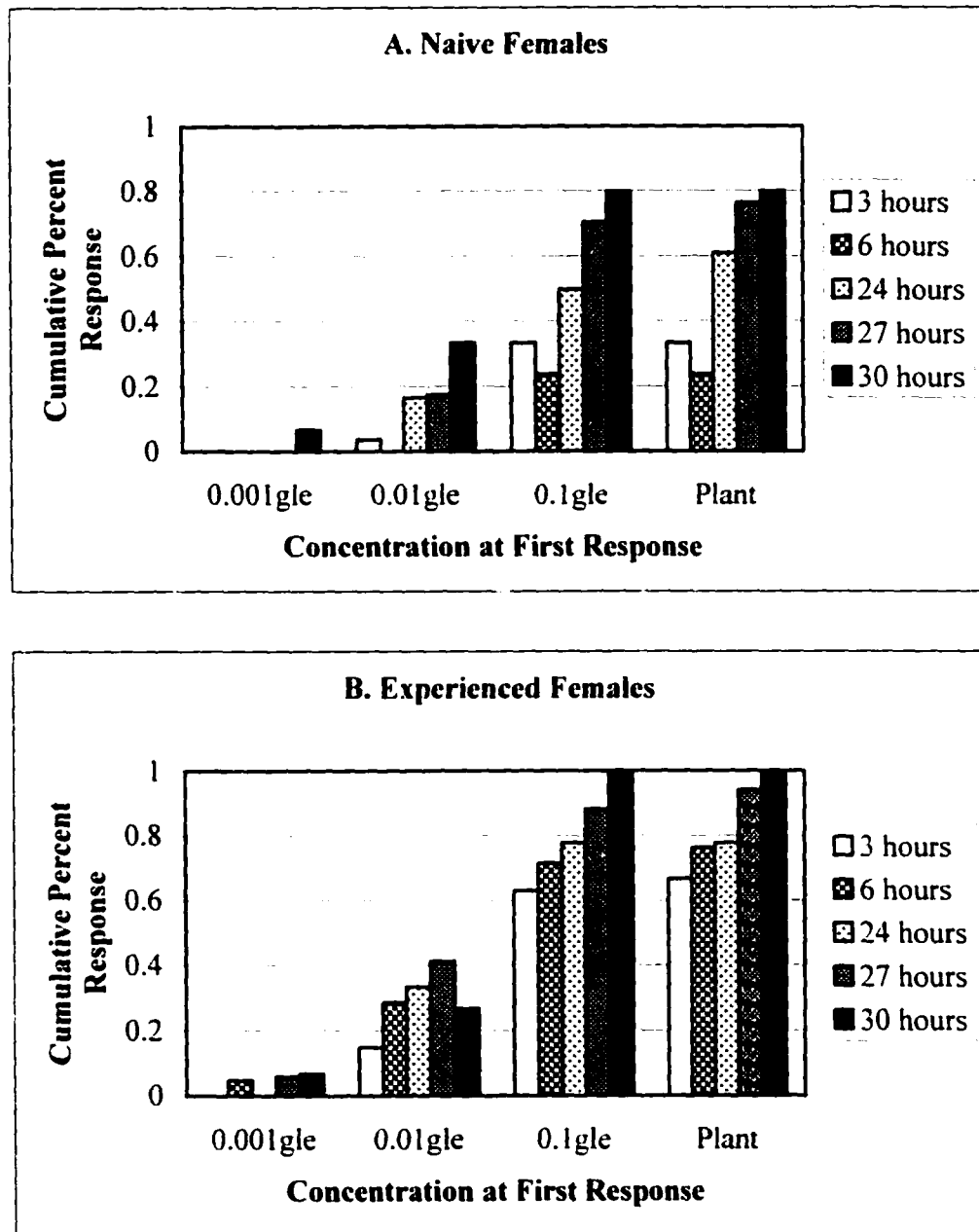


Figure 2.1 Cumulative first responses of naïve (A) and experienced (B) females to *D. carota* extracts in Experiment 1. There are significant effects of temporal treatment and experience (compounded by age — see text). N=27, 21, 20, 18, and 15 for Treatments 3, 6, 24, 27, and 30 respectively.

females, those in Treatments 24, 27, or 30 were more responsive than those in treatments 3 and 6.

## EXPERIMENT 2: EXPERIENCED TEST WITHOUT AGE EFFECT

### *Specific Methods: Butterflies*

Female *P. polyxenes* were the first and second generation offspring of wild females caught in central New York (Town of Locke) in August 1997. Females emerged from pupae in a room devoid of plant material.

The butterflies were assigned to one of four treatments with varying combinations of plant experience and extracts. In Treatments I and II, females were given experience with *D. carota*, while in Treatments III and IV, females were given experience with *C. maculatum*. Females in Treatments I and III were tested with *D. carota* extracts, while females in Treatments II and IV were tested with *C. maculatum* extracts.

After mating, each female was placed for 48 hours in a 16 X 31 X 9cm cage with a sprig of either *D. carota* or *C. maculatum* in a water-filled aquapic and allowed to lay eggs ad libitum. These two hosts were chosen on the basis of females' equal high preference for both (P. Feeny and L. Rosenberry, unpublished data). Females were removed from the plants for 24 hours and then tested using the filter paper assay with extracts of either *D. carota* or *C. maculatum*, dependent on the treatment. Treatment I had 35 females, while treatments II, III, and IV had 31 females each.

### *Bioassay*

The bioassay for activity of the extract for experienced *P. polyxenes* followed Feeny et al. (1988). The extracts were removed from the freezer and allowed to thaw to room temperature. Serial dilutions of each host-plant extract, *D. carota* and *C. maculatum*, were applied to confined areas (6.45cm<sup>2</sup>) of 2.54cm-wide strips of filter paper (Whatman #1) in the following concentrations: 0.001gle, 0.01gle, and 0.1gle. A negative control using only distilled water was also made. Strips were hung from a wooden frame and misted with distilled water. Females were placed individually on the control strips for five seconds and the response (accept or reject, as measured by the curling of the abdomen) was recorded. The order of presentation of strips was distilled water, 0.001gle, 0.01gle, and 0.1gle of the appropriate host extract, followed by a sprig of the appropriate plant. Experienced females not accepting either an experimental strip or the plant were discounted; likewise, any female accepting distilled water was discounted.

### *Analysis*

SAS software was used to analyze data (SAS Institute, Inc.). First responses to extracts were used for analysis: all females responded to all higher concentrations after the initial response. Kruskal-Wallis tests were used to determine differences between female responses to the two plant extracts and the responses of females experienced on each of the two plants.

### *Results*

Females responded to the *D. carota* and *C. maculatum* extracts differentially, with *D. carota* receiving more and greater responses than *C. maculatum* (Figure 2.2; Kruskal-Wallis test:  $\chi^2_1 = 25.441$ ,  $P = 0.0001$ ). These responses were not affected

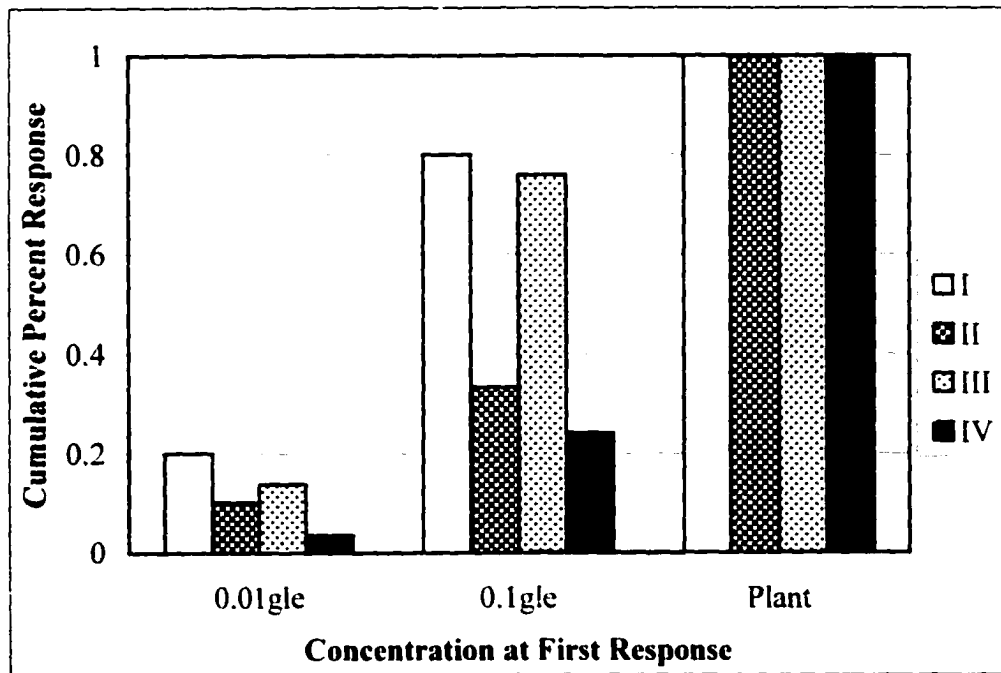


Figure 2.2 Cumulative first responses of females to plant extracts for each of the four treatments in Experiment 2. Treatments I and II received *D. carota* plants for experience, while Treatments III and IV received *C. maculatum*. Treatments I and III were tested with *D. carota* extract; II and IV, *C. maculatum*. There is a significant effect of plant extract (see text), but no effect of experience. N for each treatment, with non-responders removed, is 30, 25, 26, and 27 for Treatments I, II, III, and IV, respectively.

by experience; females responded to an extract in a similar way regardless of their previous experience (Figure 2.2; Kruskal–Wallace test:  $\chi^2_1 = 1.0611$ ,  $P = 0.3030$ ).

### EXPERIMENT 3: BEAN DETERRENCY TEST

#### *Specific Methods: Butterflies*

Female *P. polyxenes* were first generation offspring of wild females caught in eastern Canada in the fall of 1998. These butterflies had been used for another experiment testing responses to volatile host–plant cues (Chapter 3). Females were tested for their response to *D. carota* extract applied to *V. faba* plants and filter paper strips when they were 9 or 10 days old. A total of 37 females was tested for their responses.

#### *Bioassay*

Each *D. carota* extract was applied to one leaf of a small *V. faba* plant (6–12 leaves) as well as to a confined area of a filter paper strip. Three concentrations of extract were used, 0.001, 0.01, and 0.1gle, each diluted to a total volume of 20 $\mu$ l. On *V. faba* leaves, extract was applied to one leaf of a plant, in several droplets near the tip of the leaf. The extract was allowed to dry on the leaves and was not moistened with distilled water. Extracts were applied to filter paper strips as above, and were misted frequently with distilled water during testing of females. One *V. faba* leaf and one filter paper strip were treated with distilled water only, as negative controls.

Females were fed before testing and were deprived of host plants for at least 3 hours prior to the tests. The order of presentation was as follows: distilled water, 0.001gle *D. carota* extract, 0.01gle *D. carota* extract, 0.1gle *D. carota* extract. Within each concentration level, extract was presented first on the *V. faba* leaf, then on the filter paper, then again on the *V. faba* leaf. Females were allowed to lay eggs during

the bioassay; curling of the abdomen was scored as a positive response, and egg-layings were also recorded. Each female was allowed to curl for one minute; if no egg had been laid, she was scored as a positive response with no egg.

### *Analysis*

SAS Software was used to analyze the data (SAS Institute, Inc.). Mantel–Haenszel statistics (Stokes, et al. 1995) were used to determine whether there was a difference between a female's response to the *D. carota* extract on the *V. faba* leaves and the response to the extract on the filter paper.

### *Results*

Females responded positively to the *D. carota* extracts applied to *V. faba*. They were significantly more likely to curl on the second presentation of *V. faba* plants than on the filter paper of the same concentration at each dose, with the first presentation of *V. faba* being intermediate (Figure 2.3: Mantel–Haenszel statistics:  $Q_{SMH2} = 10.889$ ,  $P = 0.004$ ).

## DISCUSSION

Without any prior adult experience with host plants, female *P. polyxenes* will respond to contact chemical cues, favoring host–plant over non–host extracts. Of the females that did not respond to anything, most were from the earlier temporal treatments (3 or 6 hours after the start of mating); as mating can take from 20 minutes to several hours, this is probably too soon for a typical female to be motivated to lay an egg. Blau (1981) found that *P. polyxenes* females from central New York laid the highest percentage of their eggs on the third day after mating, with a slightly smaller slope in the decline after day 3 than in the increase from day 2 to 3, which is consistent with



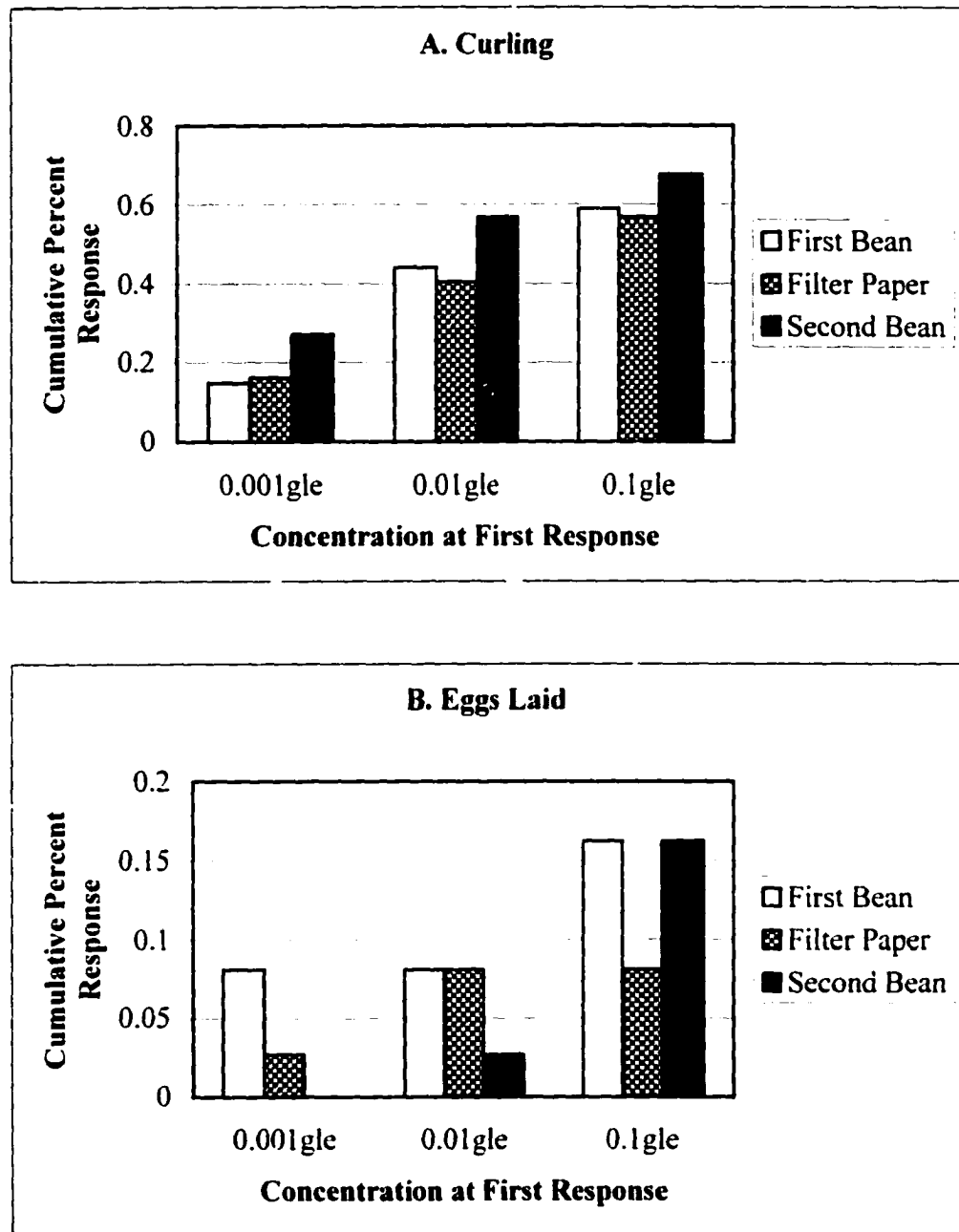


Figure 2.3 The cumulative percent curling (A) or egg-laying (B) response of females to *D. carota* extract applied to bean leaves or filter paper strips. There is a significant effect of the presentation medium on the response (see text). N=37.

these findings. No females responded to *V. faba* extract, and Experiment 3 showed that *V. faba* is apparently neutral, becoming acceptable to females when host extract is applied to the leaf surface. The *D. carota* extract was in fact more attractive to female *P. polyxenes* when it was applied to *V. faba* leaves than when presented on filter paper strips at the same concentration. This indicates that the females may have been responding to more appropriate microclimate, surface texture, color, and gestalt of the *V. faba* leaves as opposed to the filter paper strips.

That female *P. polyxenes* will respond to contact chemical cues prior to adult host-plant experience is consistent with the earlier work of Papaj (1986), who showed that naïve *Battus philenor* females could learn the leaf shape of a non-host plant treated with host-plant extract in a single trial. While he did not directly test whether naïve females would respond to the extracts, prior to any adult host-plant experience, the females were clearly mistaking the non-host plants for hosts when the non-hosts were treated with the host extracts. For *B. philenor*, the contact chemical cues act as an unconditioned stimulus for the conditioning of leaf shape (Papaj 1986; Papaj and Prokopy 1989). Because contact chemical cues are only available to a searching butterfly after landing, they would be ineffective for increasing the efficiency of the search via learning, as efficiency would be better increased via a pre-landing cue. However, they are dependable indicators of the acceptability of the plant, and thus could be reliably used as unconditioned stimuli for the learning of other cues.

In the first experiment of the present study, females were tested both while naïve and also after exposure to *D. carota* plants. After this experience, females were even more likely to respond to the *D. carota* extract and responded at lower concentrations (lower threshold). It is important to note, however, that due to the

design of this experiment, the experienced females were inevitably older than the naïve females, and the effect of age could not be factored out of the results.

Although the age of the experienced females compounds the discussion of the differences between naïve and experienced females, there remains an effect of time on the response. For both naïve and experienced females, females that were tested on the day after mating or access to plants (Treatments 24, 27, and 30) responded at a lower threshold than those that were tested on the same day. This suggests that motivation increases as a result of deprivation of host plants (Dethier 1982). It may also represent the time before a female is ready to lay eggs, when no plants are accepted: the time prior to a female entering the discrimination phase of Singer (1982).

The second experiment was designed to remove age as a confounding factor in the test. Instead of testing the same females twice, before and after host-plant experience, the host plant experience itself was varied. In this manner the responses of the females experienced with either host could be compared to those of females that had not previously experienced this host plant. Females responded differentially to the extracts of *D. carota* and *C. maculatum*, but with no effect of the host that had been previously experienced. The response to contact chemical cues is thus fixed and innate: It is shown by naïve females, and it is immutable by host-plant experience.

The results of Experiments 1 and 2 indicate that age affects the response to contact chemical cues. The difference between naïve and experienced females in Experiment 1 is compounded with age, but Experiment 2 demonstrates that there is no measurable effect of experience, leaving age to explain the differences in Experiment 1. This is again consistent with Blau's findings (1981).

Although the responses to contact chemical cues are innate and unaffected by host-plant experience, they do not by themselves reflect the whole-plant preferences of females. *D. carota* and *C. maculatum* were chosen because they are known to be equally and highly preferred, on average, an earlier finding (Feeny and Rosenberry, unpublished data) that is corroborated by the similar responses to the whole plants in Experiment 2. Yet females clearly responded to the *D. carota* extracts more strongly than to the *C. maculatum* extracts (Fig. 2). This observation could be due to an artifact of the extraction procedures (perhaps the active compounds in *C. maculatum* are degraded), but is more likely due to actual differences in the contact stimulant profile of the two plants (M. Haribal, personal communication). Clearly, females must thus be using at least one other cue to identify their host plants. A likely candidate for this is the volatile chemistry (Feeny, et al. 1989), which can also be used by a searching female at a greater distance than can the contact chemistry.

Feeny et al. (1983) outline the history of the attempts to understand host-plant relations of butterflies in terms of the underlying plant chemistry. At that time, the statement "we still know remarkably little about ... the role of phenotypic experience" (Feeny, et al. 1983 p. 70) in the oviposition behavior of butterflies was true for not only contact chemistry, but also volatile chemistry and visual cues. Although much progress has been made with regard to the role of experience in the use of volatile compounds and visual cues (Papaj and Prokopy 1989), contact chemical cues are most often used as a stimulus for conditioning either of these cues (e.g. Traynier 1984; Allard and Papaj 1996). Contact chemical cues for oviposition have been identified for many orders of insects on many families of plants, particularly Diptera, Lepidoptera, and Coleoptera feeding upon Cruciferae or Umbelliferae (Städler 1992). In these experiments, it has, by necessity, been assumed that oviposition responses to contact

chemical cues would be innate and not altered by experience in the bioassays leading to these identifications. Females were first exposed to whole plants and then tested for their responses to fractionations of leaf extracts, in part to compare the response to the extracts with that to the whole plant, but also to give females the opportunity to oviposit before bioassays (e.g. Feeny, et al. 1988; Haribal and Feeny 1998; Carter, et al. 1999), a design that makes comparisons between naïve and experienced females impossible. Such tests would increase the already large number of bioassays necessary to positively identify contact chemical stimulants, and could be seen as inefficient when insects are expensive in terms of time and effort.

In particular, most workers have focused on the identification of contact chemical stimulants for swallowtail butterflies (e.g. Feeny 1995; Honda and Hayashi 1995; Nishida 1995; Haribal and Feeny 1998; Carter, et al. 1999). Two general patterns in the chemical basis of oviposition behavior across the family have been identified by Feeny (1995) and Nishida (1995): the need for multiple compounds for a response, and the ability to group the compounds into several classes of compounds, namely flavonoids, carboxylic acids, basic compounds, neutral cyclitols, and zwitterions. The appearance of these patterns supported the assumption that these responses were innate and fixed, but before the current study it had not been tested experimentally. If these responses were not innate, but rather learned after host–plant experience, they would be unreliable as characters for evolutionary studies. However, that these responses are not only innate but also fixed and unalterable by experience may make them ideal for studying the evolution of host use and recognition in this family (Miller and Wenzel 1995).

Although this approach may be considered time-consuming, it is useful to ask what the initial, naïve response of an organism is before asking whether this response can be modified by experience, such as with learning. Although this approach may be more common in other systems, including the study of feeding or oviposition preference (e.g. Karowe 1989; Thompson 1998), it is uncommon in studies of the chemistry of insect oviposition behavior.

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## CHAPTER THREE

### RESPONSES TO VOLATILE CHEMISTRY

Volatile chemical cues are known to be important in insect–plant interactions (Visser 1986). In adult insects, they are generally detected at some distance from the source by sensilla in the antennae. Volatiles can also be used at closer range in combination with contact chemical cues to verify the identity of a potential host plant. The use of volatiles has been studied across the Lepidoptera, as reviewed recently by Renwick and Chew (1994), Honda (1995), and Ramaswamy (1988). Some species do not show a response to volatile cues (Ramaswamy, et al. 1987; Hora and Roessingh 1999), or show a deterrent response, even with host plants (e.g. Copp and Davenport 1978). For others, the response is positive and sometimes learned with host–plant experience, as in the case of *Trichoplusia ni* (Landolt and Molina 1996).

Within the Papilionidae, volatiles were first noted to play a role by Vaidya (1969). While studying the role of visual stimuli in *Papilio demoleus*, she found that females gave an oviposition response to certain colored papers in the presence of host plant volatiles. Some butterflies were so stimulated as to lay eggs on the papers, with no other relevant chemical stimulus, and prior to any host–plant experience. In a more thorough investigation, Saxena and Goyal (1978) found that *P. demoleus* females oriented towards a volatile–containing ether extract of the host *Citrus limettoides*, laying eggs if contact with the moistened extract was allowed. More recently, another swallowtail, *Eurytides marcellus*, was found to land more often in the presence of volatiles, leading to increased oviposition on contact chemical extracts, in addition to some oviposition without contact chemical cues (Haribal and Feeny 1998).

The first experiments on responses to volatile chemical cues by *Papilio polyxenes* showed an increase in the general activity level of females and an increase in the number of eggs laid on contact chemical extracts in the presence of host (*Daucus carota*, carrot) volatiles; volatiles from a non-host (cabbage) had an inhibitory effect on both activity levels and landing rates (Feeny, et al. 1989). Electroantennagrams (EAGs) were also examined and greater magnitude and number of responses were seen to the host than to non-host volatiles (Feeny, et al. 1989). Later, five active compounds were identified by EAG coupled with a gas chromatograph (GC) in volatiles from *D. carota* foliage (Baur, et al. 1993): (*E*)-sabinene hydrate, (*Z*)-sabinene hydrate, 4-terpineol, bornyl acetate, and (*Z*)-hexenyl acetate. Most recently, Donzé and Feeny (2001) have found evidence for learning in the behavior towards volatiles by *P. polyxenes*: Females given oviposition experience with both a host (*D. carota*) and a non-host (yarrow, *Achillea millefolium*) land less often and lay fewer eggs on model leaves treated with *D. carota* contact stimulants in the presence of yarrow volatiles than in the presence of *D. carota* volatiles. Females experienced only with *D. carota*, however, will lay eggs equally in the presence of *D. carota* or yarrow volatiles.

*P. polyxenes* is an oligophagous butterfly, with eggs laid and larvae feeding upon members of the carrot family, Apiaceae. Contact cues are thought to be required for oviposition (Feeny 1991; Nishida 1995), and have previously been shown to be innate and unaltered by previous host experience (Heinz 2001). In central New York, the most common host plant is *Daucus carota*, wild carrot; other local hosts include *Pastinaca sativa*, wild parsnip, and *Conium maculatum*, poison hemlock (Scriber and Feeny 1979). Because larvae, in general, are unable to move from one plant to another, especially in the earliest instars, the placement of eggs by the ovipositing

female ultimately determines the host plant and fate of the larvae (Rausher 1979). The larval host does not, however, have an effect on the ovipositing choice of the resulting female (Wiklund 1974 for *P. machaon*; P. Feeny and L. Rosenberry, unpublished, for *P. polyxenes*).

Despite the prior work with *P. polyxenes* and host-plant volatiles, it was not known whether the response to these cues would be innate or learned with experience. *P. polyxenes* females do not behave well in a flight tunnel, and these butterflies do not regularly fly in even moderate winds in the field (C. A. Heinz, pers. obs.). Therefore, the present experiment was designed to test the responses of *P. polyxenes* to volatile chemical extracts before and after host-plant experience using a free-flight bioassay in the absence of other relevant host-plant cues .

## MATERIALS AND METHODS

### *Butterflies*

Butterflies were first and second generation offspring of wild-caught females from New Jersey and southeastern Canada in the fall of 1998. First generation offspring were from diapausing pupae, and were kept in a refrigerator at 3°C until needed, when they were removed to an environmental growth chamber (Environmental Growth Chambers, Chagrin Falls, OH) with 16:8 LD, 27°C daytime temperature and 15.5°C nighttime temperature, at 75±5% relative humidity. The growth chamber was devoid of host-plant material at all times. Butterflies were fed and numbered as described in Carter and Feeny (1985), with two exceptions: butterflies were fed a 20% (increased from 10%) solution of honey in water and several colors of Sharpie<sup>®</sup> permanent markers (Sanford, Inc.) were used for numbering to aid in individually identifying

females in flight. Females were mated by hand pairing (Carter and Feeny 1985) more than 24 hours after emergence, on the second day after eclosion.

*Extractions: General*

The two apiaceous host plants, *Daucus carota* (wild carrot) and *Conium maculatum* (poison hemlock), were grown from seed collected near Ithaca, New York, in a greenhouse module atop Seeley Mudd Hall, Cornell University. The non-host, *Vicia faba* (Fabaceae, fava bean), was grown from commercial seed (Agway, Inc.) in the same greenhouse module.

Volatiles were collected by soaking leaves in hexane rather than by headspace collections, due to the volume of extract required. A pilot experiment determined the wild carrot, *Daucus carota*, extract with the greatest response rate to be from a five-minute soak in hexane. This elicited greater responses than one- or ten-minute soaks in hexane, one-, five-, or ten-minute soaks in methylene chloride or pentane, or a hexane extraction of leaves blended in near-boiling ethanol, though differences between most treatments were not significant (C. A. Heinz, unpublished data).

*Extractions: Surface area regressions*

A relationship between leaf surface area and weight for *D. carota* has been previously reported by Brooks et al. (1996); however, this relationship had not previously been demonstrated for *C. maculatum* or *V. faba*. Fifty leaves each of *D. carota*, *C. maculatum*, and *V. faba* were individually weighed, and measurements of length, width, and surface area were made. Petioles were not included in any measurements. Surface area was measured using a Li-cor Model 3100 area meter (Li-cor, Inc., Lincoln, NE) set at low resolution (1mm) to allow for measurement of large leaves.

Surface area of each leaf was measured three times and the largest measurement of the three was used for further calculations. To find a reliable proxy for surface area, weight, length, and width were regressed onto twice the surface area (top and bottom surfaces), as volatiles would be better correlated with the total surface area of a leaf surface than other measures, but surface area would be inefficient to measure for every leaf.

#### *Extractions: Volatiles collection*

Leaves were weighed with the petioles removed and 20–30g bunches were placed into a 2000ml Erlenmeyer flask. Hexane was added to the flask to cover all of the leaves (about 1000ml). After 5 minutes, the hexane was decanted into a clean flask and frozen at -10°C. After all of the samples of a species were extracted, the combined extract was reduced by rotoevaporation to the desired concentration (see Results: Surface area regressions). The extract was stored in a -10°C freezer in 1ml aliquots in 2ml vials until needed.

#### *Bioassay*

Bioassays took place in a 3.66 X 3.66 X 1.83m cage placed in a large greenhouse module atop Seeley Mudd Hall at Cornell University. This greenhouse module, and all adjacent modules, contained no apiaceous plants at any time during the experiment. The cage had nylon “no-see-um” mesh walls and the floor was covered in black landscape fabric (Agway, Inc.). Forty-nine model plants were arranged in a seven-by-seven array on the floor, and one nectar plant (*Pentas lanceolata* (Rubiaceae) or *Lantana camera* (Verbenaceae)) was placed in each of the four corners.

Each model plant consisted of a 23cm length of 1.5cm–diameter green wooden dowel attached to a black wooden base (15 X 13.5 X 3.75cm). A 3 X 3 X 4.5cm green block with a 1.5cm diameter and 3cm–deep hole was attached to the dowel; a vial of volatile extract fits into this hole. Model leaves were cut from green card stock (Hammermill, Bright Hue® Cover) and four were attached to the block at right angles to each other on each of the 49 model plants. The leaf shape (Figure 3.1) was adapted from a shape found to be attractive to the carrot root fly, *Psila rosae* (Degen and Städler 1997). Model leaves were scored on the bottom side to allow for more realistic three–dimensionality. Model leaves were also given additional support by stapling a 10.2cm long X 1.6cm wide strip of the same paper, folded in half lengthwise, to the back side of the “stem” of the model leaf.



Figure 3.1: The leaf shape used in this experiment. The leaves were 12.2cm wide and 17.2cm tall.

Extracts were arranged in the array as in Figure 3.2; 41 of the plants were “empty” and held only empty vials while 8 held extracts. There are four possible arrangements of the extracts; the arrangement was rotated between trials. At the



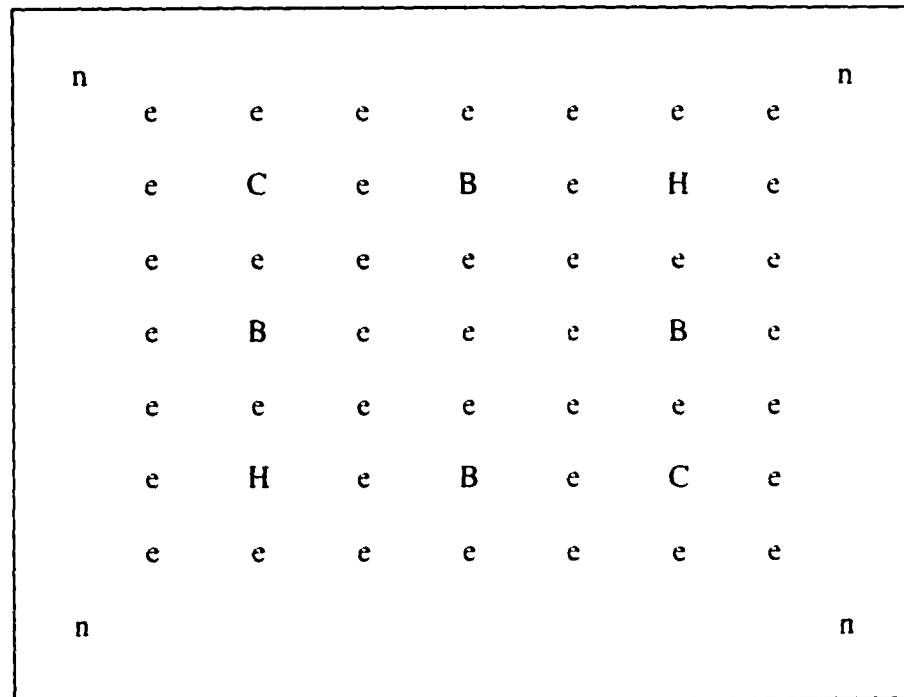


Figure 3.2: The arrangement of model plants, nectar plants, and extracts in the array. *C*'s are model plants with *Daucus carota* extract, *H*'s are model plants with *Conium maculatum* extract, *B*'s are model plants with *Vicia faba* extract, *e*'s are model plants with no extract (empty vial), and *n*'s are nectar plants (*Pentas lanceolata* or *Lantana camera*). Four arrangements of the extracts are possible by rotating the extracts. Each side of the cage is 3.66m.

beginning of a trial, two *D. carota*, two *C. maculatum*, and four *V. faba* vials were removed from the freezer and 1 ml of mineral oil was added to each to slow evaporation. The vials were then transported to the array and placed in the appropriate plants. Vials were uncapped immediately before butterflies were released into the array.

Butterflies were transported to the array from the growth chamber in butterfly envelopes (BioQuip) and released onto nectar plants. At least three but no more than 13 butterflies were tested at any one time. Activity was sporadic, such that it was possible to watch as many butterflies at one time. A total of 53 trials was used to test 84 butterflies, with a mean of 6.34 butterflies per trial. Trials lasted for 90 minutes and there was no interference during a trial. All behaviors towards the model plants, as well as transitional behaviors and the identities of the behaving butterflies, were recorded using a tape recorder and transcribed no later than two days after the trial. For analysis, behaviors were classified into ten discrete categories, as shown in Table 3.1. Trials were started between 11:00 and 16:40. The greenhouse temperature was set at 25.5°C, and lights (Sylvania Lumalux LU400, sodium high intensity discharge) were used to supplement daylight on cloudy days.

Each female butterfly was subjected to four trials, two “naïve” and two “experienced”, as seen in Figure 3.3. For some analyses, the first two trials were combined into one “naïve” category. After the second trial, each female was placed into an individual 16 X 31 X 9cm cage with a sprig of either *D. carota* or *C. maculatum* in a water-filled aquapic; an equal number of females received each plant species. These females were kept in a distant greenhouse chamber and were fed at least once per day. Females were allowed to lay eggs *ad libitum* until the morning of

the fourth day after mating, at which point they were returned to the growth chamber devoid of host-plant material. At least three hours after removal from the plants, females were given their third test, the first “experienced” test. The fourth and final test occurred on the fifth day after mating. Again, the two experienced trials were combined into a single “experienced” category for some analyses. Females were then returned to the individual cage with a sprig of each of the two host plants attached to opposite sides of the cage with green time tape. Caged females were returned to the distant greenhouse chamber and allowed to lay eggs *ad libitum* for 48 hours. All eggs were counted for each female.

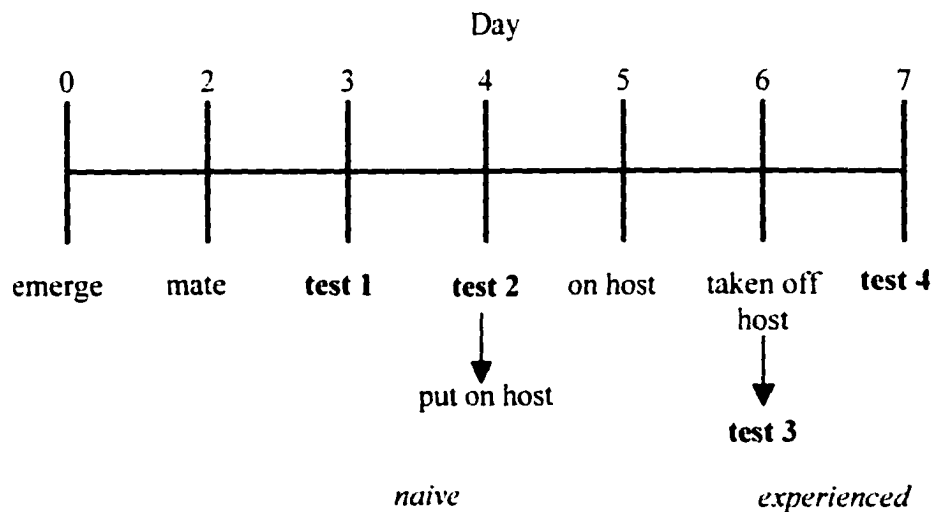


Figure 3.3: Timeline for an individual female.

### *Analysis*

Regressions of leaf surface area were analyzed with JMP statistical exploration software (SAS Institute, Inc). Data with butterflies were analyzed using SAS software, version 6.12 (SAS Institute, Inc.). Non-parametric statistics were used for behavioral analyses due to the non-normality of the data (Conover 1980). Approaches and

landings were primarily used for analysis due to the large number of both types of behavior (Table 3.1), as well as the accuracy for identifying the model plant at which the behavior was directed. Only females displaying a tested behavior at least once were included in the analyses.

Responses of naïve females to host and non-host volatile extracts and the effect of host-plant extract on the responses of experienced females were both analyzed by the sign test. The null hypothesis was that there was no effect of plant extract or plant experience on behavior. For naïve females, approaches and landings on model plants containing volatiles from the non-host, *V. faba*, and on model plants containing volatiles of either of the hosts, *D. carota* or *C. maculatum*, were each summed for each female. For each approaches and landings, the sum for the non-hosts was subtracted from the sum for the hosts and the sign of the result was scored. The same procedure was used for experienced females, except that comparisons were made between the two host-plant extracts, rather than between host and non-host volatiles.

The behaviors of experienced females toward host-plant volatiles with respect to host-plant experience and host-plant extracts were analyzed using Fisher's exact test. Approaches and landings were each tested in a 2 x 2 table of experience type versus the type of model plant eliciting the behaviors. To test the effect of host-plant experience on experienced females, a median test was used, again with a null hypothesis of no effect. The number of approaches and landings on model plants containing host-plant volatiles was compared between females who had experienced each of the two host-plant species.

Table 3.1 Categories of behaviors observed during trials with the overall frequency of each.

<b>Category of Behavior</b>	<b>Examples of Behavior</b>	<b>Frequency</b>
Non-plant directed behaviors	Fly high (>1m over model plants) around cage, Bother other females on side of cage; often transitional between array activity and inactivity	1551
Nectaring behaviors	Nectar, Land on nectar plant	1435
Fly mid over plants	Fly mid-level (<1m, >10cm) over model plants, may be directed to particular model plants	32
Fly low over plants	Fly low (<10cm) over model plants, may be directed to particular model plants	658
Approach	Approach a model plant	1785
Inspect	Flutter low (<5cm) over a model plant	71
Land	Land on a model plant	1145
Drum	Drumming behavior on a part of a model plant	17
Curl	Abdomen curl while on a model plant	49
Oviposit	Lay an egg on a model plant	3

Fisher's exact test was used to look for a relationship between the first host-plant extract approached or landed on during a trial and the overall preference during the trial for each female for each of the four trials. Chi-square tests were used to examine the data taking the sequence of behaviors into account. 2 x 2 tables were used to examine the relationship between approaches and landings for hosts and non-hosts for naïve females, and for the two different hosts for experienced females. The Mantel-Haenszel test was used to simultaneously examine the effects of experience, behavior following an approach or landing, and host-plant cues (Stokes, et al. 1995).

## RESULTS

Female *Papilio polyxenes* behaved in this bioassay in a similar manner to those in a bioassay using whole host plants rather than model plants (Chapter 6). Females flew over the array, approached and inspected the model plants. They also landed frequently, drummed as they would on a natural leaf (e.g., Ilse 1955), curled their abdomens in preparation to lay an egg, and, on three occasions, laid eggs on the model plants (Table 3.1). Naïve females were more active than experienced females, but the current data do not allow for discrimination between effects of age and experience when comparing females before and after host-plant experience.

### *Surface area regressions*

Regression parameters for each of the three plant species are shown in Table 3.2. For *D. carota* and *C. maculatum*, weight was the best predictor of surface area, whereas width was the best predictor for *V. faba*. However, as weight was also highly correlated with surface area for *V. faba*, weight was used for all three species. For the pilot study, a concentration of 3 gram leaf equivalents per ml (1gle = extract from 1

Table 3.2 Leaf surface area regression parameters for each species.

Species	Measure	Intercept	Slope	$r^2$
<i>D. carota</i>	Length	-47.503	8.25401	0.828302
	Width	-8.3019	9.2284	0.771244
	Weight	7.61246	69.6376	0.922384
<i>C. maculatum</i>	Length	-81.788	14.0726	0.80295
	Width	-70.372	13.5182	0.771912
	Weight	10.218	79.7505	0.968334
<i>V. faba</i>	Length	-45.600	12.2275	0.918486
	Width	-45.219	18.4481	0.93308
	Weight	2.71806	117.129	0.901326

gram of foliage) of *D. carota* was sufficiently stimulating; this corresponds to a total surface area of nearly 215cm<sup>2</sup>, based on these regressions. Thus, all three extracts were concentrated to 215cm<sup>2</sup> worth of leaf foliage per ml of extract; the corresponding weights are *D. carota*, 2.99g; *C. maculatum*, 2.57g; and *V. faba*, 1.81g.

#### *Responses of naïve females*

Naïve females responded preferentially to host–plant cues. Females approached and landed significantly more often on the model plants with host (*D. carota* or *C. maculatum*) extracts than on model plants with the non–host *V. faba* extract (Figure 3.4; approaches: sign test: N=49, T=14.5, p<0.0001; landings: sign test: N=44, T=15.5, p<0.0001). Naïve females were sufficiently stimulated on at least three occasions to lay eggs on a model plant (each time, on a model plant with *C. maculatum* extract), with no contact chemical cues available. Approaches by naïve females to model plants treated with host–plant volatiles were significantly more likely to be followed by landing than were approaches to model plants treated with non–host volatiles (Figure 3.5; Chi–square test: N=348,  $\chi^2_1=6762$ , p=0.009).

#### *Responses of experienced females*

Host–plant experience did not increase the number of approaches or landings on model plants with the extract of the host–plant experienced (Figure 3.6; Fisher’s exact test: approaches: N=40,  $\chi^2_1=0.556$ , p=0.482; landings: N=30,  $\chi^2_1=3.3333$ , p=0.169). However, females approached and landed more frequently on model plants with *C. maculatum* extract than on those with *D. carota* extract (Figure 3.7; approaches: sign test: N=19, T=6, p=0.0075; landings: sign test: N=15, T=5, p=0.0129). There was a slightly significant effect of the host plant experienced on approaches (Figure 3.8; Median test: N=19,  $\chi^2_1=4.0011$ , p=0.0455), with females experienced on *C.*



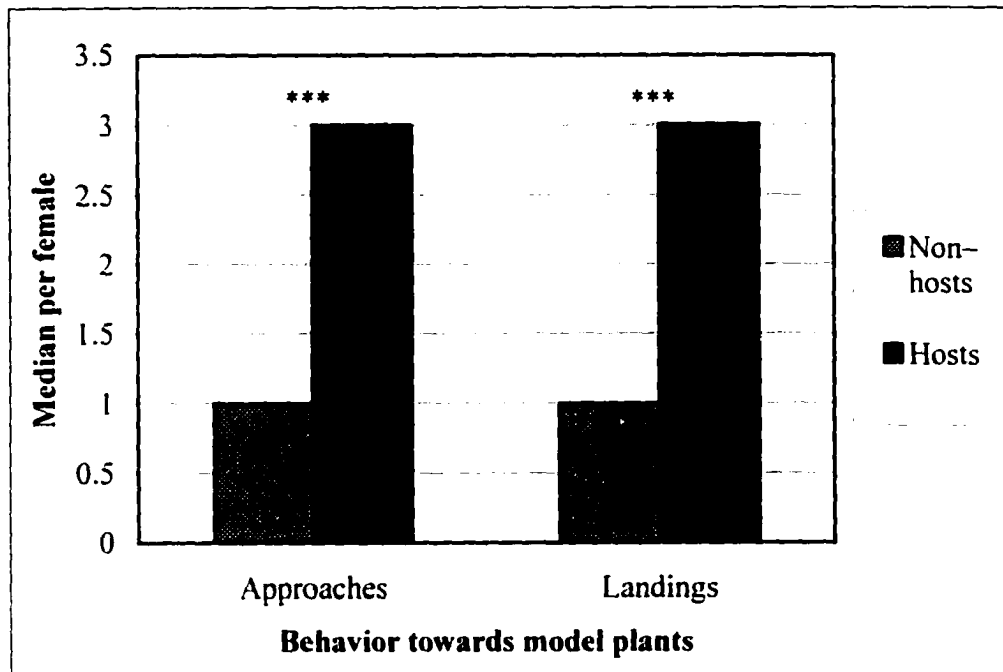


Figure 3.4: Median number of approaches and landings per female in naïve trials (trials 1 and 2). Data for the two host extracts (*D. carota* + *C. maculatum*) are combined; the non-host is *V. faba*. (\*\*\*) significant difference at  $p < 0.01$ .)

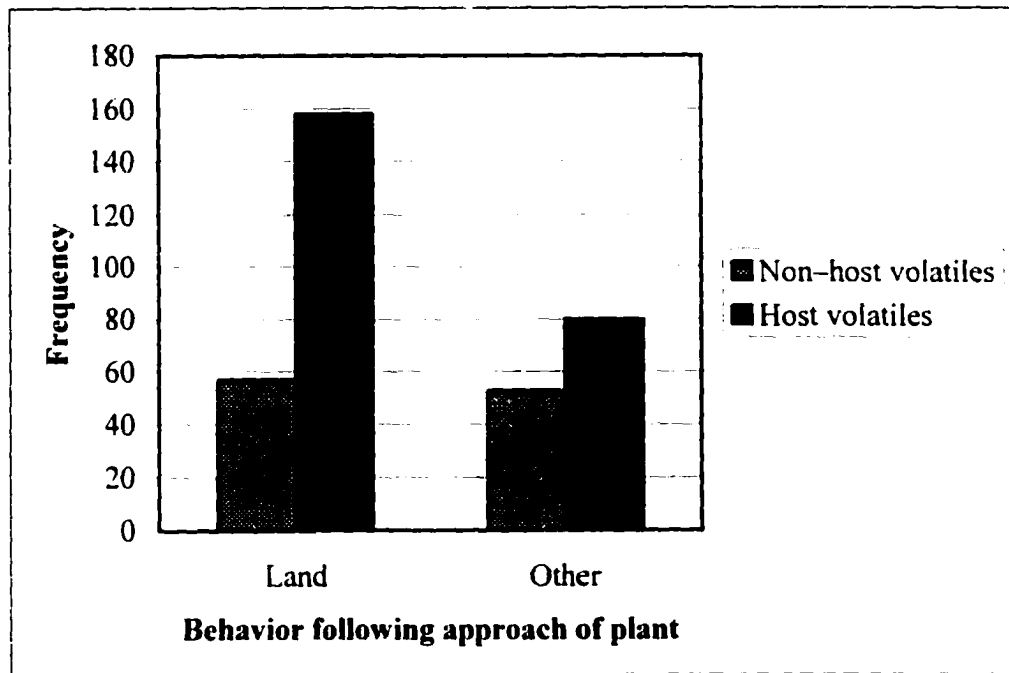


Figure 3.5: Frequency of landings or other behaviors immediately following approaches to model plants with non-host or host-plant volatiles by naïve females.

Other behaviors include all pre-landing behaviors seen in Table 3.1.

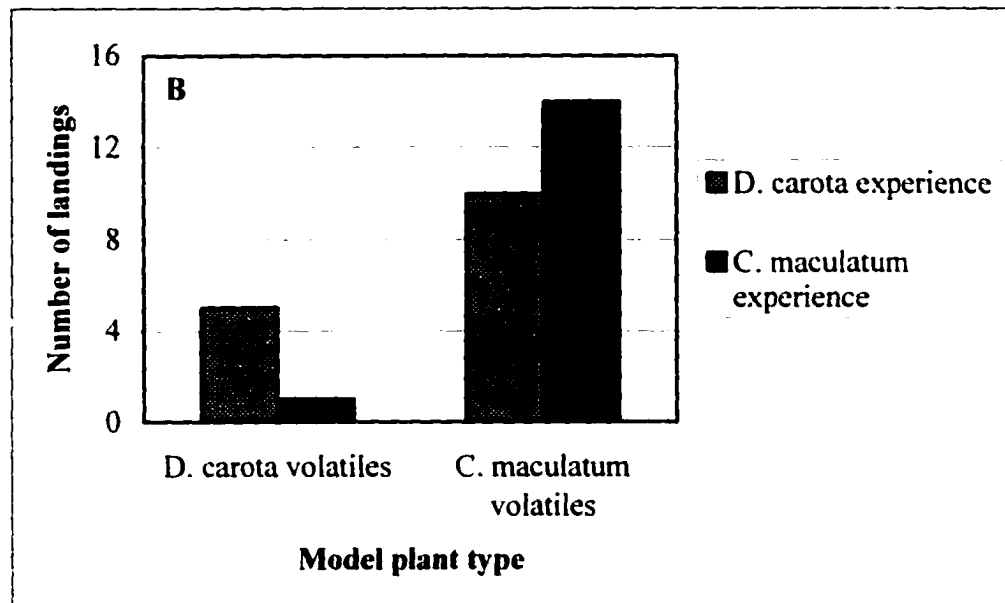
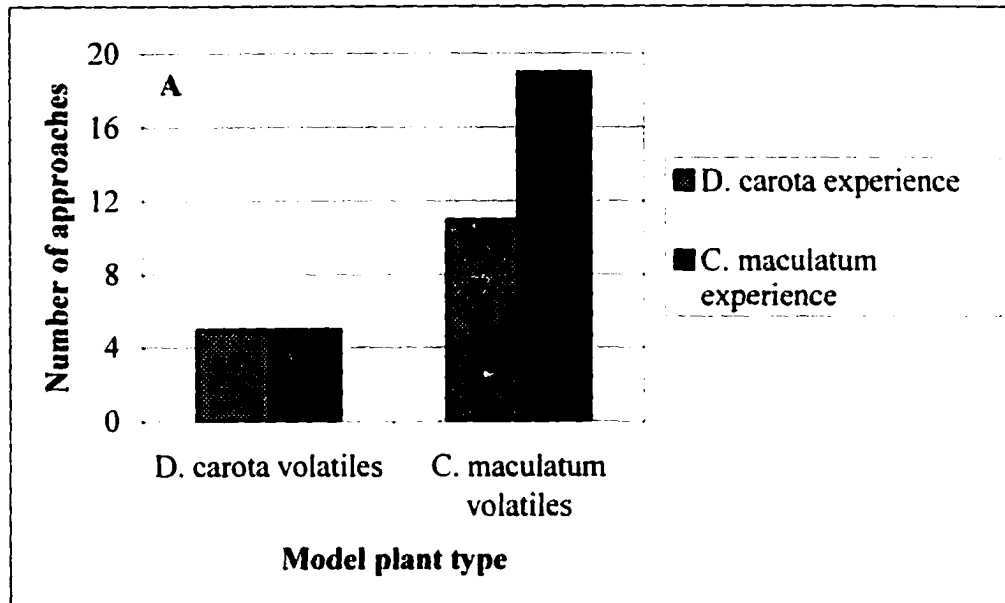


Figure 3.6: Number of approaches (A) and landings (B) by females in experienced trials on model plants with host-plant volatiles with respect to host-plant experience and host-plant volatiles.

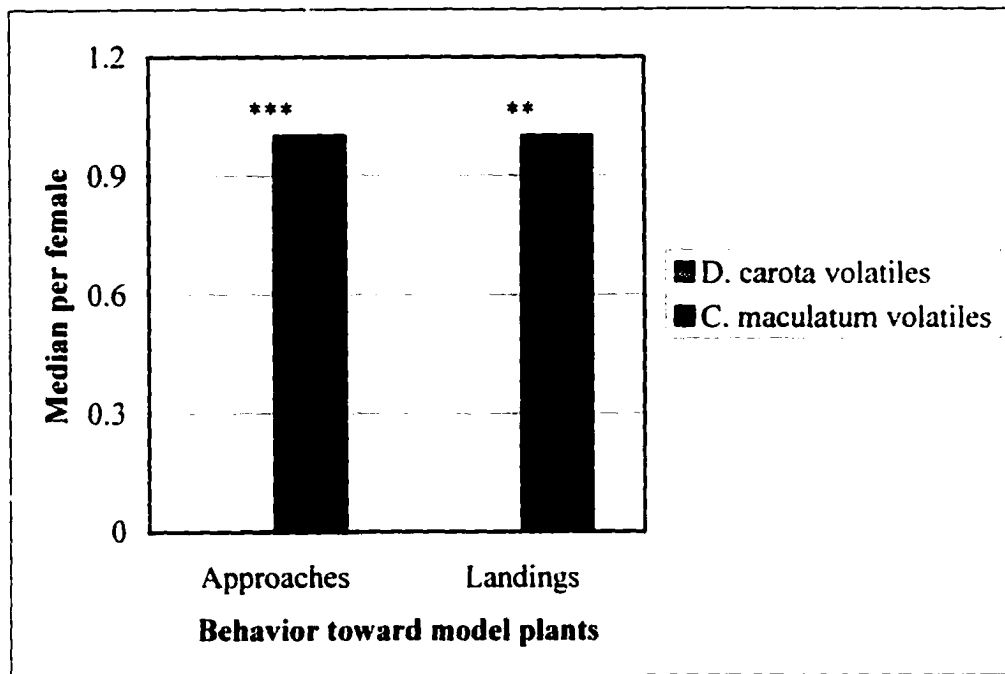


Figure 3.7: Median number of approaches and landings by experienced females on model plants with respect to the two host-plant volatile extracts. (\*\*\*)  $p < 0.01$ ; \*\*  $p = 0.0129$ .)

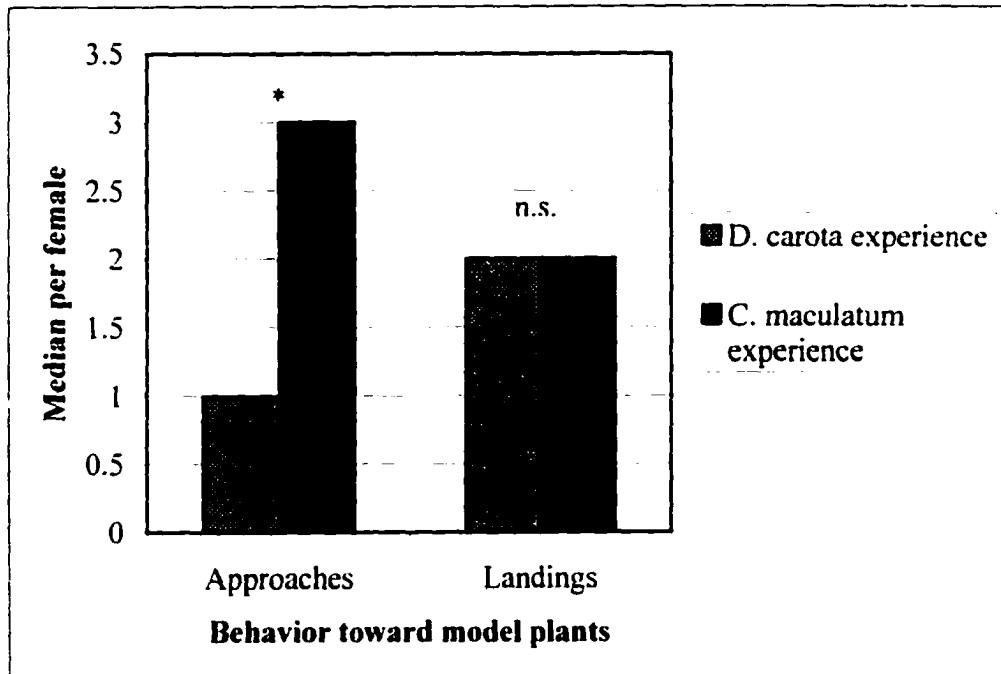


Figure 3.8: Median number of approaches and landings by experienced females on model plants with host-plant volatile extracts with respect to the host plant experienced. (\*  $p < 0.05$ , n.s. not significant.)

*maculatum* more likely to approach model plants with host–plant extracts than females experienced on *D. carota*. This effect was not seen for landings (Figure 3.8; Median test:  $N=15$ ,  $\chi^2_1=0.42105$ ,  $p=0.5164$ ).

#### *Within–trial experience effects*

To look at the effect of within–trial experience, Fisher’s exact tests were used (Conover 1980). There was a significant relationship between the first model plant with host volatiles approached or landed upon and the model plant most frequently approached or landed upon during a trial (Figure 3.9A; Fisher’s exact test: approaches:  $N=81$ ,  $\chi^2_1=30.519$ ,  $p<0.001$ ; landings:  $N=66$ ,  $\chi^2_1=27.477$ ,  $p<0.001$ ). Females overwhelmingly approached and landed on model plants with *C. maculatum* volatiles, making a distinction between preference and learning unclear.

There was a significant effect of female experience and the behavior following an approach, while controlling for the volatiles of the model plant (Figure 3.10; Mantel–Haenszel test:  $N=40$ ,  $MH_1=4.995$ ,  $p=0.025$ ). *D. carota*–experienced females were more likely to land following an approach to a model plant with *D. carota* volatiles, while *C. maculatum*–experienced females were less likely to land following an approach to a model plant with *D. carota* volatiles.

#### *Within–trial preferences and whole–plant preferences*

To look further at the apparent preference for *C. maculatum*, a preference index for *C. maculatum* was derived for landings in the trial (Figure 3.11), and for eggs laid in the choice test after the fourth trial (Figure 3.12). Though females clearly prefer *C. maculatum* model plants in the trials (as seen above), the distribution of eggs laid on

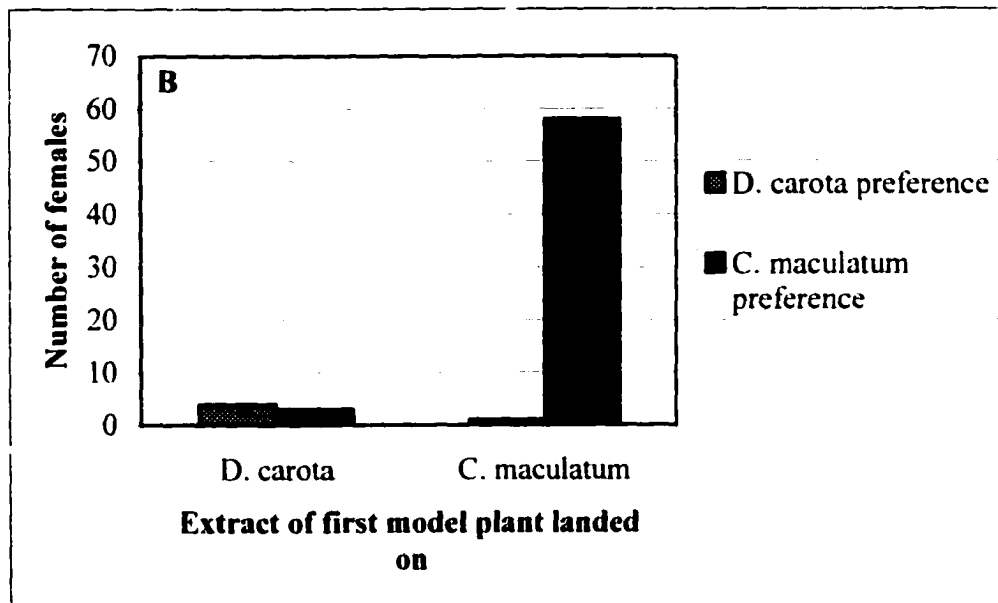
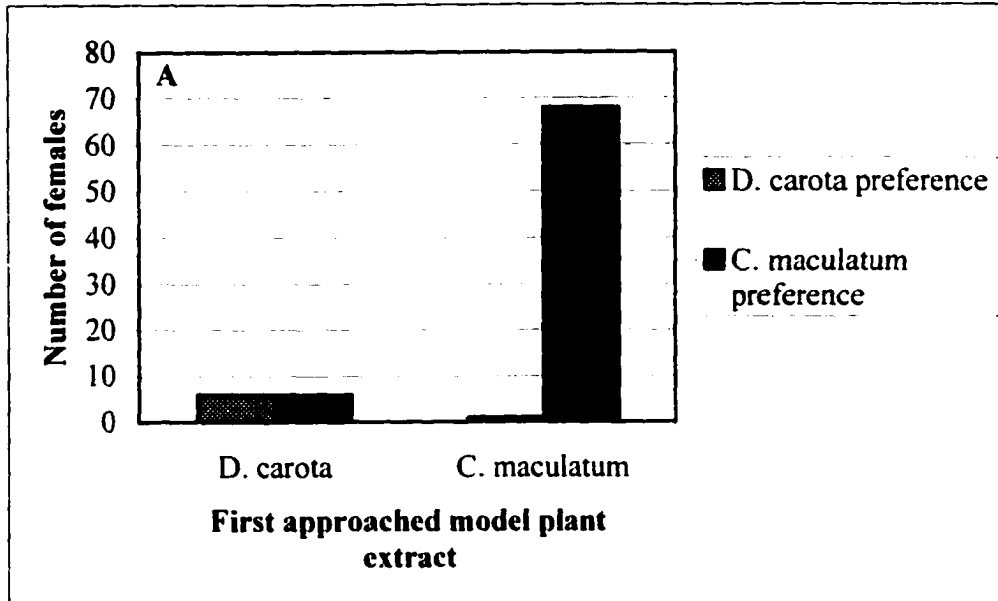


Figure 3.9: Number of females approaching (A) and landing (B) on model plants bearing each of the two host-plant extracts (*D. carota* and *C. maculatum*) with respect to the type of model plant most frequently landed upon within a single trial.

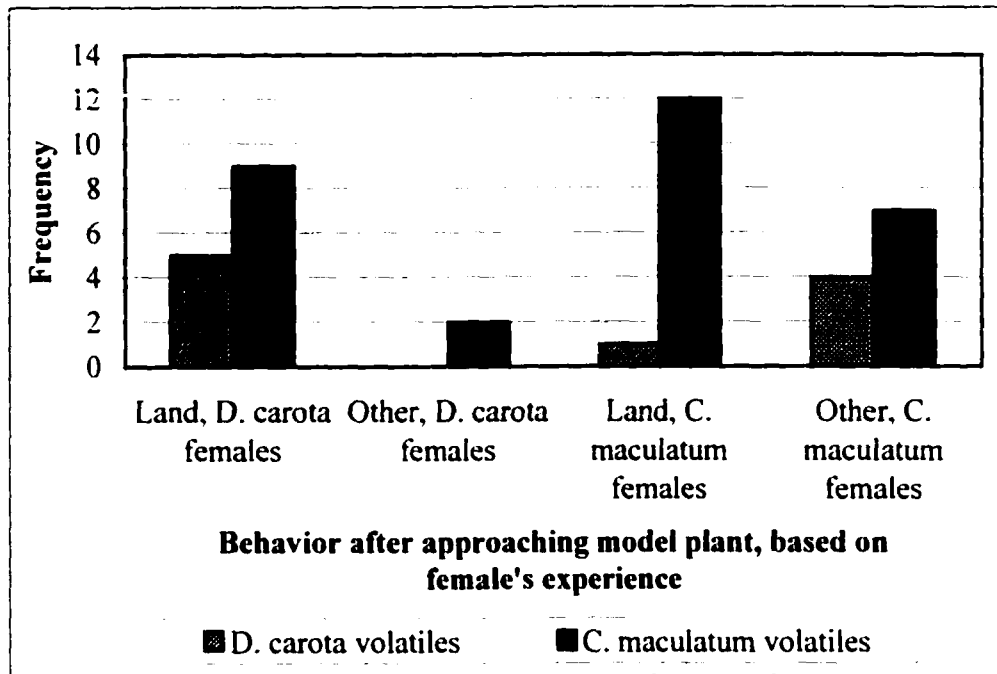


Figure 3.10: Frequency of landings or other behaviors immediately following approaches to either of the two host plants, *D. carota* or *C. maculatum*, by experienced females, with respect to the experience of the female.



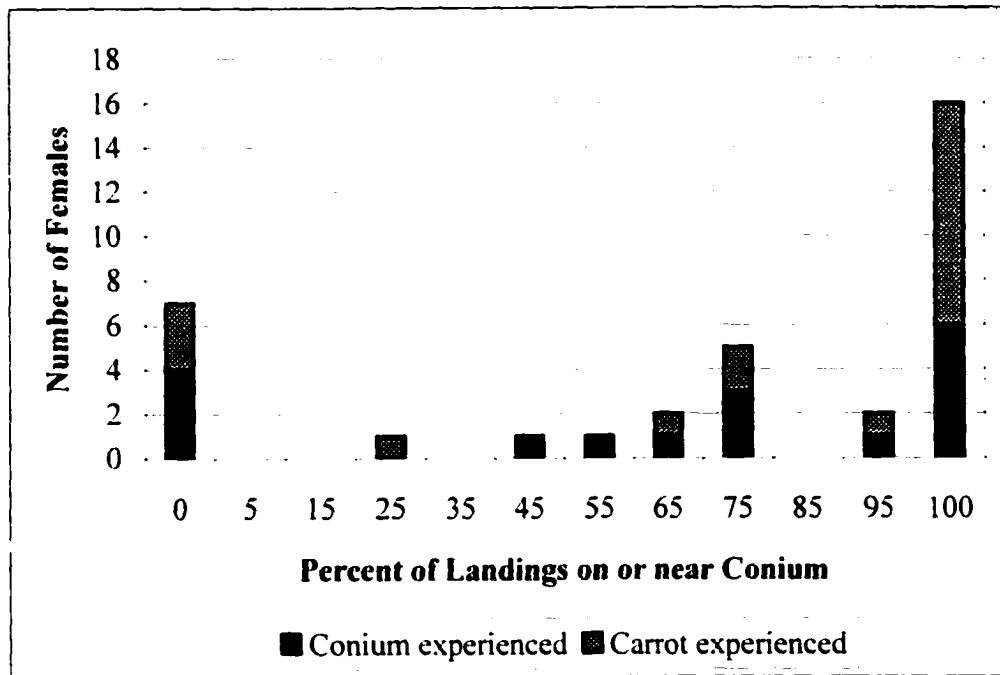


Figure 3.11: Distribution of landings by experienced females on *C. maculatum* model plants. “Near” model plants are empty plants immediately surrounding an extract-containing plant. Females land more frequently on *C. maculatum* model plants, regardless of plant experience.

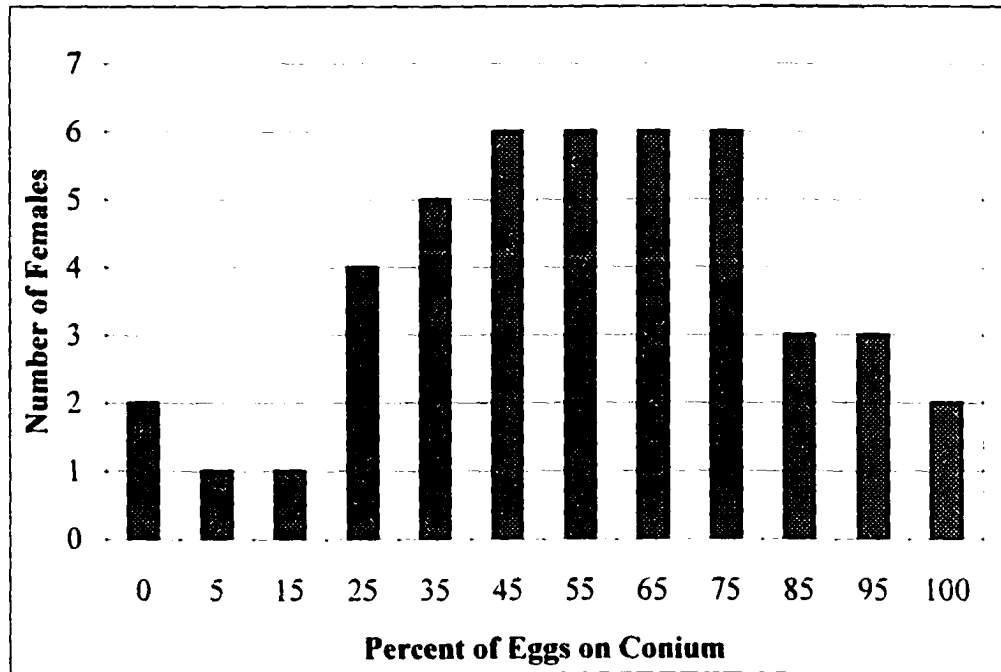


Figure 3.12: Distribution of eggs laid on *C. maculatum* by females after all trials. The distribution is not significantly different from a normal distribution (Shapiro–Wilk test:  $W = 0.97148$ ,  $p = 0.4656$ ).

plant sprigs by individual females is normal (Shapiro–Wilk test:  $W = 0.97148$ ,  $p = 0.4656$ ).

## DISCUSSION

Female *Papilio polyxenes* exhibit clear attraction and stimulatory responses to volatile host–plant cues, even without prior adult host–plant experience. Although the early work of Vaidya demonstrated a naïve response in *P. demoleus* (Vaidya 1969), more recent studies have expressed doubt, both experimentally and by way of discussion, that such responses were innate in swallowtail butterflies (Åhman, unpublished manuscript; Feeny, et al. 1983; Haribal and Feeny 1998; Donzé and Feeny 2001). Although previous experiments have found volatile chemical cues to be insufficient for stimulating oviposition behavior in *P. polyxenes* (Feeny, et al. 1989; Baur, et al. 1993), there is clearly much variation in the responses to these cues within the species. For several naïve females, volatile chemicals alone stimulated females to drum on the paper leaves, curl their abdomens, and for at least a few, lay an egg, all with no host contact chemicals available. Variation of this sort has also been seen in the graphiine papilionid *Eurytides marcellus* (M. Haribal, pers. comm.), where some females will lay eggs in response to only a volatile chemical stimulus, even without prior adult host–plant experience. It has been stated that papilionids require contact chemical cues to lay eggs (Feeny 1991; Nishida 1995); clearly, this is not the case for all individuals.

The responses of naïve females are greater for host–plant cues, as females approached and landed significantly more often on model plants with host–plant extracts than on model plants treated with the extract of a non–deterrent non–host, *V. faba* (Figure 3.4). Once approached, females were also significantly more likely to land on a plant if it contained host–plant volatiles (Figure 3.5). Although females will

respond to green-leaf volatiles (Baur, et al. 1993), there are clearly host-specific compounds that are attractive to female *P. polyxenes*. Although the role, in a more natural context, of the volatile compounds previously identified via GC-EAG by Baur et al. (1993) is still unknown, this finding of host-specificity and a naïve response suggests that the identification of these volatile compounds may be as important as for contact chemical cues in the explanation of host specificity in swallowtail butterflies (Nishida 1995). When examining the patterns of contact chemicals used as oviposition stimulants by swallowtail butterflies for the purpose of explaining evolutionary host shifts within the family (Feeny 1995; Nishida 1995), it may be equally important to examine the volatile compounds acting as attractants, and, where appropriate, oviposition stimulants. To fully understand host shifts within the family, it will be important to examine the attractant volatile compounds, as a plant bearing such compounds would likely be attractive to an ovipositing female, barring the presence of deterrents. If the same compounds are also stimulatory (and deterrents lacking), eggs may be laid even in the absence of host-specific contact chemical cues.

The results of this experiment suggest that host-plant experience does not modify the response to host-plant volatile chemical cues in this species. Females were more likely to land on model plants treated with *C. maculatum* extracts than on those with *D. carota* extracts, regardless of their previous host-plant experience. The role of experience is not ruled out, however. Within-trial results suggest a combination of preference and learning, with most females approaching or landing first and most frequently on model plants with *C. maculatum* extracts (Figure 3.9). There is also a trend for females experienced on *D. carota* were more likely to land after approaching a model plant with *D. carota* volatiles than are females experienced on *C. maculatum* (Figure 3.10). These same females were still more likely to approach a *C. maculatum*

model plant than *D. carota*, and most females landed after approaching either plant type.

Although these results do not show clear evidence of learning of host–plant volatile cues by *P. polyxenes*, work by Donzé and Feeny (2001) seems to suggest that these females are capable of learning volatile chemical cues. In that case, females may either have learned host–plant (*D. carota*) cues when presented with a deterrent non–host plant (*Achillea millefolium*, yarrow), or learned to avoid the volatiles of the deterrent non–host. These results are consistent with those of this study, as a non–deterrent host was used specifically to distinguish between green–leaf and host–specific volatiles. Further experimentation is certainly needed to determine the nature of the learning observed by Donzé and Feeny.

Females given *C. maculatum* experience were more likely than those given *D. carota* experience to approach model plants with host–plant extracts during the experienced trials (Figure 3.8). This is unlikely to have resulted from a relative deprivation of eggs laid during the day with the host plant, as female preference was distributed normally between the two hosts (Figure 3.12). More likely explanations are the high amount of variation in this behavior, or a sensitization of *C. maculatum*–experienced females to volatile chemical cues. In addition, there is no difference in the median number of landings per female, which should also be elevated in the *C. maculatum*–experienced females if they were deprived of egg–laying opportunity.

Female preference was tested with a choice bioassay, with eggs counted to determine a preference between the two hosts, *D. carota* and *C. maculatum*. Oviposition responses of butterflies were distributed normally between the two hosts,

with roughly equal numbers strongly preferring one host or the other, and most females accepting both hosts nearly equally (Figure 3.12). This verifies the result that females prefer *C. maculatum* volatile cues to those of *D. carota*; the differences are not due to a sample population with skewed preference for one of the two host plants. In an earlier experiment, (Chapter 2) females were more likely to respond to contact chemical cues of *D. carota* than those of *C. maculatum*. This suggests that both volatile and contact chemical cues may determine whole plant preference, as the preference between the two plants is normally distributed.

At least some female zebra swallowtail, *Eurytides marcellus*, females will oviposit in response to volatile host–plant cues prior to adult host–plant experience (M. Haribal, pers. comm.). This suggests that for *E. marcellus*, like *P. polyxenes*, volatile chemical cues can act as oviposition stimulants, even in the absence of contact chemical cues. Despite the difference in physiological pathways, both volatile chemical cues and contact chemical cues can be utilized by a female in making a decision of whether to oviposit on a plant or not. In fact, for *P. polyxenes*, the evidence, though currently circumstantial, suggests that different modalities of host–plant cues may be the key for the recognition of different plant species as hosts. Although *E. marcellus* is an exception among swallowtail butterflies in having a single contact chemical stimulant in at least one host–plant (Haribal and Feeny 1998), more cues may be present in the form of volatiles. Alternately, *P. polyxenes* uses a suite of contact chemicals as stimulants, with at least some stimulants from one host plant absent in other hosts (M. Haribal, pers. comm.; Carter, et al. 1998). This state may either be one of increasing complexity, as *E. marcellus* is a member of the tribe Graphiini, in an ancestral position to the sister tribes Troidini and Papilionini, of which *P. polyxenes* is a member (Miller 1987). Or, it may be a case of losses along the

phylogenetic branch to *E. marcellus*. These hypotheses cannot yet be tested, as the contact cues are not yet known for another graphiine swallowtail, although multiple, synergistic contact chemical cues have been found in all other species studied thus far (Honda 1995; Nishida 1995).

The current evidence for *P. polyxenes* would suggest that volatile chemical cues may be as stimulatory as contact chemical cues, even in highly preferred hosts, as for *C. maculatum* in the current study. It is not yet clear what physiological mechanisms allow the apparent interchangeability of gustatory and olfactory cues; the decision of whether to oviposit may take place in the central nervous system, where cues from the gustatory and olfactory centers may be summed, as in Dethier's across-fiber patterning model (Dethier 1973). Summation may also take place at the sensory periphery within one or more sensory modalities (Dethier 1982). Once a threshold level of stimulants has been reached, in the absence of a similar threshold level of deterrents, oviposition occurs. More work is needed to fully understand the underlying mechanisms at work; this could lead to the verification of current theories of host-plant recognition, or the generation of new ones.

Across the Lepidoptera, there seems to be little pattern to the ability of females to recognize olfactory host-plant cues. Positive responses to at least some host-plant odors have now been shown in a number of species, including the moths *Agrius convolvuli* (Shimoda and Kiuchi 1998), *Cideria albulata* (Douwes 1968), *Cochylis hospes* (Barker 1997), *Dioryctria amatella* (Hanula, et al. 1985), *Epiphyas postvittana* (Suckling, et al. 1996), *Helicoverpa armigera* (Hartlieb and Rembold 1996; Jallow, et al. 1999), *Mamestra brassicae* (Rojas and Wyatt 1999), *Ostrinia nubalis* (Binder and Robbins 1997), *Plutella xylostella* (Justus and Mitchell 1996), and *Trichoplusia ni*

(Landolt and Molina 1996). Only one butterfly species outside of the Papilionidae has been tested decisively: the heliconiid *Agraulis vanillae incarnata*, which does use olfactory cues to positively identify some hosts, although odors of some hosts elicit deterrent responses (Copp and Davenport 1978). Host-finding of *Pieris rapae* has received much study, but the question of the role of volatile host-plant cues has not been answered conclusively (Hern, et al. 1996).

In at least one of the species listed above, *Agrius convolvuli*, olfactory cues alone are sufficient for oviposition (Shimoda and Kiuchi 1998), while *Plutella xylostella* requires olfactory cues as well as gustatory cues for the maximum oviposition response (Justus and Mitchell 1996). Few studies compared experienced or mated females with those that were not: only mated *Mamestra brassicae* females will fly upwind to host odors in a flight chamber (Rojas 1999), while *Trichoplusia ni* females are significantly more likely to fly upwind to a host plant that was previously experienced, even after only a single contact with the host (Landolt and Molina 1996). In two reported cases, volatile cues were not at all important for the oviposition response of the studied species: *Yponomeuta cagnagellus* (Hora and Roessingh 1999) and *Heliothis virescens* (Ramaswamy, et al. 1987).

In one attempt to see a pattern in the use of different types of cues for host location, Ramaswamy (1988) hypothesized that although all moths may use volatile cues for host-habitat location, only oligophagous and monophagous species should use olfactory cues for short-range host location, while highly polyphagous species should not. Although Ramaswamy did not attempt to extend his hypothesis to include butterflies, *P. polyxenes*, as an oligophagous species, does seem to fit with this pattern. Unfortunately, data to fully test this hypothesis are still lacking, as only one highly



polyphagous lepidopteran, *Heliothis virescens*, has been tested to date (Ramaswamy, et al. 1987). This pattern does appear to hold for aphids, however, as oligophagous aphid species were found to use olfaction and vision to find hosts, while polyphagous species used vision alone (Hori 1999).

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## CHAPTER FOUR

### RESPONSES TO LEAF SHAPES, WITH AND WITHOUT CHEMICAL CUES

Visual cues can be of primary importance to searching phytophagous insects, such as the butterfly *Battus philenor* in east Texas (Rausher 1978). Moreover, such cues can be part of a complex series that also includes other kinds of plant cues, as in the host-finding behaviors of the flies *Rhagoletis pomonella*, *Delia radicum*, and *D. antiqua* (Harris and Miller 1982; Prokopy 1986). Shapes, colors, and contrasts have all been shown to play a role in the ability of insects to find their host plants (Gilbert 1975; Rausher 1978; Traynier 1979; Harris and Miller 1982; Wiklund 1984; Mackay and Jones 1989; Aluja and Prokopy 1993). Shapes are of particular interest in a number of well-studied systems, such as several species of phytophagous flies (Prokopy 1986; Degen and Städler 1996), *Heliconius* butterflies (Gilbert and Singer 1975), and the pipevine swallowtail butterfly, *B. philenor* (Rausher 1978; Papaj 1986).

Most of the early work with vision in swallowtail butterflies (Lepidoptera: Papilionidae) was concerned with color vision, perhaps as a natural extension of the groundbreaking work with the color vision of honey bees by von Frisch in the early part of the 20<sup>th</sup> century (von Frisch 1967). Ilse and Vaidya (1956) and Vaidya (1969a) demonstrated feeding responses of *Papilio demoleus* to colors, also investigating the effects of size and radial patterns. More recently, Arikawa et al. (1987) have determined that *P. xuthus* is at least pentachromatic, with peaks in photoreceptor spectral receptivity ranging from the ultraviolet to red. There have also been several demonstrations of “true” color vision within the Papilionidae: Ilse and Vaidya (1956) for *P. demoleus*, Kelber and Pfaff (1999) in *P. aegeus*, and Kinoshita et al. (1999) for *P. xuthus*. Perhaps most convincing is the recent evidence for color constancy, the

ability to distinguish a trained color under different illuminating colors, in *P. xuthus* (Kinoshita and Arikawa 2000).

The role of vision in the oviposition behavior of a swallowtail butterfly was first explored by Vaidya (1969b). This was again a study of the color vision of *P. demoleus*, but this time using oviposition responses instead of feeding responses. Most drumming, a typical behavior for butterflies after landing on a host plant and before egg-laying (e.g., Ilse 1955), occurred on blue-green to yellow-green papers, with none on gray, yellow, orange, red, purple, or blue papers. Later, Saxena and Goyal (1978) showed that visual cues in the form of glass-screened leaves were highly attractive to *P. demoleus*, although this attraction was not host-specific and did not elicit any oviposition behavior. The first clear demonstration of the use of leaf shape for host-finding by a swallowtail butterfly was by Rausher (1978). He studied the pipevine swallowtail butterfly, *B. philenor*, in eastern Texas and found that individual females would land mostly on either narrow- or broad-leaved plants, corresponding to the shapes of the two local host-plant species. This system has since been well-studied, with evidence for the learning of leaf shape after contact with methanolic host-plant extracts, even without oviposition (Papaj 1986). There is even strong evidence for the butterfly acting as the selective force for the two different leaf shapes in this population (Rausher and Feeny 1980).

The role of visual cues in oviposition by *P. polyxenes* has received little attention, compared to that of contact and volatile chemical cues. Inger Ahman (unpublished manuscript) has noted that females released near a mixed patch of two hosts, *Pastinaca sativa* (wild parsnip) and *Daucus carota* (wild carrot), were more likely to land on the species that was in bloom. This suggests that searching females



are using visual or volatile chemical properties of the flowers as cues for finding the host plants, although this observation has never been tested experimentally. In recent laboratory experiments examining the learning of volatile chemical cues, Donzé and Feeny (2001) reported that females were significantly more likely to land on green versus yellow–orange sponge “leaves”. Females in these experiments also showed a significant preference for a pinnate over an ovate leaf shape.

The present experiments were designed to determine whether *P. polyxenes* could use leaf shape as a cue for host–finding. An experimental array of model host plants was used in place of real plants to allow for the presentation of leaf shapes with no relevant chemical cues present (Experiment 1). In addition, the responses to the leaf shapes in combination with contact (Experiment 2) or volatile (Experiment 3) chemical cues were also examined, to determine the relative roles of the cues within each pair.

## GENERAL METHODS

### *Plants*

The apiaceous host plants, *Daucus carota* (wild carrot), *Conium maculatum* (poison hemlock), and *Pastinaca sativa* (wild parsnip), were grown in a greenhouse atop Seeley Mudd Hall at Cornell University. Seed was wild–collected from sites near Ithaca, New York. A non–deterrent non–host, *Vicia faba* (fava bean, Fabaceae), was grown from commercial seed (var. Broad Windsor, Agway).

### *Surface area regressions*

Weight was previously shown to be a reliable proxy for surface area (Chapter 3, Brooks, et al. 1996) for *D. carota*, *C. maculatum*, and *V. faba*, but this had not been

demonstrated for *P. sativa*. Previous measures were used for *D. carota* and *C. maculatum*, as plants were grown from the same seed collection as used earlier (Chapter 3), but new measures were made for *V. faba*, as a new seed variety had been purchased. Fifty leaves each of *P. sativa* and *V. faba* were individually weighed, and measurements for length, width, and surface area were taken. Petioles were not included in any measurements. Surface area was measured using a Li-cor Model 3100 area meter (Li-cor, Inc., Lincoln, NE) set at low resolution (1mm). Surface area of each leaf was measured three times and the largest measurement of the three was used for further calculations. Weight, length, and width were regressed onto twice the surface area (top and bottom surfaces), as volatiles would be better correlated with the total surface area of a leaf than with other measures, but surface area would be impractical to measure for every leaf extracted.

#### *Model leaf shapes*

The model leaf shapes used in these experiments are shown in Figure 4.1. For all three experiments, the surface area of one side of the leaf shape (not including any stem) was 53.75 cm<sup>2</sup>, such that the total top surface area of the four leaves on a model plant would be equal to 215cm<sup>2</sup>. The amounts of contact or volatile chemical extracts were also set at an equivalent of 215cm<sup>2</sup> of leaf surface area per model plant so that the relative amounts would be the same despite the differences in shape between plant species. The *D. carota*/*C. maculatum* leaf shape was the same as that used in Chapter 3 and was originally based on a shape found attractive by female carrot root flies, *Psila rosae* (Degen and Städler 1997). The shapes used for *P. sativa* and *V. faba* were based on tracings of actual leaves; this was not done for *D. carota* or *C. maculatum* due to the high complexity of these leaf shapes and the need for more than one hundred total leaves of each leaf shape.

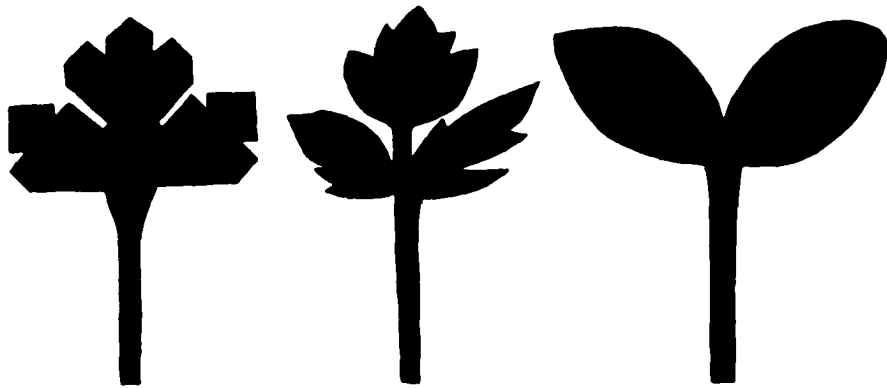


Figure 4.1 The leaf shapes used in these experiments. The left shape represents *D. carota* / *C. maculatum* (11.6cm wide and 16.7cm tall). The center shape represents *P. sativa* (12.8cm wide and 18.2cm tall). The right shape represents *V. faba* (14.1cm wide and 14.8cm tall).

Leaves for the model plants were cut from green card stock (Hammermill, Bright Hue® Cover). Additional stability was added by stapling an additional 10.2cm long x 1.6cm wide strip of the same paper, folded in half lengthwise, to the back side of the stem of the leaf. Leaves were scored with a blade to add dimensionality. Scores of *V. faba* model leaves were on the top side of the leaf, so that the surface was convex, matching the natural curvature of a *V. faba* leaf; all other leaves were scored on the bottom side, such that leaves were concave, again matching the natural curvature.

*Extractions: Contact chemicals*

*D. carota*, *P. sativa*, and *V. faba* leaves were collected from greenhouse plants. Leaves were weighed, and 100g batches were blended in 500ml boiling 95% ethanol. The slurry was filtered and the ethanol removed by evaporation under reduced pressure. The remaining aqueous solution was centrifuged and the supernatant was extracted three times each with diethyl ether, chloroform, and ethyl acetate. This aqueous extract contained virtually all of the stimulant activity of the parent ethanolic extract (Feeny, et al. 1988; Brooks, et al. 1996). The extract was evaporated to a concentration of 5 gram leaf equivalents ('gle') per ml and frozen in 0.5ml aliquots.

To prepare extracts for application to leaf shape models, the extracts were diluted to a concentration equivalent to 215cm<sup>2</sup>/4ml with HPLC-grade water. One ml of extract was painted evenly on the top surface of each leaf, with eight leaves each of *D. carota* and *P. sativa*, and 16 leaves of *V. faba* per trial. The extract was allowed to nearly dry and leaves were flattened, as the moisture caused them to curl. Leaves were prepared ahead of trials and frozen until needed due to the time needed to apply extracts and to dry leaves.

*Extractions: Volatile chemicals*

Volatile extracts were collected as in Chapter 3. *C. maculatum*, *P. sativa*, and *V. faba* leaves were collected from greenhouse-grown plants. Leaves were weighed with the petioles removed, and 20–30g from a single species were placed in a large (2000ml) Erlenmeyer flask. Hexane was added to cover all of the leaves (1000ml), and allowed to soak. After five minutes, the hexane was decanted into a clean flask and frozen at -10°C. After all of the samples of a single plant species were extracted, the combined

extract was reduced to the desired concentration (see Results (this section)). The extract was stored in a -10°C freezer in 1ml aliquots in 2ml vials until needed.

#### *Extractions: Spectral properties of extracts*

An S1000 fiber optic spectrophotometer (Ocean Optics, Inc.) with CSPEC software was used to sample the reflectance of extract-coated model leaves. Reflectance at wavelengths from 274nm to 821nm was measured at every 0.535nm. A model leaf with no extract was sampled as a reference standard. The reference standard was subtracted from each sample, and the resulting reflectance is shown in Figure 4.21.

#### *Butterflies*

Butterflies were diapausing, first generation offspring of wild females caught during 1999 in southeastern Canada. Butterflies were kept in an environmental growth chamber (Environmental Growth Chambers, Chagrin Falls, OH) with 16:8 LD, 27°C daytime and 15.5°C nighttime temperatures, and 75±5% relative humidity. The growth chamber was devoid of host-plant material at all times. Butterflies were fed and numbered as described in Carter and Feeny (1985), with two exceptions: butterflies were fed a 20% (in place of 10%) solution of honey in water and several colors of Sharpie<sup>®</sup> permanent markers (Sanford, Inc.) were used for numbering to aid in individually identifying females in flight. Females were mated by hand pairing (Carter and Feeny 1985) more than 24 hours after emergence, on the second day after eclosion.

Each female butterfly was subjected to four trials, two “naïve” and two “experienced”, as seen in Figure 4.2. For some analyses, the first two trials were combined into one “naïve” category. After the second trial, each female was placed

into an individual 16 x 31 x 9cm cage with a sprig of one of the two host species used in the particular experiment (*D. carota* and *P. sativa*, or *C. maculatum* and *P. sativa*) in a water-filled aquapic, with the plant species alternating by female so that an equal number of females would receive each plant species. Cages were kept in a greenhouse chamber away from that of the bioassays, and females were fed at least once per day. Females were allowed to lay eggs *ad libitum* until the morning of the third day after mating, at which point they were returned to the growth chamber devoid of host-plant material. The following day, females were given their third test, the first “experienced” test. The fourth and final test occurred on the fifth day after mating. Again, the two experienced trials were combined into a single “experienced” category for some analyses.

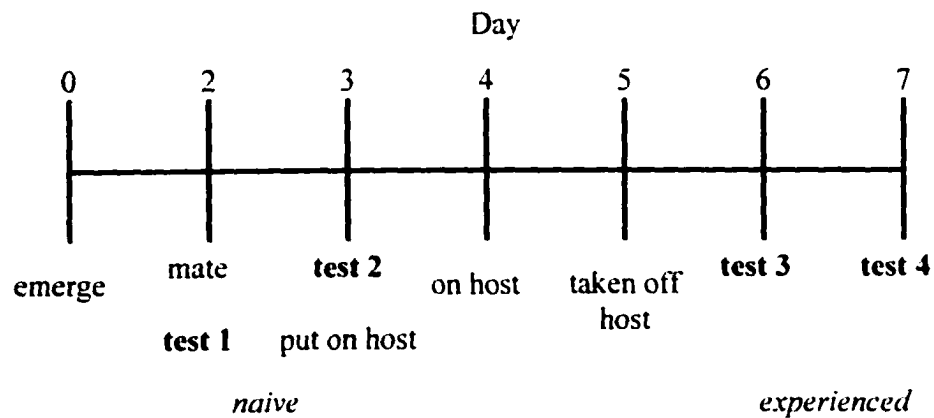


Figure 4.2 Timeline for individual females in all three experiments.

### *Bioassay*

Bioassays took place in a 3.66 x 3.66 x 1.83m cage placed in a large greenhouse module atop Seeley Mudd Hall at Cornell University. The greenhouse module, and all adjacent modules, contained no apiaceous plants at any time during the experiment. The cage had nylon mesh walls and the floor was covered in black landscape fabric (Agway, Inc.). Eight model plants were arranged in a three-by-three array on the floor (with no center plant), and one nectar plant, *Pentas lanceolata* (Rubiaceae), was placed in each of the four corners. Model plants were the same as first used in Chapter 3, four leaves were attached to each model plant, and a vial of volatiles could be inserted in the center at the leaf attachment points. Two model plants received leaves of the *D. carota*/*C. maculatum* leaf shape, two model plants received leaves of the *P. sativa* leaf shape, and four model plants received leaves of the *V. faba* leaf shape. The plants were arranged within the cage in an array as in Figures 4.3–4.5, for Experiments 1–3, respectively.

Butterflies were transported to the array from the growth chamber in butterfly envelopes (BioQuip) and released onto nectar plants. Female *P. polyxenes* do not interact during oviposition or searching behavior (pers. obs.), allowing for multiple females to be tested simultaneously. Trials lasted for 90 minutes and there was no interference during a trial. All behaviors towards the model plants, as well as transitional behaviors and the identities of the behaving butterflies, were recorded using a tape recorder and transcribed on the day of the trial. For analysis, behaviors were classified into ten discrete categories, as shown in Table 4.1. The greenhouse temperature was set at 25.5°C, and lights (Sylvania Lumalux LU400, sodium high intensity discharge) were used to supplement daylight on cloudy days.

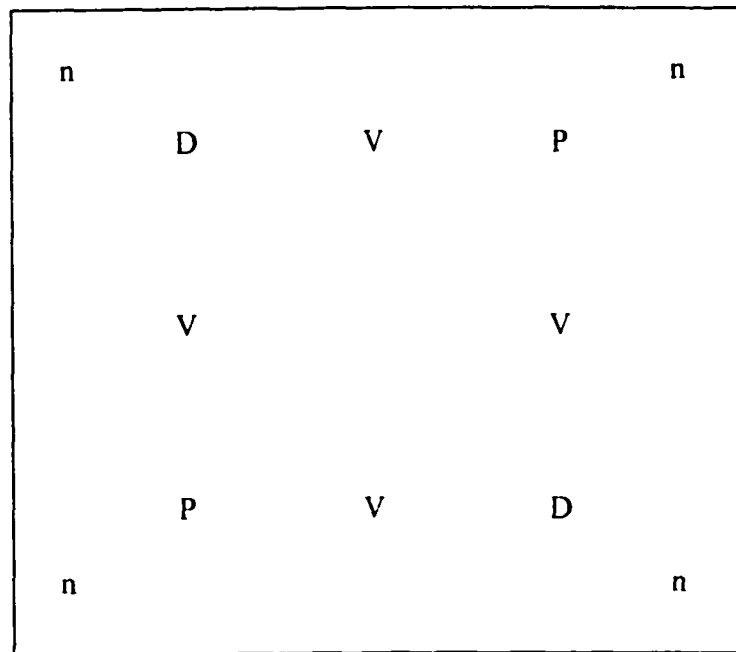


Figure 4.3 The arrangement of model and nectar plants in Experiment 1 (leaf shapes).

*D*: Model plants with the *D. carota* leaf shape; *P*: Model plants with the *P. sativa* leaf shape; *V*: Model plants with the *V. faba* leaf shape; *n*: Nectar plants (*Pentstemon lanceolatus*). Two arrangements are possible by rotating the model plants bearing host-plant leaf shapes. Each side of the cage is 3.66m.



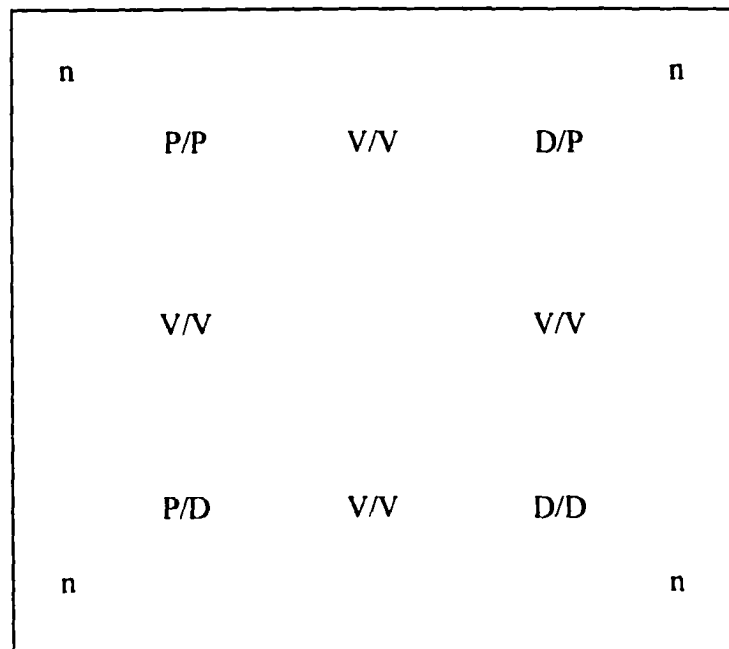


Figure 4.4 The arrangement of model and nectar plants in Experiment 2 (leaf shape and contact chemical cues). For each model plant, the first letter specifies the leaf shape, while the second specifies the contact chemical cues. *P*: *P. sativa* cues; *D*: *D. carota* cues; *V*: *V. faba* cues; *n*: Nectar plants (*Pentas lanceolata*). Eight arrangements are possible by rotating the extracts in this and one other permutation (exchanging the “hybrid” cued plants).

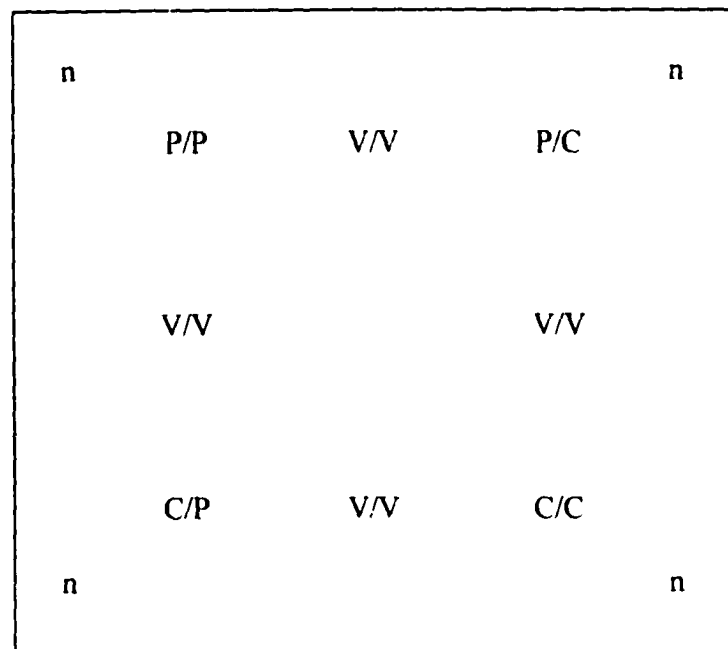


Figure 4.5 The arrangement of model and nectar plants in Experiment 3 (leaf shape and volatile chemical cues). For each model plant, the first letter specifies the leaf shape, while the second specifies the volatile chemical cues. *P*: *P. sativa* cues; *C*: *C. maculatum* cues; *V*: *V. faba* cues; *n*: Nectar plants (*Pentas lanceolata*). Eight arrangements are possible by rotating the extracts in this and one other permutation (exchanging the “hybrid” cued plants).

Table 4.1 Categories of behaviors observed during trials.

<b>Category of Behavior</b>	<b>Examples of Behavior</b>
Non-plant directed behaviors	Fly high (>1m over model plants) around cage. Bother other females on side of cage, often transitional between array activity and inactivity
Nectaring behaviors	Nectar, Land on nectar plant
Fly mid over plants	Fly mid-level (<1m, >10cm) over model plants, may be directed to particular model plants
Fly low over plants	Fly low (<10cm) over model plants, may be directed to particular model plants
Approach	Approach a model plant
Inspect	Flutter low (<5cm) over a model plant
Land	Land on a model plant
Drum	Drumming behavior on a part of a model plant
Curl	Abdomen curl while on a model plant
Oviposit	Lay an egg on a model plant

### *Analysis*

Regressions of leaf surface area for *P. sativa* and *V. faba* were made using JMP statistical exploration software (SAS Institute, Inc.). SAS software version 6.12 (SAS Institute, Inc.) was used for all analyses. Non-parametric statistics were used for behavioral analyses due to the non-normality of the data (Conover 1980). Approaches and landings were primarily used for analyses due to the large number of both types of behavior, as well as the accuracy for identifying the model plant at which the behavior was directed. Only females displaying a behavior at least once were included in analyses of such behavior.

Responses of naïve females to host and non-host extracts and the effect of host-plant cues on the responses of experienced females were both analyzed by the sign test. The null hypothesis was that there was no effect of plant cues or plant experience on behavior. For naïve females, approaches and landings on model plants containing cues of the non-host, *V. faba*, and on model plants containing cues of either host, *D. carota* or *C. maculatum*, were each summed for each female. For each set of approaches and landings, the sum for the non-hosts was subtracted from the sum for the hosts and the sign of the result was scored. The same procedure was used for experienced females, except that the extracts of the two host plants were compared, using separate analyses for each plant cue.

The behaviors of experienced females toward host-plant cues with respect to host-plant experience and host-plant cues were analyzed using Fisher's exact test. Approaches and landings were each tested in a 2 x 2 table of experience type versus the host-plant cues of the model plant receiving the behaviors, with separate analyses for contact and volatile extracts. To test the effect of host-plant experience on

experienced females, a median test was used, again with a null hypothesis of no effect. The numbers of approaches and landings on model plants containing host–plant cues were compared between females that had experienced each of the two host–plant species.

Fisher’s exact test was used to look for a relationship between the first host–plant extract approached or landed on during each trial and the cues most often approached or landed on during the same trial for each female. Again, separate analyses were used for each plant cue. Fisher’s exact test was also used to examine the data while taking the sequence of behaviors into account. 2 x 2 tables were used to examine the relationship between approaches and landings for hosts and non–hosts for naïve females, and for the two different hosts for experienced females. The Mantel–Haenszel test was used to examine the effects of experience, behavior following an approach or landing, and host–plant cues simultaneously (Stokes, et al. 1995).

### *Results*

Regression coefficients for *P. sativa* and *V. faba* are shown in Table 4.2. Like *D. carota* and *C. maculatum* (Chapter 3), weight was the best predictor of surface area for *P. sativa*. Width was again the best predictor for *V. faba*, though there was a significant difference from the variety used in Chapter 3. However, as weight also had a high correlation for *V. faba*, weight was used for both species. All concentrations were set to the equivalent of 215cm<sup>2</sup> of leaf foliage per ml of extract. The corresponding weights for *P. sativa* and *V. faba* are 1.65g and 2.84g, respectively. From Chapter 3, the corresponding weights of *D. carota* and *C. maculatum* leaves are 2.99g and 2.57g, respectively.

Table 4.2 Leaf surface area regressions. Parameters for the regression of length, width, and weight on surface area for *Pastinaca sativa* and *Vicia faba*.

	Intercept	Slope	$r^2$
<hr/>			
<i>P. sativa</i>			
Length	-60.973	16.4829	0.824758
Width	-42.18	18.7812	0.892483
Weight	11.0141	123.394	0.973947
<hr/>			
<i>V. faba</i>			
Length	-21.385	8.90838	0.85415
Width	-18.405	13.3759	0.932509
Weight	-1.1823	76.9117	0.904548

Female *Papilio polyxenes* behaved in these bioassays in a similar manner to those in a bioassay using whole plants instead of model plants (Chapter 6). Females flew over the arrays, approached and inspected the model plants. In Experiments 2 and 3, females also landed frequently, drummed as they would on a natural leaf (e.g., Ilse 1955), curled their abdomens in preparation to lay an egg, and occasionally, laid an egg on a model plant.

## EXPERIMENT 1: LEAF SHAPES

### *Specific Methods: Bioassay*

The two host plants utilized in this experiment were *D. carota* and *P. sativa*. No extracts were used in the bioassay; leaves were untreated and there were no vials in the model plants. The array was set up as in Figure 4.3. The bioassay followed the general methods described above. A total of 30 trials was used to test 35 butterflies, with a mean of 4.73 butterflies per trial. No more than 13 butterflies were tested at any one time. Activity was sporadic, such that it was possible to watch so many butterflies at one time. Trials were started between 10:45 and 16:30 hrs.

### *Results*

#### *Responses of naïve females*

Naïve females did not respond preferentially to model plants with leaf shapes intended to look like host-plant leaf shapes. Females were as likely to land on model plants with the host-plant (*D. carota* or *P. sativa*) leaf shapes as on the model plants with the non-host *V. faba* leaf shape (Figure 4.6; Sign test: approaches: N=6, T=1, p=1; landings: N=5, T=0, p=1). Females were also no more likely to follow an approach with a landing on a host-plant leaf shape than on a non-host leaf shape (Figure 4.7; Fisher's exact test: N=33,  $\chi^2_1=0.029$ , p=1).

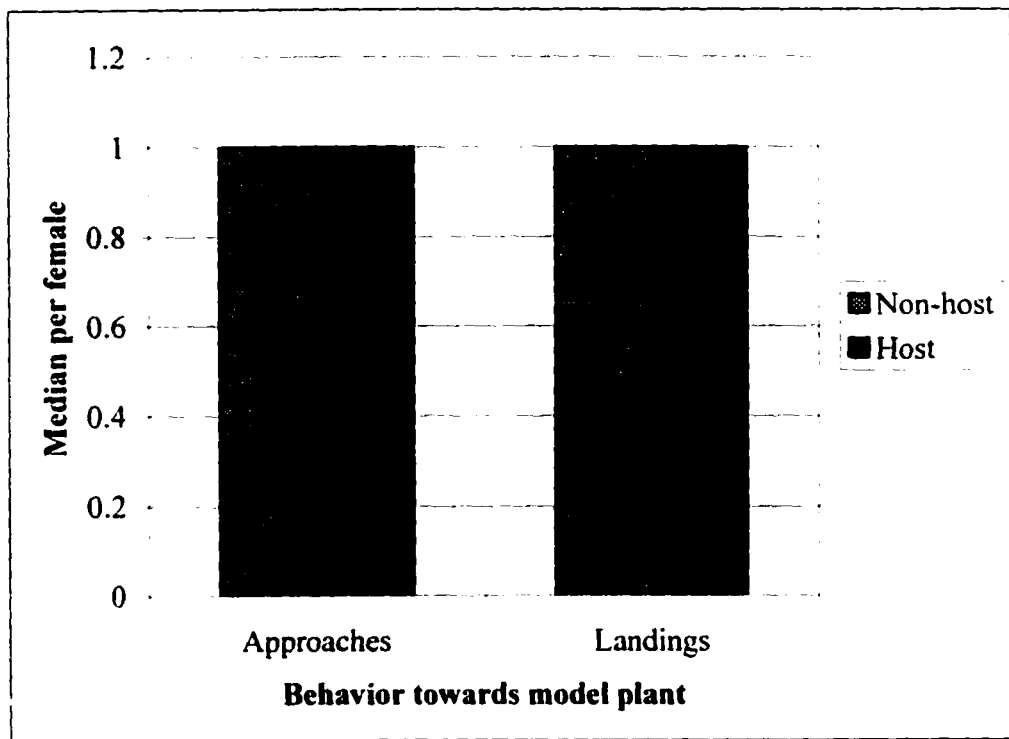


Figure 4.6 Median number of approaches and landings per female in naïve leaf shape trials (trials 1 and 2). Host leaf shapes are *D. carota* + *P. sativa*, the non-host is *V. faba*.



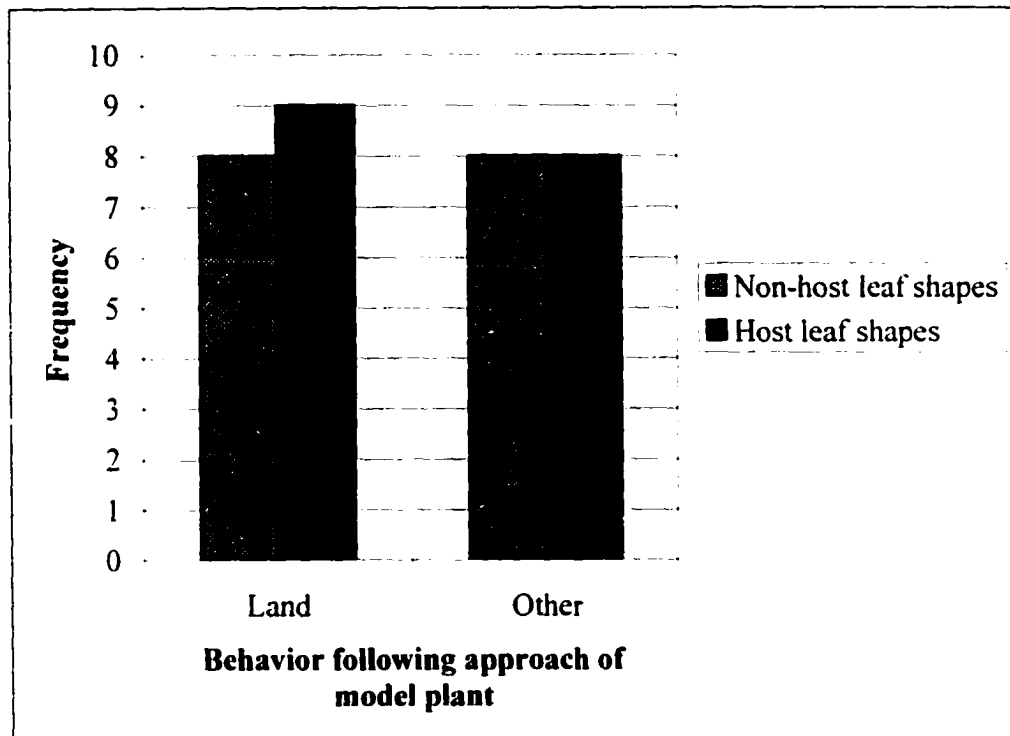


Figure 4.7 Frequency of landings or other behaviors immediately following approaches to model plants with non-host or host-plant leaf shapes by naïve females.

*Responses of experienced females*

Host-plant experience did not increase approaches or landings on model plants with the leaf shape of the host experienced by a female (Figure 4.8; Fisher's exact test: approaches:  $N=23$ ,  $\chi^2_1=0.958$ ,  $p=1$ ; landings:  $N=10$ ,  $\chi^2_1=0.278$ ,  $p=1$ ). Females approached and landed more often on the *P. sativa* leaf shape than the *D. carota* leaf shape, though the differences were only slightly significant for approaches and too few for a statistical test for landings (Figure 4.9; Sign Test: approaches:  $N=6$ ,  $T=-7.5$ ,  $p=0.0625$ ; landings:  $N=3$ ). There was a non-significant trend for females experienced on *P. sativa* to be more likely than females experienced on *D. carota* to approach or land on model plants with host-plant leaf shapes during the trials (Figure 4.10; Median test: approaches:  $N=6$ ,  $\chi^2_1=0.9375$ ,  $p=0.3329$ ; landings:  $N=3$ ).

*Within-trial experience effects*

To look at the effect of within-trial experience, Fisher's exact tests were used (Conover 1980). There was a significant relationship between the first model plant with a host-plant leaf shape approached and the type of host-plant leaf shape most often approached during an individual trial (Figure 4.11A; Fisher's exact test:  $N=12$ ,  $\chi^2_1=8$ ,  $p=0.018$ ). All females landed mostly on model plants with the *P. sativa* leaf shape (Figure 4.11B). There was no effect of female experience and the behavior following an approach while taking the leaf shape into account (Figure 4.12; Mantel-Haenszel test:  $N=21$ ,  $T=0$ ,  $p=1$ ).

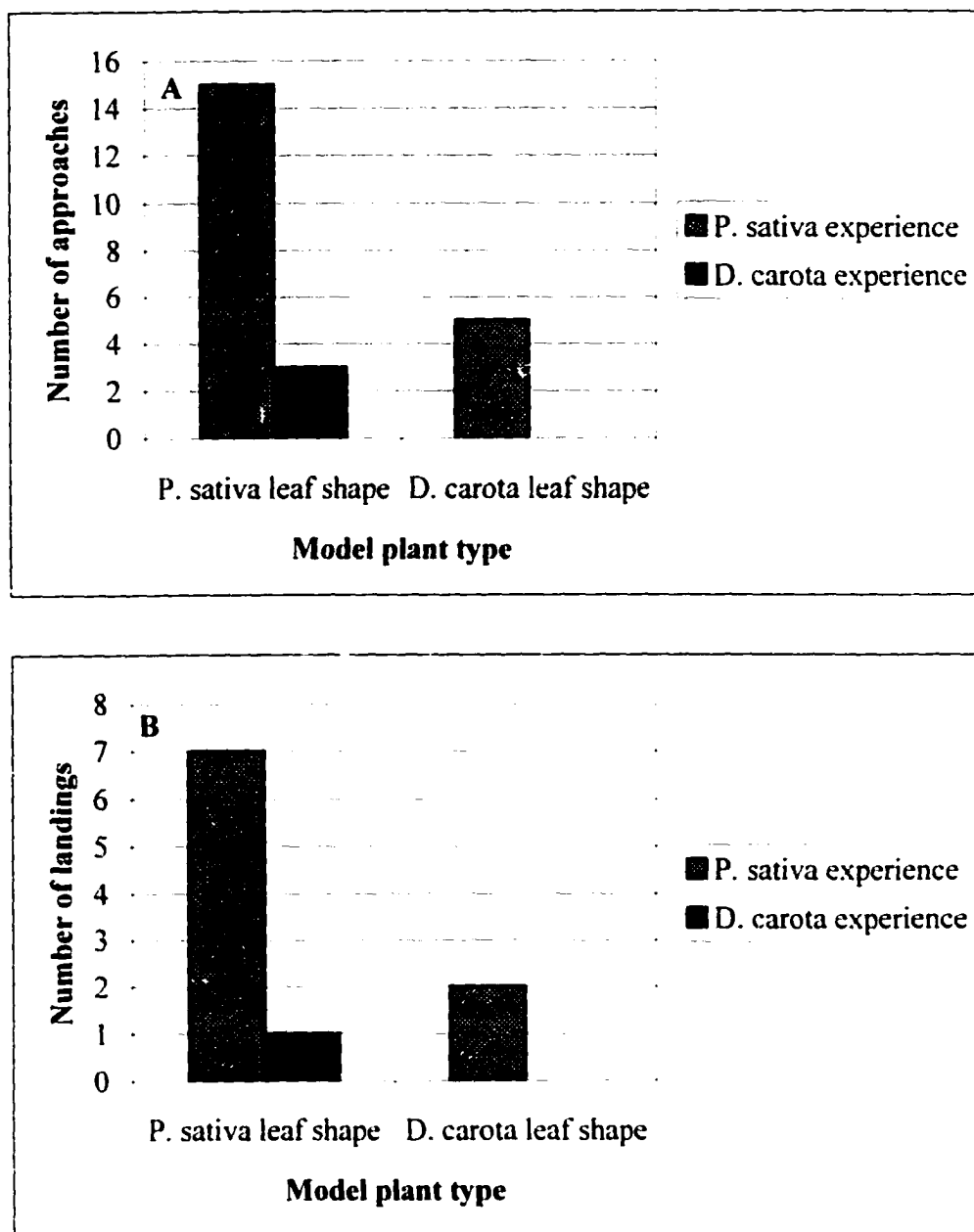


Figure 4.8 Number of approaches (A) and landings (B) by females in experienced trials on model plants with host-plant leaf shapes with respect to host-plant experience and host-plant leaf shape. (No females with *D. carota* experience approached or landed on a model plant with the *D. carota* leaf shape.)

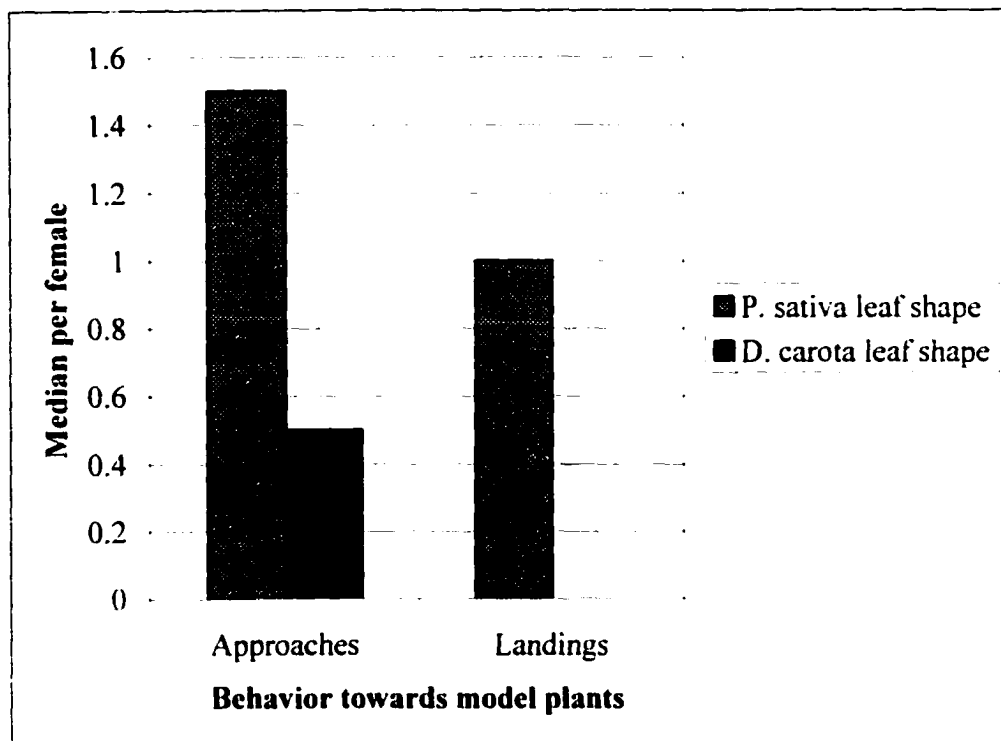


Figure 4.9 Median number of approaches and landings by females in experienced trials on model plants with host-plant leaf shapes with respect to the two host-plant leaf shapes. (The median number of landings on model plants with the *D. carota* leaf shape was zero.)

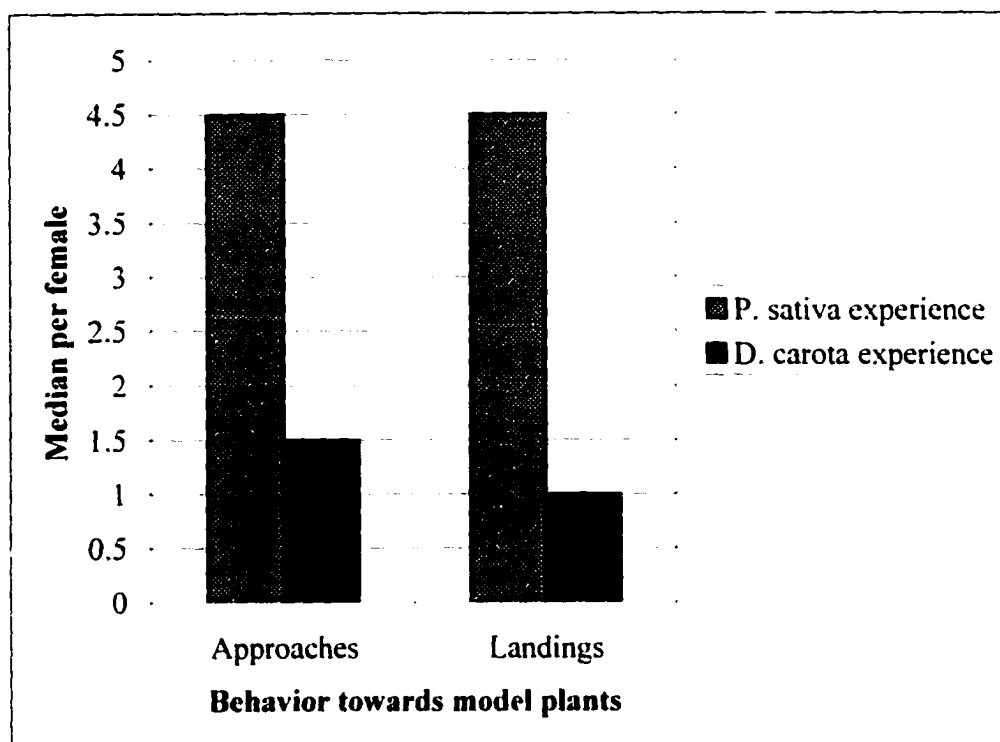


Figure 4.10 Median number of approaches and landings by experienced females on model plants with host-plant leaf shapes (*P. sativa* + *D. carota*) with respect to the host plant experienced.

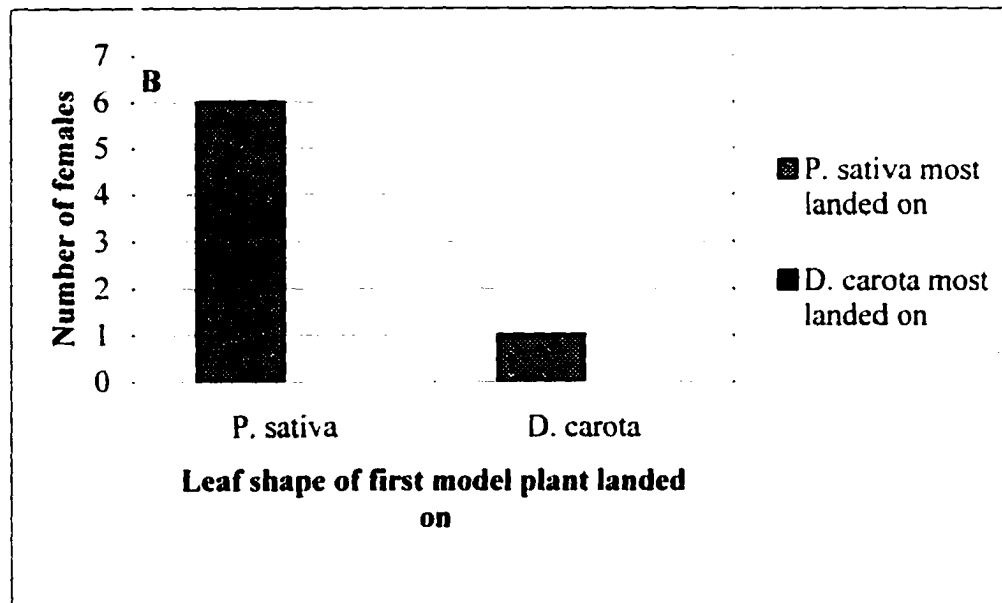
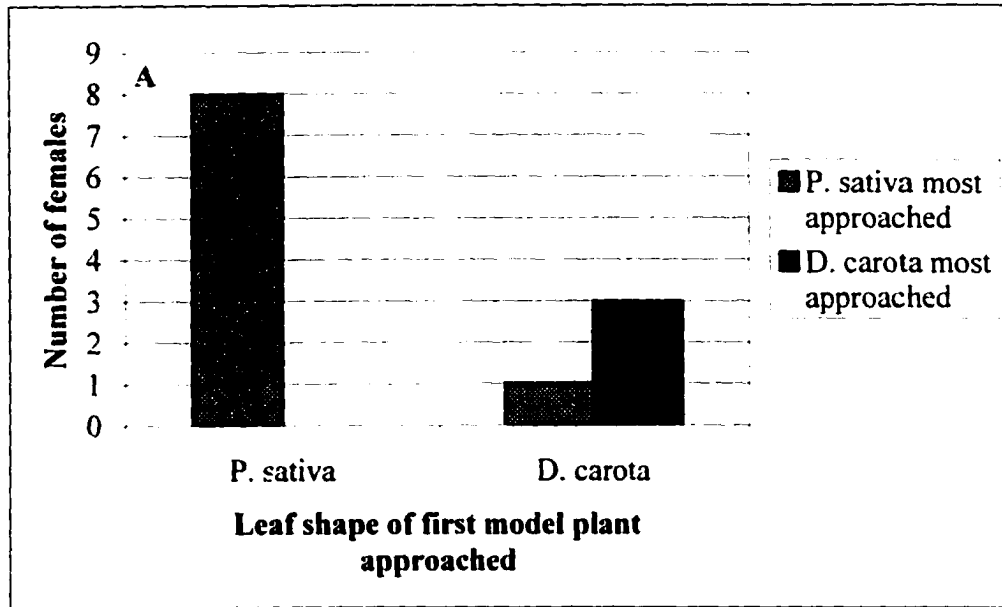


Figure 4.11 Number of females approaching (A) and landing on (B) model plants bearing each of the two host-plant leaf shapes (*P. sativa* and *D. carota*) with respect to the type of model host plant most frequently landed upon during a single trial. (No female first approached a model plant with *P. sativa* leaf shape and then approached the *D. carota* shape most often. No females landed most often on model plants with *D. carota* leaf shapes.)

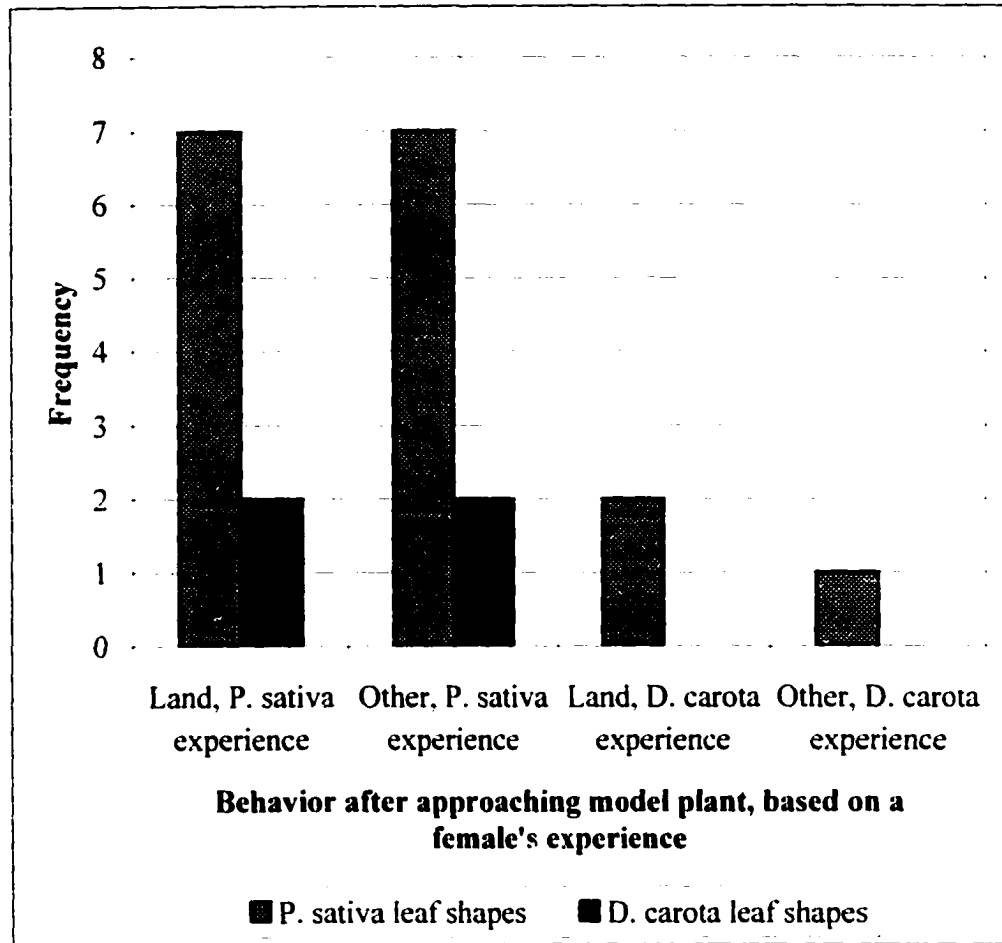


Figure 4.12 Frequency of landings or other behaviors immediately following approaches to model plants with host-plant leaf shapes by experienced females, with respect to the experience of the female. An “other” behavior is any other than landing, as listed in Table 1. (Behaviors listed below landing in the table cannot occur without landing, and are thus not included in the “other” category.) (No females with *D. carota* experience approached a model plant with the *D. carota* leaf shape.)

## EXPERIMENT 2: LEAF SHAPES AND CONTACT CHEMICAL CUES

### *Specific Methods: Bioassay*

The two host plants utilized in this experiment were *D. carota* and *P. sativa*. The model leaves were treated with contact stimulant extracts, as described above. Model plants were arranged as in Figure 4.4. The bioassay followed the general bioassay methods described above. A total of 16 trials was used to test 32 butterflies, with a mean of 8.625 butterflies per trial. No more than 24 butterflies were tested at any one time. Activity was sporadic, such that it was possible to watch so many butterflies at one time. Trials were started between 14:00 and 17:00 hrs.

### *Results*

#### *Responses of naïve females*

There was a trend for naïve females to respond more frequently to model plants with host-plant (*D. carota* or *P. sativa*) cues than non-host (*V. faba*) cues for both approaches and landings (Figure 4.13; Sign test: approaches: N=8, T=10.5, p=0.1719; landings: N=6, T=9.5, p=0.0625). Naïve females were also significantly more likely to land following an approach of a model plant with host-plant cues, as opposed to non-host cues (Figure 4.14A; Fisher's exact test: N=93,  $\chi^2_1=7.051$ , p<0.01). This trend was statistically insignificant for post-landing behaviors (Figure 4.14B; Fisher's exact test: N=33,  $\chi^2_1=1.886$ , p=0.284), but non-host model plants only received three landings overall.

#### *Responses of experienced females*

There was no effect of host-plant experience on the behavior of females in the form of increasing the approaches or landings on model plants with the leaf shape of the host-plant experienced (Figure 4.15; Fisher's exact test: approaches: N=103,



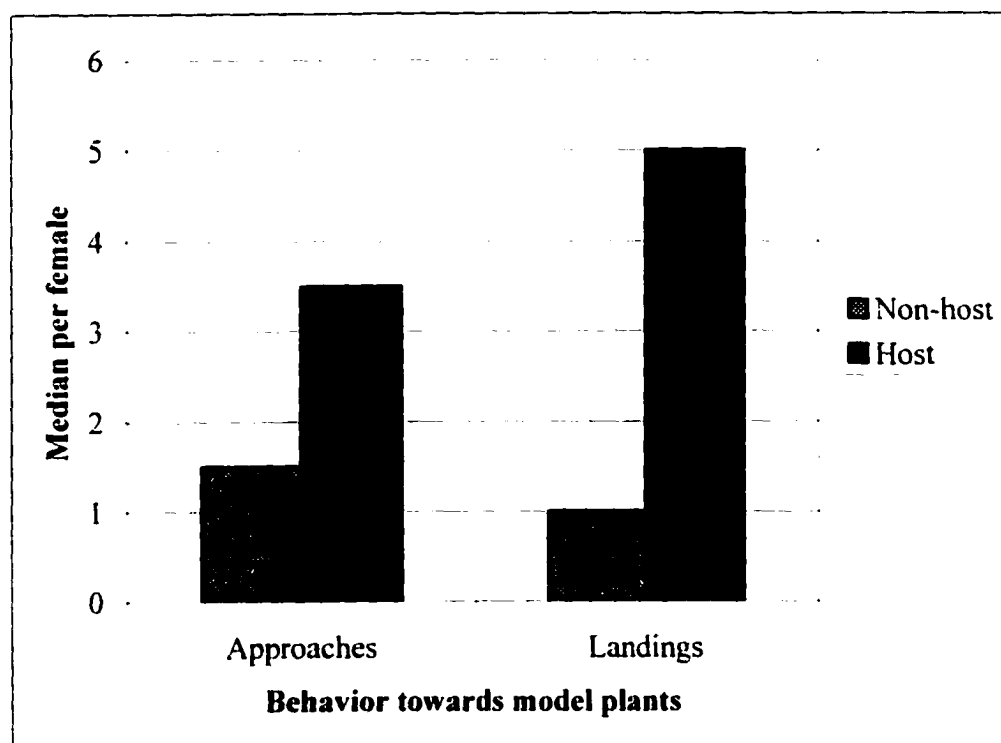


Figure 4.13 Median number of approaches and landings per female in naïve trials (trials 1 and 2) with leaf shapes and contact chemical cues. Host extracts are *P. sativa* + *D. carota*, the non-host is *V. faba*.

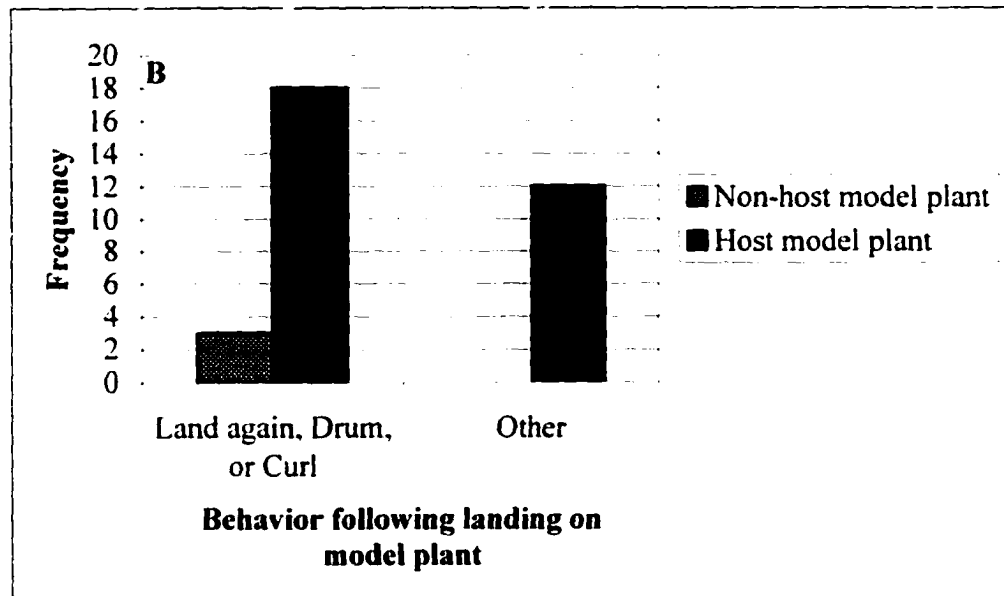
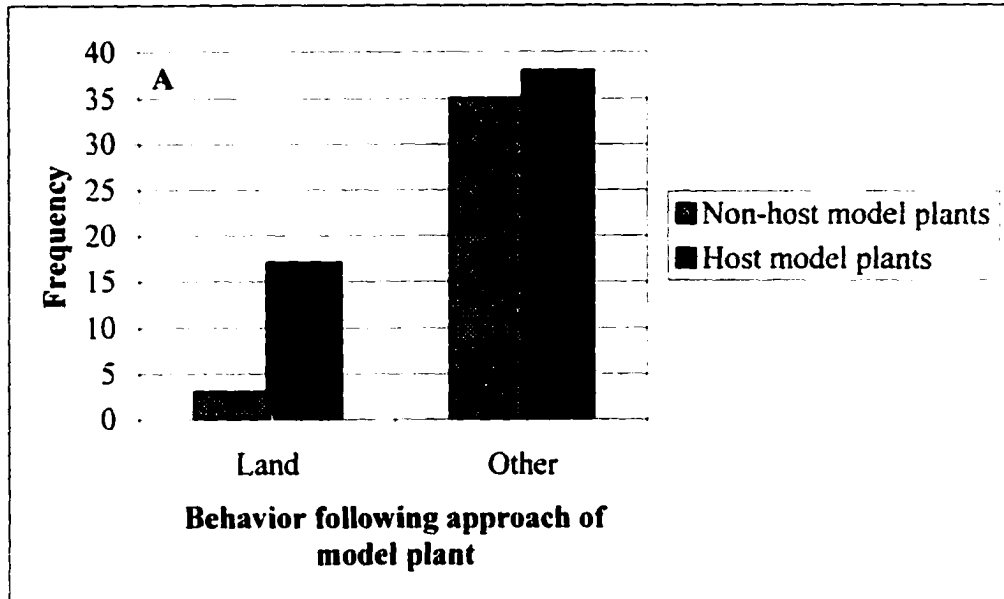


Figure 4.14 Frequency of landings or other behaviors immediately following approaches to (A) and landings on (B) model plants with non-host or host-plant cues (leaf shape and contact chemicals) by naïve females. (No females displayed pre-landing behavior after landing on a model plant with non-host cues.)

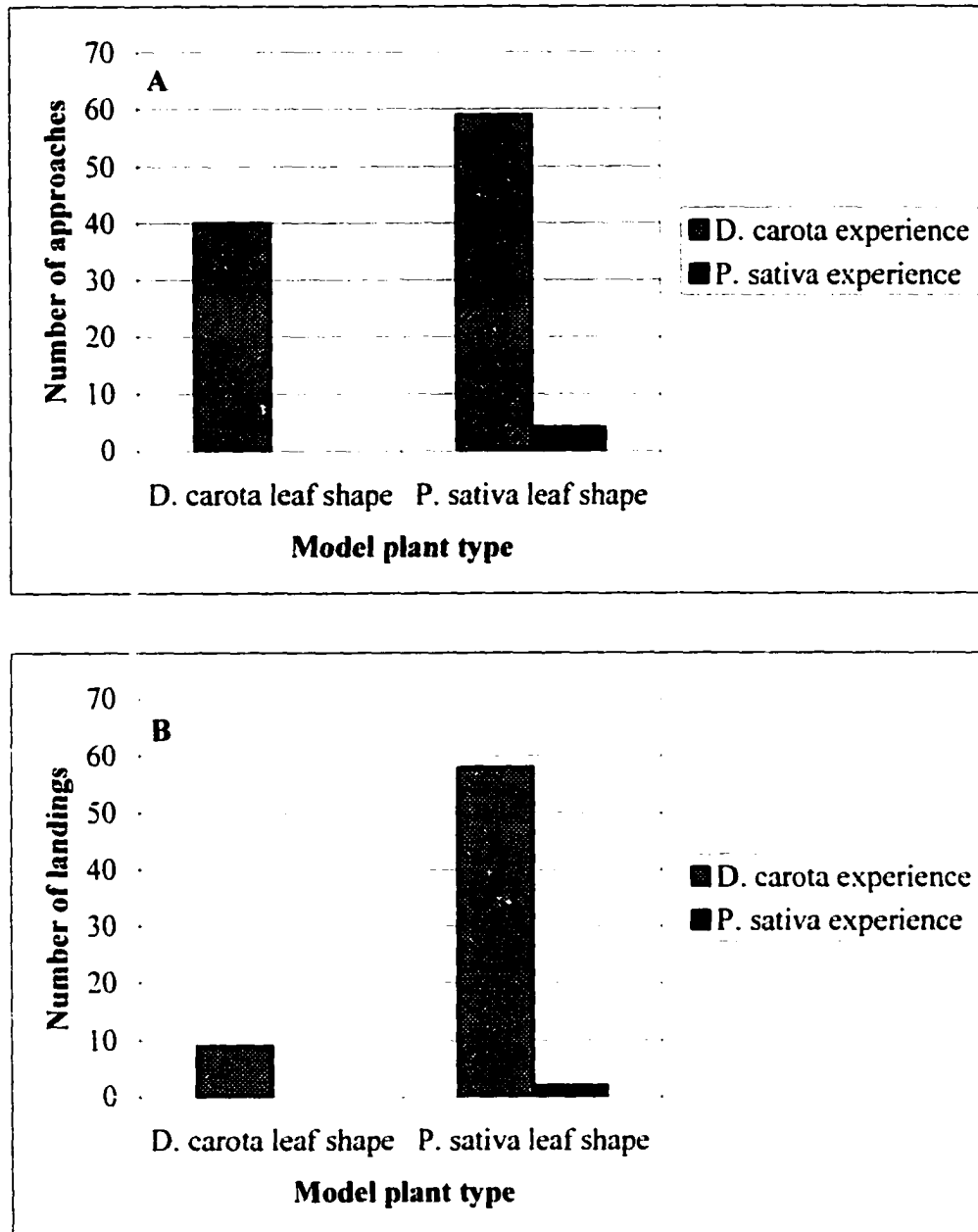


Figure 4.15 Number of approaches (A) and landings (B) by females in experienced trials on model plants with host plant cues (leaf shapes and contact chemicals) with respect to the two host-plant leaf shapes. (No females with *P. sativa* experience approached or landed on a model plant with *D. carota* leaf shape.)

$\chi^2_1=2.642$ ,  $p=0.155$ ; landings:  $N=69$ ,  $\chi^2_1=0.309$ ,  $p=1$ ). There was a significant interaction between host–plant experience and the contact chemical extract of the model plant approached, but this was not in a direction suggesting learning of the contact chemical cues (Figure 4.16A; Fisher’s exact test:  $N=103$ ,  $\chi^2_1=5.58$ ,  $p=0.031$ ). Rather, females were more likely to approach a model plant with the contact chemical extract they had not experienced. This effect was reduced for landings, however (Figure 4.16B; Fisher’s exact test:  $N=69$ ,  $\chi^2_1=2.678$ ,  $p=0.185$ ).

Females did not approach or land on model plants with either host–plant leaf shape more frequently than the other (Figure 4.17A; Sign test: approaches:  $N=6$ ,  $T=3$ ,  $p=0.6563$ ; landings:  $N=5$ ,  $T=4$ ,  $p=0.375$ ). The same was true for contact chemical extracts (Figure 4.17B; Sign test: approaches:  $N=6$ ,  $T=-0.5$ ,  $p=1$ ; landings:  $N=5$ ,  $T=2$ ,  $p=0.625$ ). Females with *D. carota* experience were more likely to approach or land on the model plants with host–plant cues than were females with *P. sativa* experience (Figure 4.18; Median test: approaches:  $N=6$ ,  $\chi^2_1=1.25$ ,  $p=0.2636$ ; landings:  $N=5$ ,  $\chi^2_1=0.667$ ,  $p=0.4142$ ).

#### *Within-trial experience effects*

Fisher’s exact test was used to examine the possibility of within–trial learning. There was a strong relationship between the first model plant with host–plant cues approached and the model plant with host–plant cues most often approached by an individual female during a single trial (Table 4.3; Fisher’s exact test:  $N=14$ ,  $\chi^2_9=26.833$ ,  $p<0.001$ ). All females approached most frequently the model plants with the same leaf shapes as the first leaf shape approached, and only two mostly approached model plants with different contact chemical cues than the first model plant approached. Landings showed the same relationship (Table 4.4; Fisher’s exact

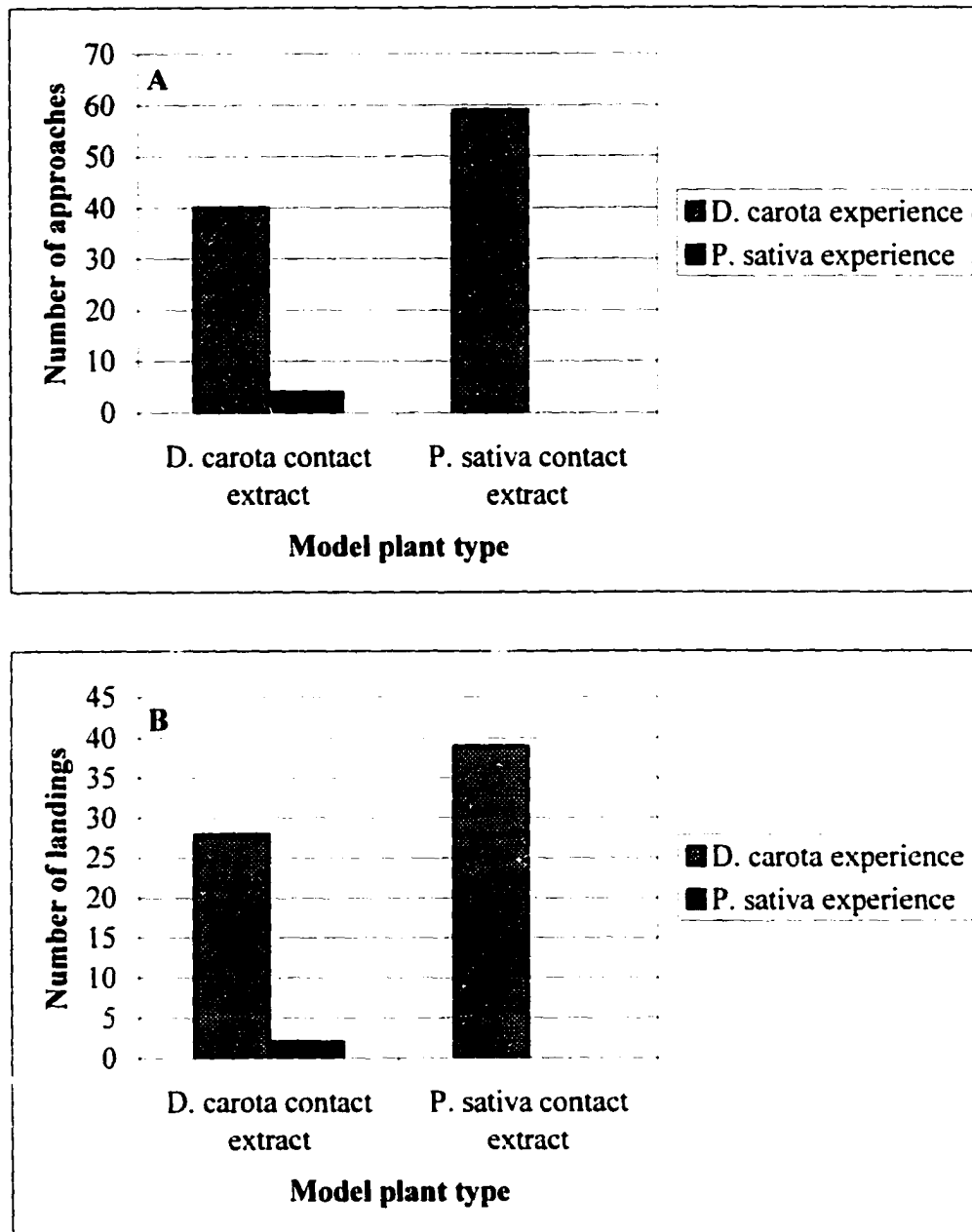


Figure 4.16 Number of approaches (A) and landings (B) by females in experienced trials on model plants with host plant cues (leaf shapes and contact chemicals) with respect to the two host-plant contact extracts. (No females with *P. sativa* experience approached or landed on a model plant with *P. sativa* contact cues.)

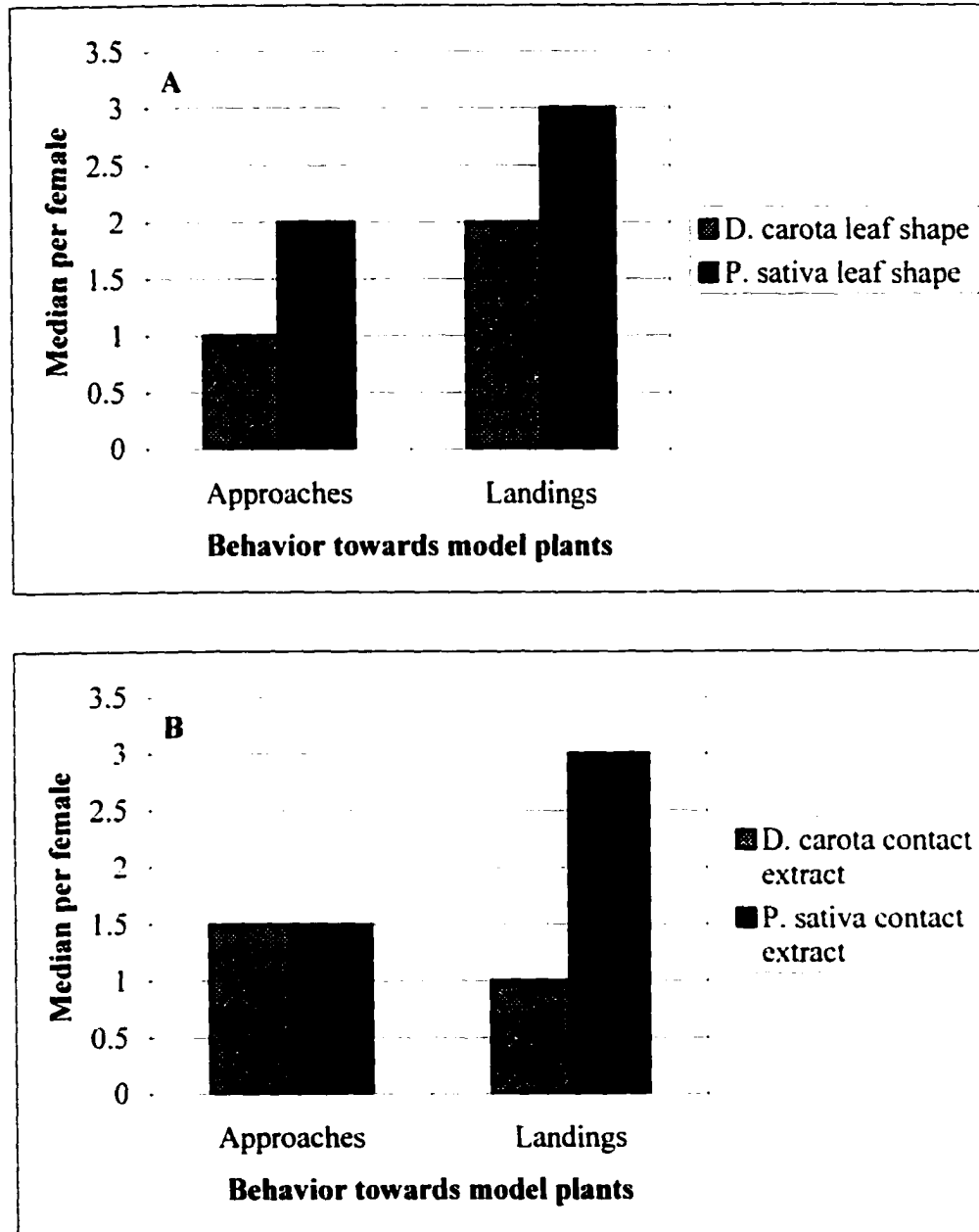


Figure 4.17 Median number of approaches and landings by experienced females on model plants with host-plant cues (leaf shapes and contact extracts). Data for leaf shapes are shown in A; for contact chemical extracts, in B.

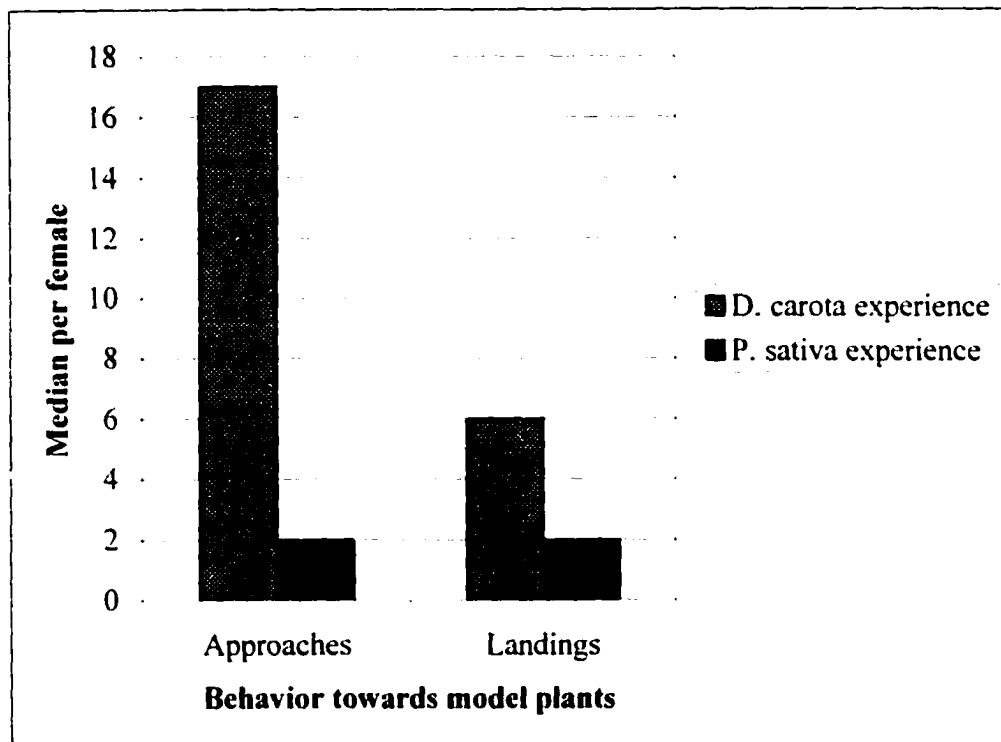


Figure 4.18 Median number of approaches and landings by experienced females on model plants with host-plant cues (leaf shape and contact chemical extracts) with respect to the host plant experienced.

Table 4.3 First model plant approached and model plant most often approached within a trial during Experiment 2. Numbers are number of females for each combination of leaf shape and contact chemical cues.

First approached		Most approached			
		<i>D. carota</i> shape		<i>P. sativa</i> shape	
Leaf shape	Contact	<i>P. sativa</i>	<i>D. carota</i>	<i>D. carota</i>	<i>P. sativa</i>
<i>D. carota</i>	<i>P. sativa</i>	1	1	0	0
	<i>D. carota</i>	0	2	0	0
<i>P. sativa</i>	<i>D. carota</i>	0	0	7	0
	<i>P. sativa</i>	0	0	1	2

test:  $N=10$ ,  $\chi^2_9=22.50$ ,  $p=0.0013$ ), with one female landing most often on the model plant with the same leaf shape but different contact chemical cues. To verify these results, the distribution of female "preference" within a trial was checked in Figure 4.19 and Figure 4.20. For approaches and landings by leaf shape, the distribution of females appears bimodal, with most of the females approaching or landing on model plants of a single leaf shape (Figure 4.19). This was less clear for contact chemical cues. Females were more likely to approach model plants with *D. carota* contact chemical cues rather than model plants with *P. sativa* cues (Figure 4.20A). The distribution for landings is least clear, with an additional peak of females that landed equally on model plants with *D. carota* and *P. sativa* contact chemical cues (Figure 4.20B).



Table 4.4 First model plant landed on and model plant most often landed on within a trial during Experiment 2. Numbers are number of females for each combination of leaf shape and contact chemical cues.

First landed on		Most landed on			
		<i>D. carota</i> shape		<i>P. sativa</i> shape	
Leaf shape	Contact	<i>P. sativa</i>	<i>D. carota</i>	<i>D. carota</i>	<i>P. sativa</i>
<i>D. carota</i>	<i>P. sativa</i>	1	1	0	0
	<i>D. carota</i>	0	1	0	0
<i>P. sativa</i>	<i>D. carota</i>	0	0	5	0
	<i>P. sativa</i>	0	0	0	2

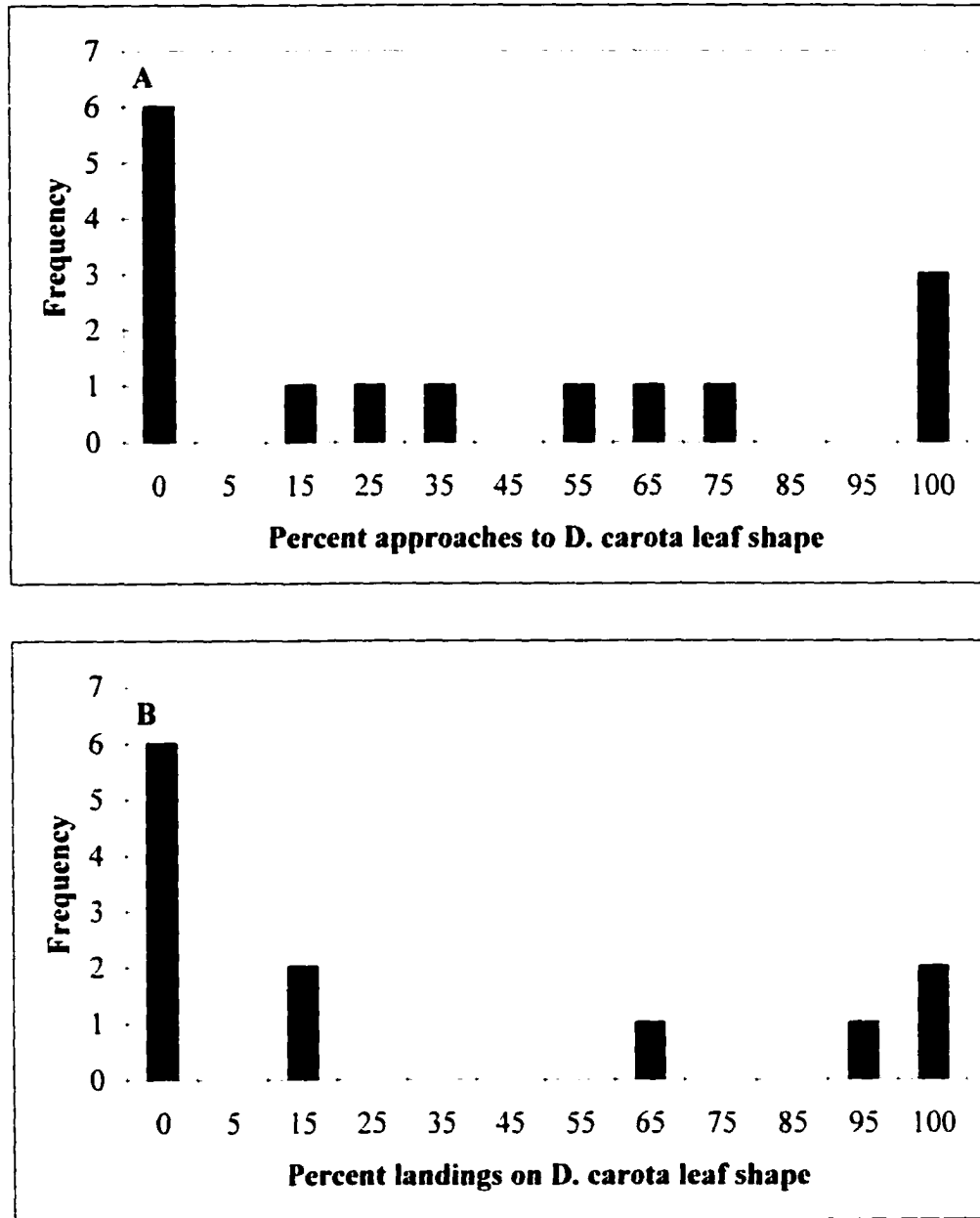


Figure 4.19 Distribution of approaches (A) and landings (B) of individual females within independent trials on model plants with *D. carota* leaf shapes.

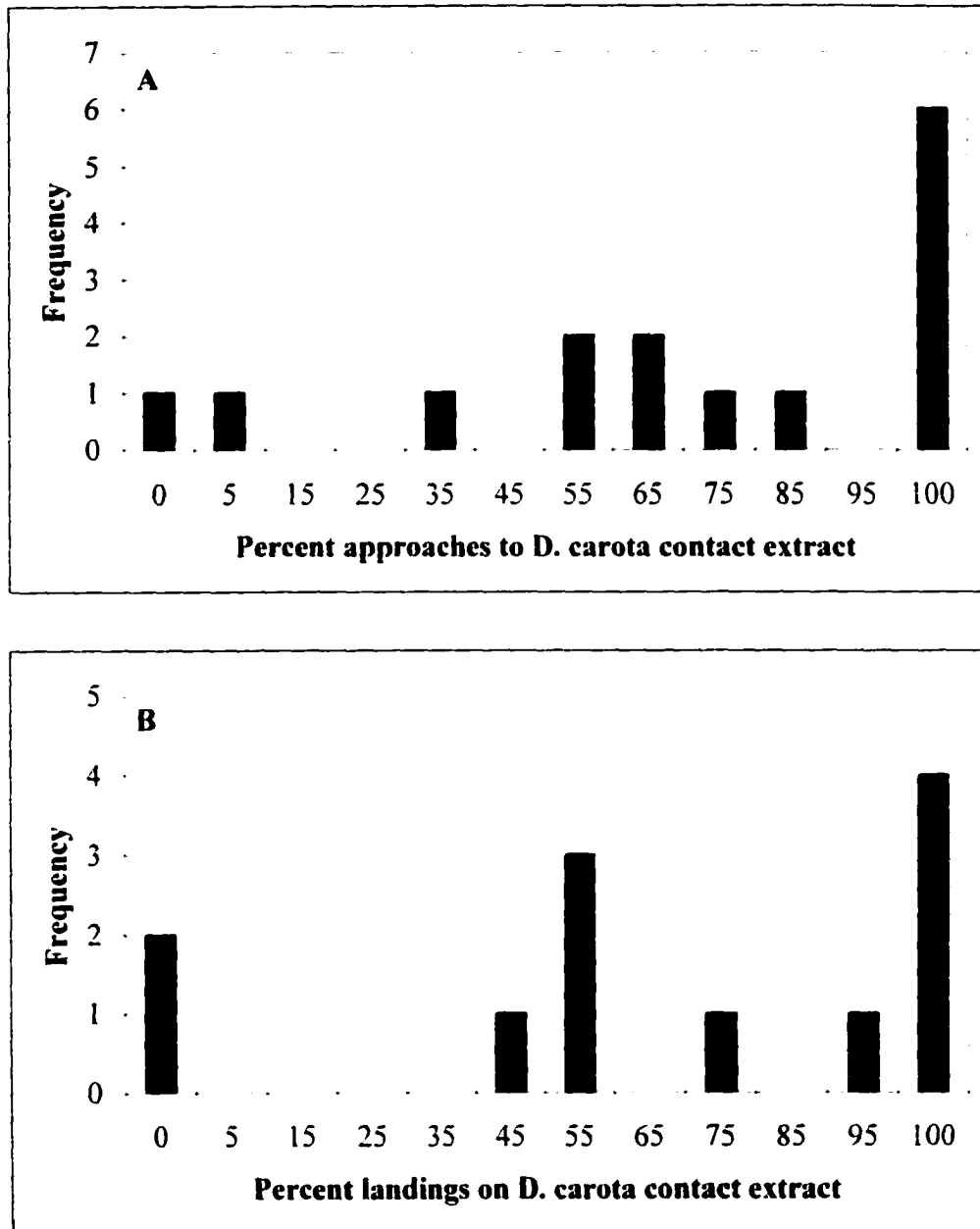


Figure 4.20 Distribution of approaches (A) and landings (B) of individual females within independent trials on model plants with *D. carota* contact chemical extracts.

There was also a significant interaction between experience and leaf shape on the behavior of females after approaching a model plant with host-plant cues (Table 4.5; Mantel-Haenszel test for general association:  $N=93$ ,  $T=11.562$ ,  $p=0.009$ ). Although few females with experience on *P. sativa* exhibited behaviors, the *D. carota*-experienced females were more likely to land on a model plant with *P. sativa* leaf shapes than expected, and less likely to land on model plants with the *D. carota* leaf shape. There did not appear to be an effect of contact chemical cues on this behavior. Once a female had landed on a plant, the situation was somewhat more complex. Again, not many *P. sativa*-experienced females were active during the trials, but females experienced on *D. carota* were significantly more likely to follow a landing on a model plant with *P. sativa* leaf shapes and contact chemical cues with another landing, drumming, or curling, than would be expected (Table 4.6; Mantel-Haenszel test for general association:  $N=60$ ,  $T=13.356$ ,  $p=0.004$ ). These females were less likely than expected to exhibit these behaviors on any other model plant.

#### *Spectral properties of contact chemical extracts on model leaves*

The absorbances of the model leaves treated with *D. carota* and *P. sativa* contact extracts are shown in Figure 4.21. Absorbances were examined to verify whether there were any visual color cues inherent in the extracts, although none were visible to the human eye. There does appear to be a difference in the two host-plant extracts in the ultraviolet range, centered around 425nm.

Table 4.5 Within-trial effects of experience and model plant on post-approach behaviors in leaf shape and contact chemical cues experiment. (“Other” behaviors include all pre-landing behaviors (see Table 4.1).)

Experience	Leaf Shape	Contact Chemicals	Observed		Expected	
			Land	Other	Land	Other
<i>D. carota</i>	<i>D. carota</i>	<i>P. sativa</i>	7	19	12.13	13.87
		<i>D. carota</i>	2	8	4.67	5.33
	<i>P. sativa</i>	<i>D. carota</i>	18	10	13.07	14.93
		<i>P. sativa</i>	15	11	12.13	13.87
<i>P. sativa</i>	<i>D. carota</i>	<i>P. sativa</i>	0	0	0	0
		<i>D. carota</i>	0	0	0	0
	<i>P. sativa</i>	<i>D. carota</i>	2	1	2	1
		<i>P. sativa</i>	0	0	0	0

Table 4.6 Within-trial effects of experience and model plant on post-landing behaviors in leaf shape and contact chemical cues experiment. ("Land +" refers to landing again, drumming, or curling; "other" includes all pre-landing behaviors (see Table 4.1).)

Experience	Leaf Shape	Contact Chemicals	Observed		Expected	
			Land +	Other	Land +	Other
<i>D. carota</i>	<i>D. carota</i>	<i>P. sativa</i>	0	6	2.85	3.15
		<i>D. carota</i>	0	2	0.95	1.05
	<i>P. sativa</i>	<i>D. carota</i>	7	13	9.49	10.51
		<i>P. sativa</i>	21	10	14.72	16.29
<i>P. sativa</i>	<i>D. carota</i>	<i>P. sativa</i>	0	0	0	0
		<i>D. carota</i>	0	0	0	0
	<i>P. sativa</i>	<i>D. carota</i>	0	1	0	1
		<i>P. sativa</i>	0	0	0	0

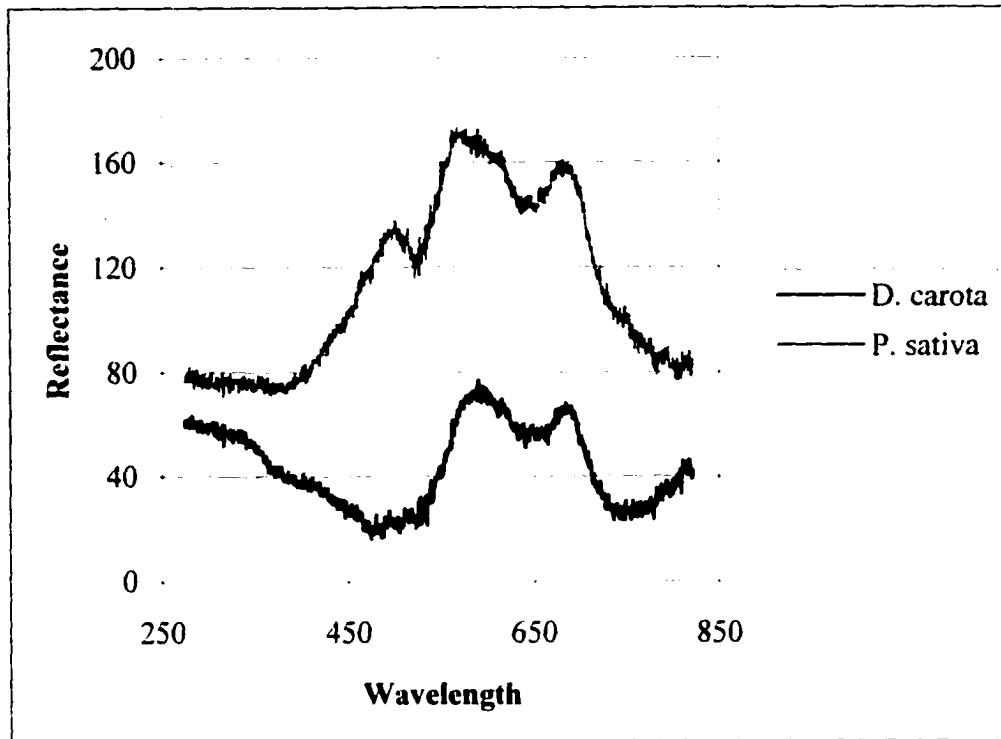


Figure 4.21 Reflectance spectra of *D. carota* and *P. sativa* extracts on model leaf surfaces. Note the strong divergence in the two extracts.

### EXPERIMENT 3: LEAF SHAPES AND VOLATILE CHEMICAL CUES

#### *Specific Methods: Bioassay*

The two host plants utilized in this experiment were *C. maculatum* and *P. sativa*.

Leaves were not treated with contact stimulant extracts, but volatiles were present in vials inserted in the center of the model plants. The model plants were arranged in the array as shown in Figure 4.5. The bioassay followed the general bioassay methods described above. A total of 18 trials was used to test 38 butterflies, with a mean of 8.5 butterflies per trial. No more than 15 butterflies were tested at any one time. Activity

was sporadic, such that it was possible to watch so many butterflies at one time. Trials were started between 12:30 and 16:35 hrs.

## *Results*

### *Responses of naïve females*

Naïve females were slightly but not significantly more likely to approach model plants with non-host (*V. faba*) cues than host-plant (*C. maculatum* or *D. carota*) cues (Figure 4.22; Sign test: N=24, T=5.5, p=0.8463). However, naïve females were significantly more likely to land on model plants with host-plant cues (Figure 4.22; Sign test: N=22, T=73.5, p=0.0042). Naïve females were also significantly more likely to follow an approach to a model host plant with a landing (Figure 4.23A; Fisher's exact test: N=338,  $\chi^2_1=18.023$ , p<0.001), as well as to follow a landing on a model host plant with another landing, drumming, or curling (Figure 4.23B; Fisher's exact test: N=193,  $\chi^2_1=17.655$ , p<0.001).

### *Responses of experienced females*

Host-plant experience did not increase the number of approaches or landings on model plants with the leaf shape of the host plant experienced by a female (Figure 4.24, Figure 4.25; Fisher's exact test: Leaf shape: approaches: N=295,  $\chi^2_1=1.172$ , p=0.284; landings: N=243,  $\chi^2_1=2.191$ , p=0.085; Volatiles: approaches: N=295,  $\chi^2_1=2.191$ , p=0.157; landings: N=240,  $\chi^2_1=4.426$ , p=0.043). Although for both types of cue, the test is significant or nearly-significant for landings, and this trend is in the direction of reducing the number of landings on *C. maculatum*-cued plants by *C. maculatum*-experienced females.



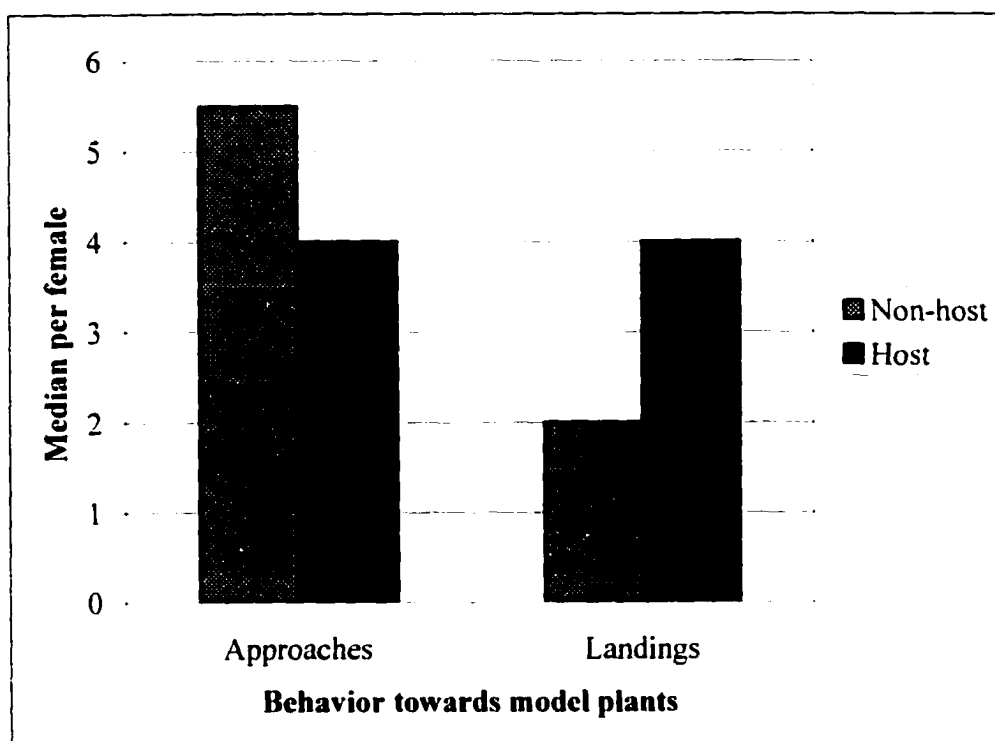


Figure 4.22 Median number of approaches and landings per female in naïve trials (trials 1 and 2) with leaf shapes and volatile chemical cues. Host extracts are *P. sativa* + *C. maculatum*, the non-host is *V. faba*.

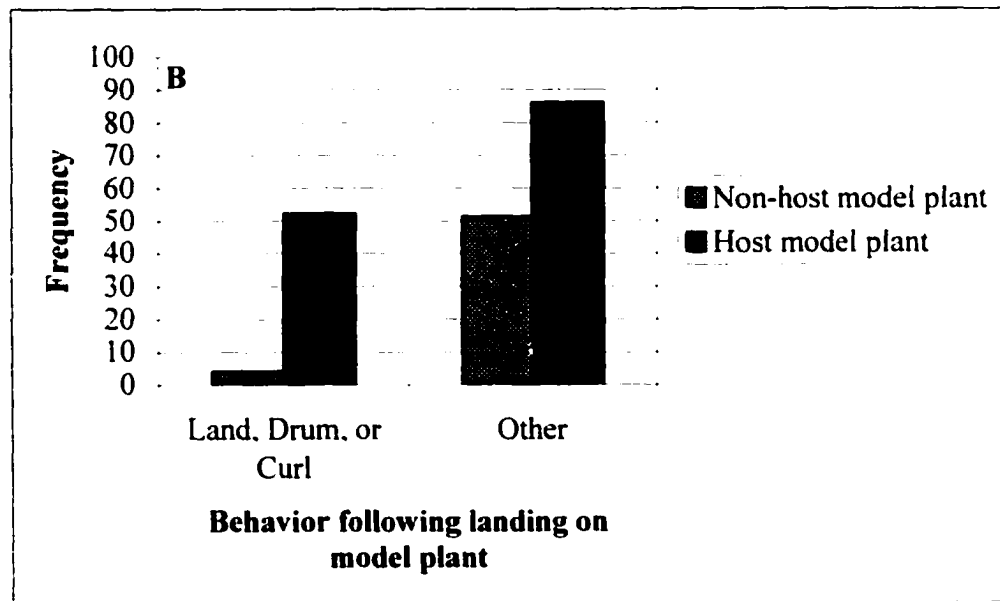
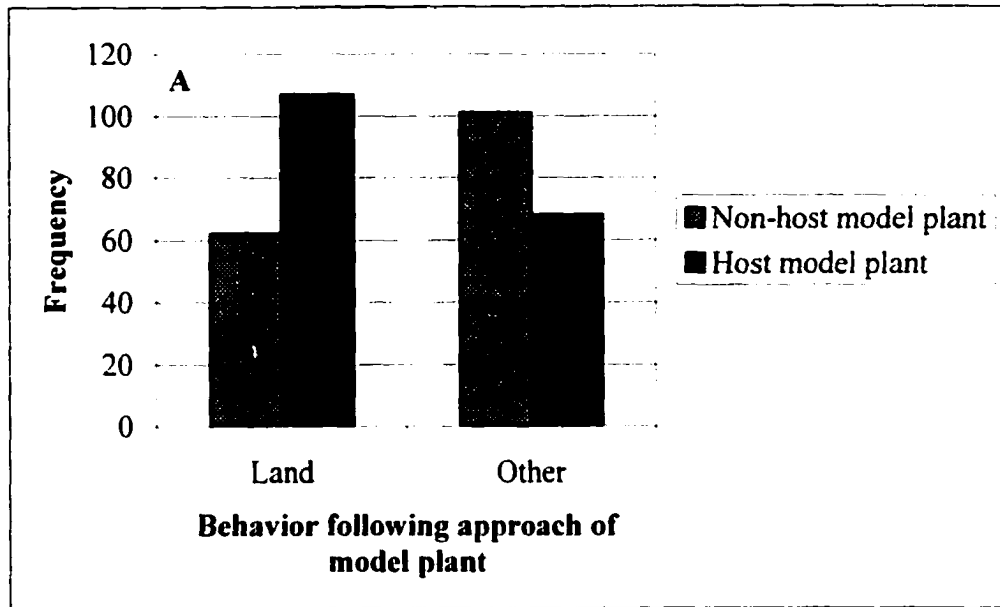


Figure 4.23 Frequency of landings or other behaviors immediately following approaches (A) and landings (B) to model plants with non-host or host-plant cues (leaf shape and volatile chemicals) by naïve females.

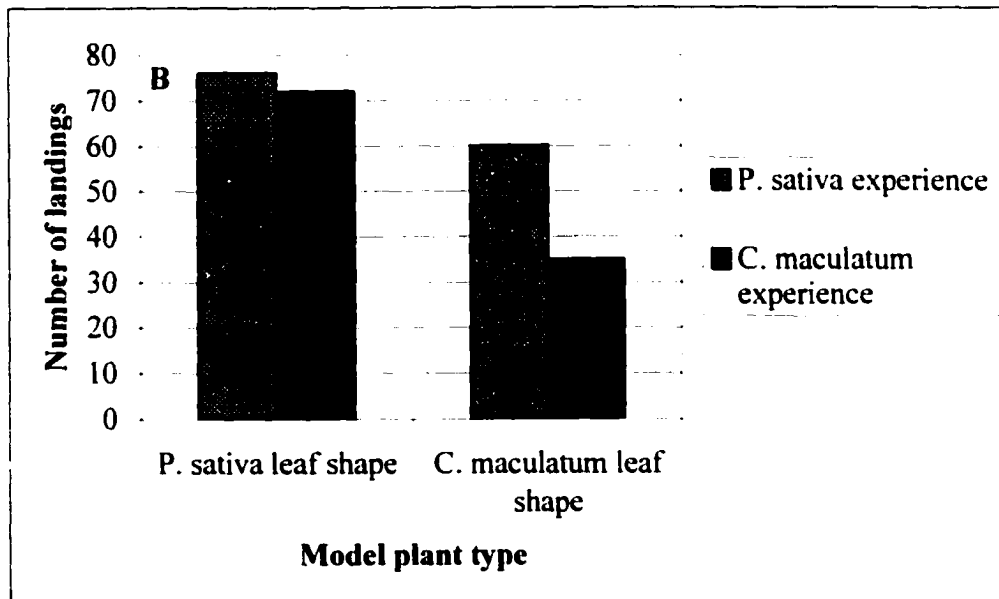
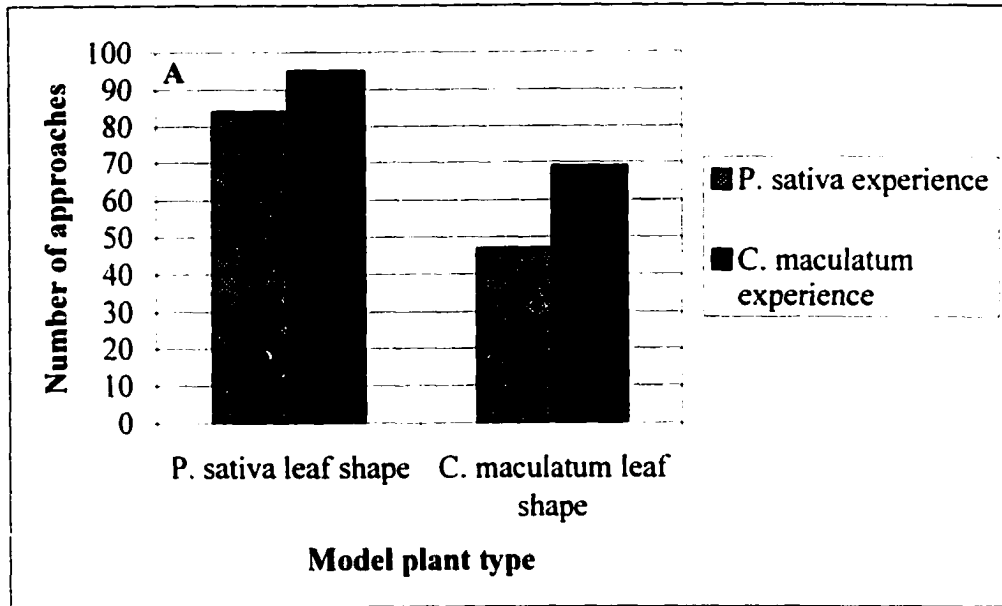


Figure 4.24 Number of approaches (A) and landings (B) by females in experienced trials on model plants with host plant cues (leaf shapes and volatile chemicals) with respect to the two host-plant leaf shapes.

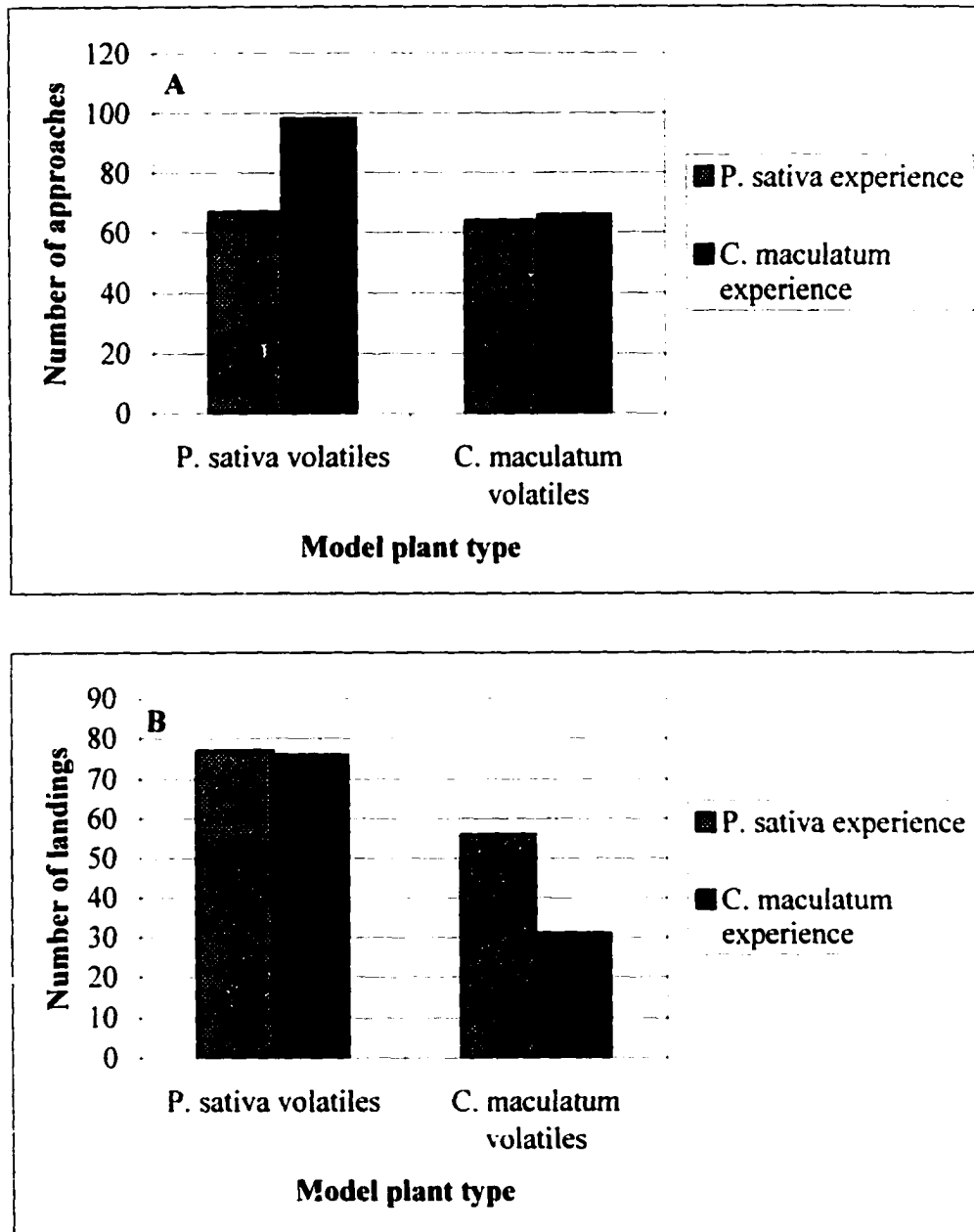


Figure 4.25 Number of approaches (A) and landings (B) by females in experienced trials on model plants with host plant cues (leaf shapes and volatile chemicals) with respect to the two host-plant volatile extracts.

Females did approach and land more frequently on model plants with *P. sativa* than *C. maculatum* leaf shape (Figure 4.26A; Sign test: approaches: N=22, T=-62, p=0.0396; landings: N=22, T=-50.5, p=0.0762). There were no significant differences for volatile chemical cues (Figure 4.26B; Sign test: approaches: N=22, T=12.5, p=0.6933; landings: N=22, T=-21.5, p=0.4345). There was no significant effect of the host-plant experienced on the behaviors towards host-plant cues overall (Figure 4.27; Median test: approaches: N=22,  $\chi^2_1=0.75$ , p=0.3865; landings: N=22,  $\chi^2_1=0$ , p=1).

#### *Within-trial experience effects*

Fisher's exact test was used to look at the effect of within-trial experience. There was a strong relationship between the host-plant cues of the first model plant approached or landed on and the host-plant cues most frequently approached or landed on. Females first approaching or landing on a model plant with *P. sativa* leaf shapes were most likely to approach or land most often on model plants with *P. sativa* leaf shapes, with the same being true for females first approaching or landing on a model plant with *C. maculatum* leaf shapes (Table 4.7; Fisher's exact test: approaches: N=55,  $\chi^2_1=30.583$ , p<0.001; landings: N=44,  $\chi^2_1=44$ , p<0.001). The same was also true for volatile chemical cues (Table 4.8; Fisher's exact test: approaches: N=51,  $\chi^2_1=29.973$ , p<0.001; landings: N=43,  $\chi^2_1=25.868$ , p<0.001). To verify these results, the distribution of female "preference" within a trial was checked in Figure 4.28 and Figure 4.29. In all cases, the distribution tends to be bimodal, with the peaks at 100% of the behaviors on *P. sativa* (100% on graphs) or *C. maculatum* (0% on graphs) cues.

There was no significant interaction between experience and host-plant cues on the behavior of females after approaching a model plant with host-plant cues (Table 4.9; Mantel-Haenszel test for general association: N=284, T=1.977, p=0.577).

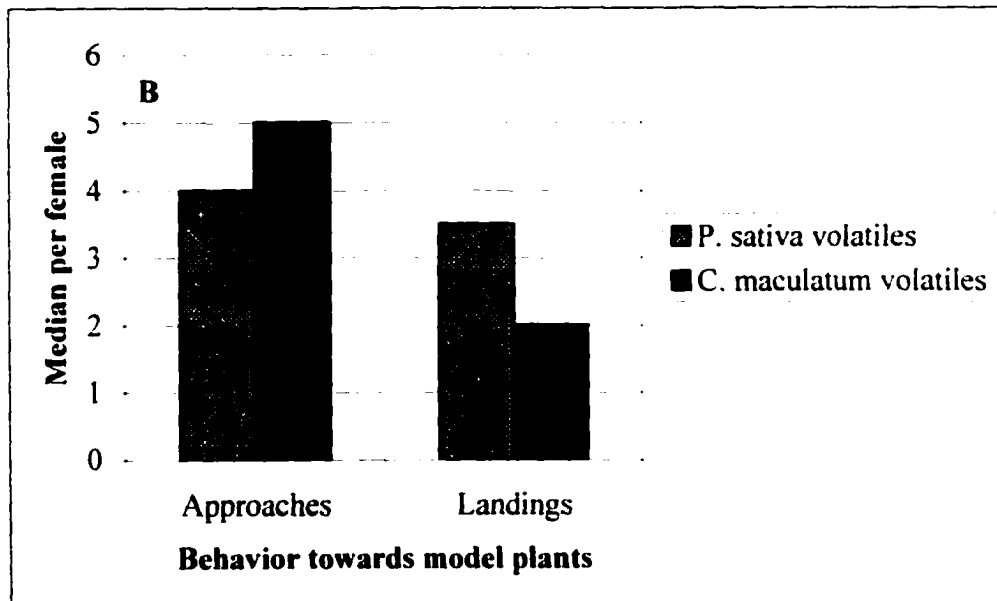
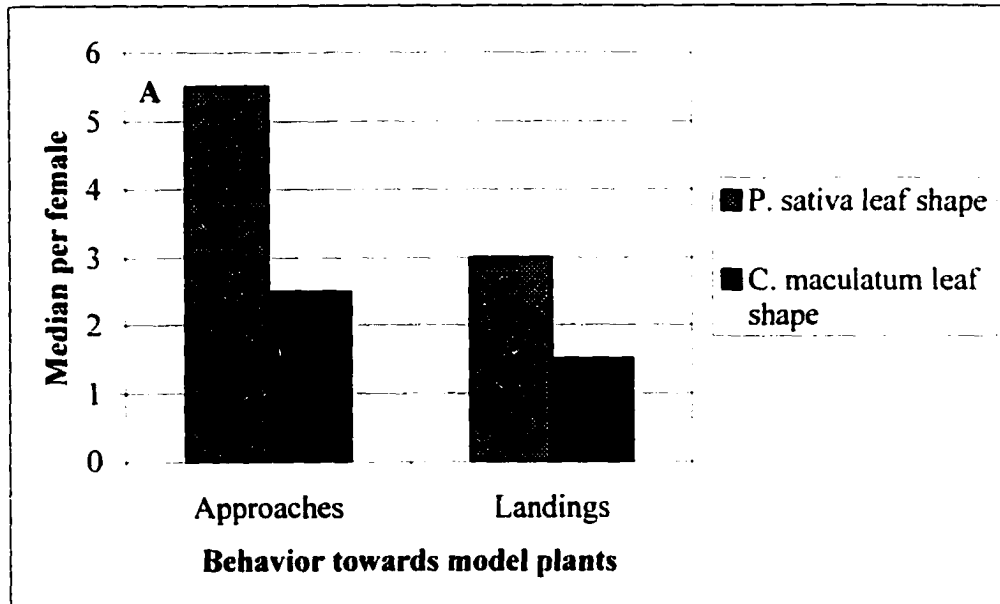


Figure 4.26 Median number of approaches and landings by experienced females on model plants with host-plant cues (leaf shapes and volatile extracts). Data for leaf shapes are shown in A; for volatile chemical extracts, in B.

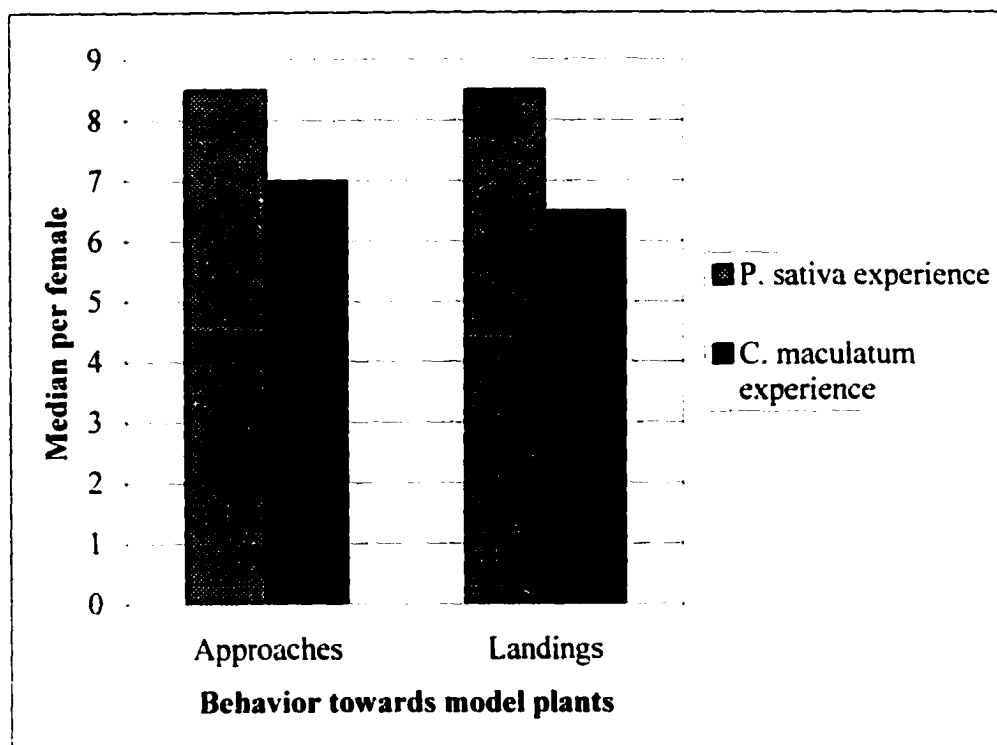


Figure 4.27 Median number of approaches and landings by experienced females on model plants with host-plant cues (leaf shape and volatile chemical extracts) with respect to the host plant experienced.

Table 4.7 First model plant approached and model plant most often approached within a trial during Experiment 3. Numbers are number of females for each combination of leaf shape and volatile chemical cues.

First approached		Most approached			
		<i>P. sativa</i> shape		<i>C. maculatum</i> shape	
Leaf shape	Volatiles	<i>C. maculatum</i>	<i>P. sativa</i>	<i>P. sativa</i>	<i>C. maculatum</i>
<i>P. sativa</i>	<i>C. maculatum</i>	11	1	1	0
	<i>P. sativa</i>	1	11	2	0
<i>C. maculatum</i>	<i>P. sativa</i>	0	0	9	0
	<i>C. maculatum</i>	0	2	1	9

Table 4.8 First model plant landed on and model plant most often landed on within a trial during Experiment 3. Numbers are number of females for each combination of leaf shape and volatile chemical cues.

First landed on		Most landed on			
		<i>P. sativa</i> shape		<i>C. maculatum</i> shape	
Leaf shape	Volatiles	<i>C. maculatum</i>	<i>P. sativa</i>	<i>P. sativa</i>	<i>C. maculatum</i>
<i>P. sativa</i>	<i>C. maculatum</i>	9	1	1	0
	<i>P. sativa</i>	1	12	0	0
<i>C. maculatum</i>	<i>P. sativa</i>	0	0	8	0
	<i>C. maculatum</i>	0	0	2	7



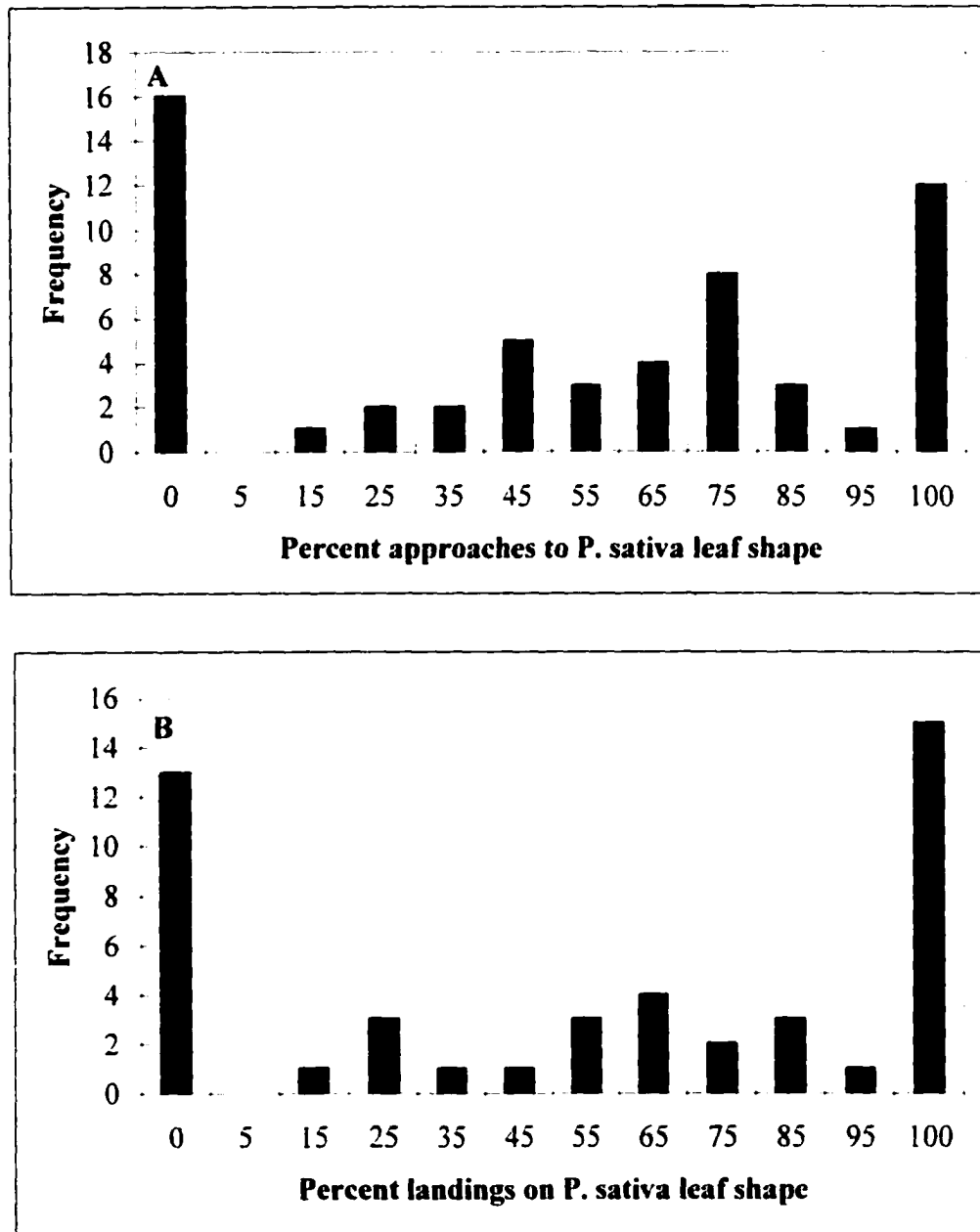


Figure 4.28 Distribution of approaches (A) and landings (B) of individual females on model plants with host-plant leaf shapes during independent trials.

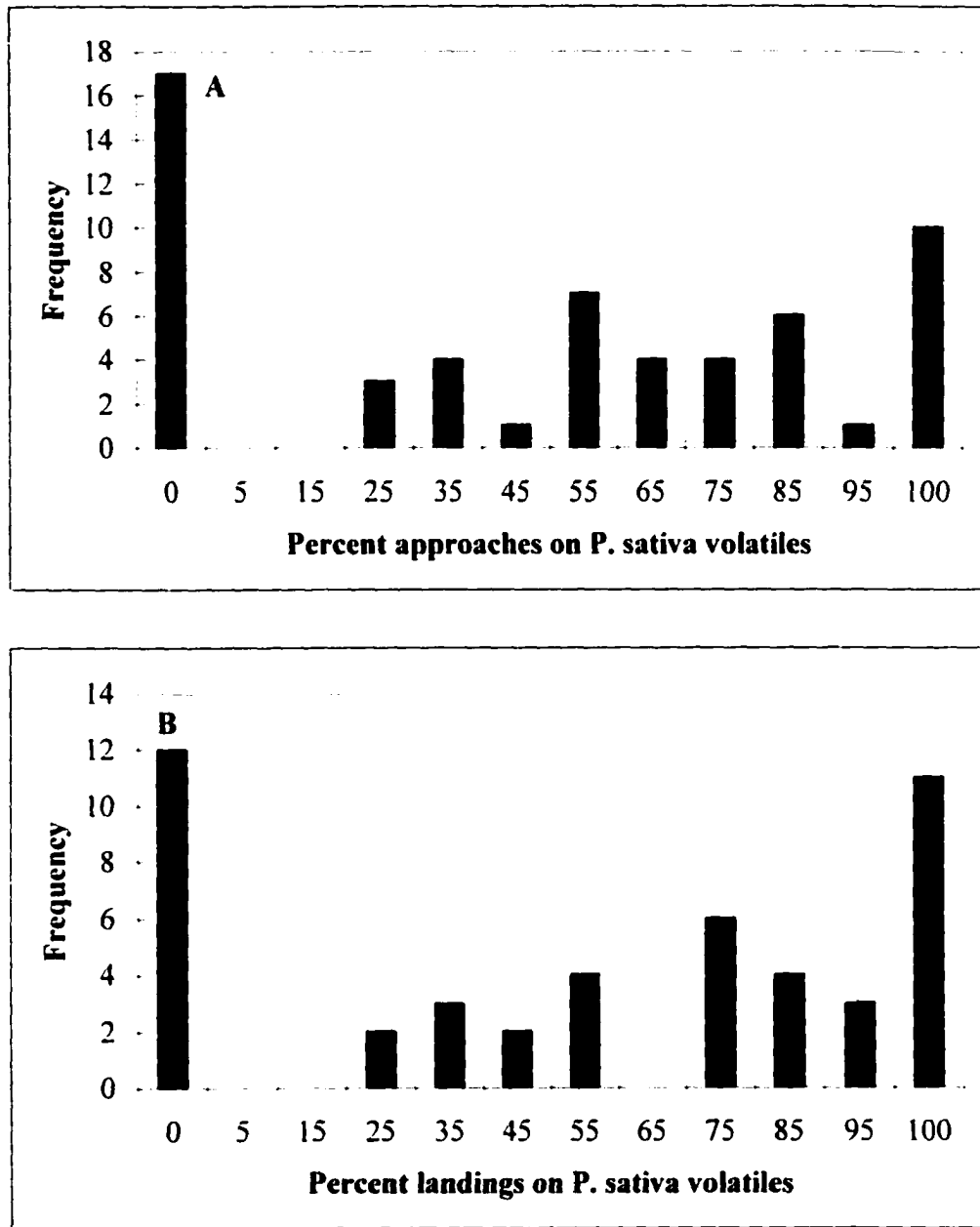


Figure 4.29 Distribution of approaches (A) and landings (B) of individual females on model plants with host-plant volatiles during independent trials.

Table 4.9 Within-trial effects of experience and model plant on post-approach behaviors in leaf shape and volatile chemical cues experiment. (See Table 4.1 for explanation of "Other" behaviors.)

Experience	Leaf Shape	Volatile Chemicals	Observed		Expected	
			Land	Other	Land	Other
<i>P. sativa</i>	<i>P. sativa</i>	<i>C. maculatum</i>	20	13	19.64	13.36
		<i>P. sativa</i>	27	19	27.38	18.62
	<i>C. maculatum</i>	<i>P. sativa</i>	11	8	11.31	18.62
<i>C. maculatum</i>	<i>P. sativa</i>	<i>C. maculatum</i>	17	11	16.67	11.33
		<i>P. sativa</i>	16	23	19.25	19.75
	<i>C. maculatum</i>	<i>P. sativa</i>	33	22	27.15	27.85
	<i>C. maculatum</i>	<i>P. sativa</i>	18	22	19.75	20.25
		<i>C. maculatum</i>	11	13	11.85	12.15

However, there was a slightly significant interaction for behaviors following a landing (Table 4.10; Mantel–Haenszel test for general association:  $N=220$ ,  $T=7.906$ ,  $p=0.048$ ). Females experienced on *P. sativa* were more likely to land, drum, or curl following a landing on a model plant with *P. sativa* volatiles, while females experienced on *C. maculatum* were more likely to land, drum, or curl following a landing on a model plant with *P. sativa* leaf shapes and volatiles.

## DISCUSSION

*P. polyxenes* females are able to use leaf shape cues for host–finding, although this result was more detectable when other cues were also available. Naïve females did not distinguish at all between host and non–host leaf shapes when there were no other cues present (Figure 4.6), but landed more often on model host plants when either contact or volatile chemical cues were present (Figure 4.13 and Figure 4.22). Once a model plant was approached, females were more likely to land (than continue with pre–landing behaviors) if host–plant cues were present, regardless of which cues were present (Figure 4.7, Figure 4.14A, and Figure 4.23A). Additionally, in Experiments 2 and 3, there were sufficient landings to examine the post–landing behavior as well. Here again, females were more likely to engage in landing, drumming, or curling after landing on a model plant with host–plant cues than one with non–host cues (Figure 4.14B and Figure 4.23B).

Although the different types of cues were not differentiated for naïve females, these results would suggest that the different leaf shapes were not recognized as “host” or “non–host” by naïve females. In fact, the results with experienced females also suggest that the females did not recognize the leaf shapes as being representative of the three host species tested. The detailing of the leaf shapes was constrained by the

Table 4.10 Within-trial effects of experience and model plant on post-landing behaviors in leaf shape and volatile chemical cues experiment. ("Land+" refers to landing again, drumming, or curling, while "other" includes all pre-landing behaviors (see Table 4.1).)

Experience	Leaf Shape	Volatile Chemicals	Observed		Expected	
			Land+	Other	Land+	Other
<i>P. sativa</i>	<i>P. sativa</i>	<i>C. maculatum</i>	8	13	12.87	8.13
		<i>P. sativa</i>	32	17	30.03	18.97
	<i>C. maculatum</i>	<i>P. sativa</i>	18	6	14.71	9.29
		<i>C. maculatum</i>	18	12	18.39	11.61
<i>C. maculatum</i>	<i>P. sativa</i>	<i>C. maculatum</i>	5	9	6.13	7.88
		<i>P. sativa</i>	28	22	21.86	28.13
	<i>C. maculatum</i>	<i>P. sativa</i>	7	15	9.63	12.38
		<i>C. maculatum</i>	2	8	4.38	5.63

time it took to make them, as many leaves were needed when contact chemical cues were being applied. Thus, it is not possible to determine whether females were learning leaf shapes during their host–plant experience. However, the within–trial tests for each experiment are an appropriate test for whether females were learning leaf shapes during the course of a trial, and these results do show learning when other host–plant cues are present.

Even with no relevant chemical cues present, in Experiment 1, females approached most often the model plants with the same leaf shape as the first model plant approached (Figure 4.11A). In Experiments 2 and 3 there were dramatic effects of the first model plant approached or landed on (Tables 4.3, 4.4, 4.8, and 4.9). In all cases, nearly all females approached or landed mostly on the same type of plant (all cues considered) as the very first model plant approached or landed on. The sample size was relatively small for Experiment 2, but the larger sample size of Experiment 3 upheld the pattern. This provides evidence for the ability of *P. polyxenes* females to learn all three types of cues, leaf shape, contact chemical, and volatile chemical, in the context of these trials. Although these results could also be explained by an ability of females to learn the position of an acceptable model plant during a trial, as plants were not rotated within a trial, there are two faults with this explanation. First, positional effects alone cannot explain these effects, as different females in the same trial approached and landed on different plants. Second, in Experiment 1 there were two of each type of model plant, so that any potential for positional effects would be reduced. Thus, the females are at least able to learn leaf shapes within a trial. Allowing this in Experiments 2 and 3 provides further support for the ability of females to learn all three cues.

A closer look at the females with discordant responses in the latter two experiments gives only some insight into the relative roles of visual and chemical cues (Tables 4.3, 4.4, 4.7, and 4.8). In Experiment 2, with the contact chemical cues, two (of fourteen) females approached mostly model plants matching the leaf shape but not the contact chemical cues of the first model plant approached. One female (of ten) landed mostly on a model plant matching the leaf shape but not the contact chemical cues of the first model plant landed on. Together, these suggest that these females may place a greater emphasis on leaf shape than contact chemical cues when deciding to approach or land on a model plant, though the evidence more strongly suggests that for most females, both cues are in use at equal strength. In Experiment 3, with leaf shapes and volatile chemical cues, eight (of 48) females most often approached a model plant other than the first model plant approached. Of these, three approached the model plant with the same leaf shape (but different volatiles), two approached the model plant with the same volatiles (but not leaf shapes), and three approached model plants with different leaf shape and volatiles. For landings, five (of 41) females landed mostly on a model plant that varied from the first model plant landed on. Four of these landed on model plants with the same leaf shape (but different volatiles), and one landed mostly on the model plant with different leaf shape and volatiles. These results suggest that the leaf shape cue may be more important for landing for a minority of females, though most females weigh leaf shape and volatiles equally.

One of the more interesting implications of these results is the apparent ability of a female to sense the contact chemical extract on the model leaf surface prior to landing on the leaf. This must be the case to explain the females' fidelity in approaches and landings on model plants with the same leaf shapes and contact chemicals in Experiment 2. As contact chemoreceptors must contact the surface, and

volatiles cues are unlikely to come from these extracts, the reflectance of the model leaf surfaces were examined, and are shown in Figure 4.21. There is no solid evidence to support the idea that females could distinguish the contact chemicals on the model leaf surface using visual cues, but the differences in reflectance may allow this possibility. Arikawa et al. (1987) have identified five color receptor types in the closely related *P. xuthus*. These receptors have peaks at 360, 400, 460, 520, and 600nm (Arikawa, et al. 1987). If the butterflies are responding to this difference in reflectance between the two host-plant extracts, it may be that the learning seen in the context of contact chemical cues is actually a result of learning of the visual cue associated with difference in colors seen by the butterfly. Other than repeating the second experiment with more females, despite the low response rates, another possibility for explaining this result lies in further examining the chemistry that may explain the difference in reflectance over this range. Three compounds have already been identified as contact chemical stimulants, two from *D. carota* (luteolin-7-O-(6''-O-malonyl)- $\beta$ -D-glucoside and *trans*-chlorogenic acid) and one from *P. sativa* (tyramine) (Feeny, et al. 1988; Carter, et al. 1998). Efforts to identify the remaining compounds responsible for the positive response to contact chemical extracts are continuing, to explore the ability of these females to see differences in the extracts (M. Haribal and P. Feeny, personal communication).

Ovipositing butterflies differ from other phytophagous insects in preferring green shades for oviposition behavior (e.g., Scherer and Kolb 1987), as opposed to yellow, which appears as a supernormal stimulus (Prokopy and Owens 1983). Kelber (1999) has shown in *P. aegeus* that this is due to the negative inputs of the blue and red receptors, with special attention paid to the red receptor, which is lacking in many other insects. Although Kelber also suggests that the ultraviolet and violet receptors



have negative inputs, this may vary across species within the Papilionidae. Haribal (unpublished results) has found seasonal variation within *Asimina triloba*, a host plant of the zebra swallowtail butterfly, *Eurytides marcellus*, for a deterrent flavonoid with a high absorbance within the ultraviolet. In contrast, she has found variation across several host plant species of *Papilio polyxenes* for stimulant flavonoids, including the flavonoid glycoside luteolin-7-O-(6''-O-malonyl)- $\beta$ -D-glucoside. The implications of this would be that *E. marcellus* may actually be deterred by high ultraviolet absorbance in host leaves, while *P. polyxenes* may be attracted to high ultraviolet absorbing leaves. Or, as suggested in the current experiments, *P. polyxenes* may be able to associate the contact chemistry of the leaf surface with visual cues in the low end of their visual spectrum, thereby giving perhaps more importance to the role of the ultraviolet receptor than suggested by Kelber (1999).

The results demonstrating the learning of volatile chemical cues by *P. polyxenes* in Experiment 3 (Tables 4.7 and 4.8) are surprising given the clear results of the experiments in Chapter 3 showing no learning of volatile chemical cues. However, the results in Experiment 3 do not demonstrate an ability for the females to learn the volatile chemical cues independently of the visual leaf shape cues. In fact, they are tightly linked, with most females showing fidelity to a particular pair of cues. This demonstrates not only the importance of examining each cue independently, but also the combination of cues, as the results are dependent on the cues being tested. It is of note that there was no apparent effect of the host plant experienced, which suggests that either the hexane extract is not a good representation of the headspace volatiles actually released by the plants (e.g., Heath and Manukian 1992), or that the association of a visual cue, such as leaf shape, with the volatile chemistry is required.

An alternate explanation for the apparent inability for *P. polyxenes* to learn the volatiles of the host plant experienced could result from the inability of a female to approach the host plant in a natural fashion within the small cages used for host-plant experience. If volatiles are learned by associative learning, it may be necessary for a volatile cue to be presented at some time prior to landing on the plant. For example, honey bees can learn odor cues during nectar foraging. Prior to the initiation of feeding, bees are receptive to learning odors that can then be used to predict the location of the next nectar source (reviewed in Menzel 1990). If *P. polyxenes* similarly requires an odor to be presented during natural flight and prior to landing, the free-flight arena may have been more suited to allowing for odor learning than the smaller cages used for host-plant experience.

There are few examples of the ability of moths to use visual cues, in part because of the large number of nocturnal species, where visual cues are less likely to be of use (Ramaswamy 1988). However, the ability of butterflies to learn leaf shapes and colors has been documented in several systems. Colors, in particular, have been shown to be learned in association with nectar finding or feeding by *Heliconius charitonius* (Nymphalidae) (Swihart and Swihart 1970; Swihart 1971), by *Pieris napi* (Pieridae) (Goulson and Cory 1993), by *Agraulis vanillae* (Nymphalidae) (Weiss 1995), and the pipevine swallowtail, *Battus philenor* (Papilionidae) (Weiss 1997). Learning of color in association with oviposition behavior has been best studied by Traynier in *Pieris rapae* (Pieridae). Females of this species are able to learn to associate several, though not all, colors with sinigrin, an oviposition stimulant (Traynier 1986). The congener *P. brassicae* can also learn to associate sinigrin with either dark or light green disks (van Loon, et al. 1992). No reported studies have included ultraviolet cues for oviposition studies.

In the two cases in which butterflies have been demonstrated to be able to learn leaf shapes, the butterflies have also been implicated in the evolutionary divergence of the shapes themselves. The first case is that of *Heliconius* butterflies and their *Passiflora* host plants in the New World tropics. Several species of *Passiflora* have egg mimics on the leaves, matching the color and location of *Heliconius* eggs almost perfectly, supplying circumstantial evidence for the ability of the butterflies to exert selection pressure on these hosts (Gilbert 1975). The case for leaf shape is not as strong, but there are several locations where the diversity of leaf shapes is striking. A far stronger case is that of *Battus philenor* in eastern Texas (Rausher 1978). Females of *B. philenor* are specialists on the genus *Aristolochia*, two species of which are represented in this area: the narrow-leafed *A. serpentaria* and the broad-leafed *A. reticulata*. Searching females can be observed to land preferentially on narrow- or broad-leafed plants in the habitats where the host species are found, and Rausher and later Papaj have very nicely demonstrated that this is due to the learning of leaf-shape cues in association with the contact chemistry of the host plant (Papaj 1986; Rausher 1995). In this manner, females are able to track seasonal changes in their host plants, such as the toughening of the leaves of *A. reticulata* by the time of the second flight of *B. philenor* (Rausher 1980). There is evidence for the butterfly being able to exert strong selection pressure on *A. reticulata* in the field (Rausher and Feeny 1980), and the narrow-leafed morph of *A. serpentaria* is restricted to areas in which it co-occurs with the broad-leafed *A. reticulata*. Thus, it seems highly likely that the butterfly is able to maintain the separation in leaf shapes between its two major host plants in this location.

It seems highly unlikely that *Papilio polyxenes* could have selected for the variance in leaf shapes in the Apiaceae of central New York, particularly as its major

contemporary host plants are all introduced species (Scriber and Feeny 1979). Nonetheless, an individual female that is able to make use of leaf shapes to find her hosts could be at an advantage compared to females that cannot. *P. polyxenes* females have already been shown to have strong responses to contact and volatile chemical cues prior to adult plant experience (Chapters 2 and 3). It is not clear whether females have an innate response to the actual leaf shapes of their host plants, but they are clearly able to learn to associate leaf shapes with the chemical cues. Whether this would translate to an increase in fitness in the field remains to be demonstrated, but it could potentially lead to an increase in efficiency. As this butterfly is limited by time in which to lay enough eggs to replace herself in the next generation (Feeny, et al. 1985), an increase in efficiency is likely to lead to an increase in the number of eggs laid, and thus an increase in the fitness of the individual.

The use of multiple cues in the context of host-finding by phytophagous insects has been examined in only a few systems, mainly within the Diptera. In one of the best-studied systems, Prokopy and colleagues have shown that *Rhagoletis pomonella*, the apple maggot fly, uses both olfactory and visual stimuli to locate fruiting host trees (see overview in Prokopy 1986). From a distance, a combination of an attractive blend of volatiles and a supernormal tree shape (a large yellow rectangle) are more attractive together than either stimulus alone. When females search for fruits to oviposit in, after finding a tree, visual cues alone are used if the fruit is abundant, with volatile cues used only if fruit is sparse. Similarly, *Delia radicum*, the cabbage root fly, uses volatile cues alone at a distance, but at close range, cue use is dependent on the density of the plants. If plants are closely spaced, visual cues alone are used. If plants are farther apart, visual and volatile cues together are used to find the plants (Prokopy 1986). There is also a synergistic effect at close range between visual stimuli

and contact chemical cues, with the presence of both the chemical cues and a model stem leading to a synergism in the response rate of female flies, although conspicuousness of the hosts is the most important cue at a distance (Kostál 1993). One of the best examples of the importance of multiple cues for host finding is that of *Delia antiqua*, the onion fly. A “stem” increases egg-laying substantially — more eggs are laid on an onion stem without a bulb than on a submerged onion bulb without a stem (Harris and Miller 1982). If onions are submerged, a glass rod is an attractive surrogate stem, and the addition of a yellow color to the glass rod is even more attractive. Clearly, multiple host stimuli are either required at different stages or are required in tandem to allow the release of oviposition behavior.

Within the *Lepidoptera*, examples of the use of visual and chemical cues simultaneously are harder to find. *Mamestra brassicae* (Noctuidae), the cabbage moth, will orient to odor, but is more likely to land when an artificial leaf is given as a visual cue (Rojas and Wyatt 1999). *Pieris rapae* (Pieridae) uses color to find hosts plants from a distance, and contact chemical cues are important after landing, though a role for volatile cues is unclear (Hern. et al. 1996). *Papilio demoleus* (Papilionidae) is attracted by the color of its host plants, and oviposition behaviors will occur when moisture and host odors are also included (Saxena and Goyal 1978). In general, visual and olfactory cues appear to be important for behaviors leading to the host plant, with contact chemical cues then taking over in importance (Renwick and Chew 1994).

The role of learning in the context of multiple cues has not received much attention within the phytophagous insects. Most of this research regarding insects has taken place with parasitoids or honey bees. Larval parasitoids, in general, are expected to be able to learn any predictable environmental cues, with particular emphasis placed

on cues from the host-plants of the larvae attacked (Vet, et al. 1995). For example, *Microplitis croceipes* (Hymenoptera: Braconidae) is a larval parasitoid on *Helicoverpa zea*. Female wasps can be trained to odor cues from the frass of caterpillars fed on specific plant parts, to colored targets, or both simultaneously. Odor cues are better learned than visual cues, though the interaction between odor and visual cues is additive, with the highest rates of learning in those wasps trained on both cues (Wäckers and Lewis 1994).

Not surprisingly, it is in the literature of the honey bee that the example most relevant to the results seen in the current experiment is found. Honey bees can be trained in the contexts of nectar foraging or hive location to both visual and odor cues with relative ease (Menzel 1990). Either of these can be paired with time of day as well, and combinations of any of the three can result in rather strong linkages of the cues, with performance seriously degraded if one or more cue is missing (Bogdany 1978). Color and scent form a particularly strong pair, as do color and time, while time and scent form a less strong pair. These results are strikingly similar to those found in the current Experiments 2 and 3, where a strong linkage between chemical cues and visual cues was apparent in the learning behavior of *P. polyxenes*. One major difference is the apparent inability of *P. polyxenes* to learn odor cues in the absence of visual cues (Chapter 3). This may be a difference in the host plants tested, as the earlier study included *D. carota* and *C. maculatum* volatiles, with the latter being far more attractive to searching females, while the current study included the approximately equally attractive volatiles of *P. sativa* and *C. maculatum* (Figure 4.26). It is possible that the overwhelming preference for *C. maculatum* volatiles in the earlier experiment eliminated the ability to detect the effects of learning.

The current results are intriguing not only as the first clear demonstration of learning in *Papilio polyxenes* females, but also in light of the apparent tight linking of cues in the learning process. These results merit further study of the role of visual cues in conjunction with chemical cues, as previous studies have focused on chemical cues alone. To fully understand the behavior of these butterflies in the field, it is clear that a more holistic approach is necessary. Only by examining the different types of cues together as well as independently can we understand the roles each plays in the host-finding behavior of a species, as well as the way the cues interact.

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## CHAPTER FIVE

### RESPONSES TO CONTACT AND VOLATILE CHEMISTRY COMBINED

Phytophagous insects are dependent on the chemistry of their host plants for proper identification (Bernays 2001). Accurate identification is of utmost importance for female lepidopterans, as the ovipositing female chooses the larval food plant (Honda 1995). The cues available to a searching insect include visual and volatile chemical cues, both accessible from a distance, and contact chemical cues, which are only available after landing. Volatile chemical cues tend to be most important prior to landing, while contact chemical cues often play the major role after a female has landed on the leaf surface (Renwick and Huang 1994, Schoonhoven 1968).

Swallowtail butterflies, family Papilionidae, are a model system for understanding the patterns of chemical cues for host-plant identification (Feeny 1995, Nishida 1995). Contact chemical cues have been particularly well-studied in this group, with many compounds identified across several different species (Carter, et al. 1999, Haribal and Feeny 1998, Honda and Hayashi 1995, Nishida 1995). Volatile chemical cues have not been studied to the same extent, but they are known to play a role in host finding as well as oviposition (Chapter 3, Feeny, et al. 1989). Saxena and Goyal (1978) examined the responses of *Papilio demoleus* to the stimuli of one of its rutaceous host plants, *Citrus limettoides*, in one of the first studies of the responses of a swallowtail butterfly to chemical cues. They were mainly concerned with the effects of odor and visual stimuli, but found that the greatest egg-laying response was to contact with a moist combination of ether (mainly volatile) and ethanolic (mainly non-volatile) extracts of the host plant. Although they did not recognize the importance of contact with the extract as a gustatory response, they clearly

demonstrated the importance of both types of chemical cues for the oviposition behavior of this butterfly. Both types of cues are also known to play a role in the oviposition behavior of the zebra swallowtail butterfly, *Eurytides marcellus*. Although the non-volatile compound 3-caffeoyl-muco-quinic acid is an important oviposition stimulant for this butterfly, females are stimulated to land more often and lay more eggs when yet-unidentified volatile chemical cues are also present (Haribal and Feeny 1998). Some *E. marcellus* females are so stimulated by the presence of volatile chemical cues that the contact chemical cues are not necessary.

*Papilio polyxenes* shows an oviposition response to both contact and volatile chemical cues prior to adult experience with a host plant (Chapters 2 and 3). Only a few females laid eggs when only volatile chemical cues were present, and most females curled their abdomen, a precursor to egg-laying, when presented with contact chemical cues, even when this is done on a white strip of filter paper. Previous work has identified several contact chemical stimulants from two host plants (Carter, et al. 1999, Feeny, et al. 1988). Earlier work with volatile chemicals showed an increase in landing rates, and thus egg-laying rates, when volatile chemical extracts were added to contact chemical extracts in a free-flight assay (Feeny, et al. 1989). The current experiment combines an examination of the relative roles of contact and volatile chemical cues with an examination of how this might be influenced by the experience of the butterfly. Although no learning was seen after host-plant experience when either chemical cue was examined alone (Chapters 2 and 3), there was an effect of experience seen when visual cues were combined with either type of chemical cue (Chapter 4). Thus, the effect of combining the two types of chemical cue remains to be tested.

The goal of this study, therefore, was to test the response of ovipositing females of *Papilio polyxenes* to contact and volatile chemicals simultaneously, both before and after host-plant experience. A response to each type of cue by naïve females of this species has already been demonstrated when either cue is presented independently of the other, and neither response is affected by host-plant experience (Chapter 2 for contact chemicals, Chapter 3 for volatiles). The role of experience in oviposition responses to chemical cues has not been examined in any other swallowtail species, although experience with visual cues plays a large role in the pipevine swallowtail, *Battus philenor* (Rausher 1995).

## MATERIALS AND METHODS

### *Butterflies*

Butterflies were diapausing, first generation offspring of wild females caught during 1999 in southeastern Canada. Butterflies were kept in an environmental growth chamber (Environmental Growth Chambers, Chagrin Falls, OH) with 16:8 LD, 27°C daytime and 15.5°C nighttime temperatures, and 75±5% relative humidity. The growth chamber was devoid of host plant material at all times. Butterflies were fed and numbered as described in Carter and Feeny (1985), with two exceptions: butterflies were fed a 20% (in place of 10%) solution of honey in water and several colors of Sharpie<sup>®</sup> permanent markers (Sanford, Inc.) were used for numbering to aid in individually identifying females in flight. Females were mated by hand pairing (Carter and Feeny 1985) more than 24 hours after emergence, on the second day after eclosion.



Each female butterfly was subjected to four trials, two “naïve” and two “experienced”, as shown in Figure 5.1. For some analyses, the first two trials were combined into one “naïve” category. After the second trial, each female was placed into an individual 16 X 31 X 9cm cage with a sprig of one of the two host species (*D. carota* or *C. maculatum*) in a water-filled aquapic, with the plant species alternating by female so that an equal number of females would receive each plant species. Cages were kept in a greenhouse chamber away from that of the bioassays, and females were fed at least once per day. Females were allowed to lay eggs *ad libitum* until the morning of the third day after mating, at which point they were returned to the growth chamber devoid of host-plant material. The following day, females were given their third test, the first “experienced” test. The fourth and final test occurred on the fifth day after mating. Again, the two experienced trials were combined into a single “experienced” category for some analyses.

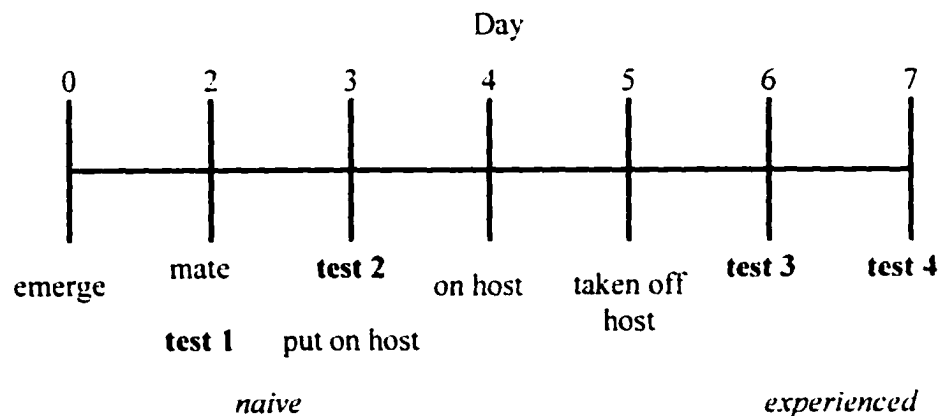


Figure 5.1 Timeline of females in this experiment.

### *Plants*

The apiaceous host plants, *Daucus carota* (wild carrot) and *C. maculatum* (poison hemlock), were grown in a greenhouse atop Seeley Mudd Hall at Cornell University.

Seed was wild-collected from sites near Ithaca, New York. A non-deterrent non-host, *Vicia faba* (fava bean, Fabaceae), was grown from commercial seed (var. Broad Windsor, Agway).

### *Bioassay*

Bioassays took place in a 3.66 x 3.66 x 1.83m cage placed in a large greenhouse module atop Seeley Mudd Hall at Cornell University. The greenhouse module, and all adjacent modules, contained no apiaceous plants at any time during the experiment. The cage had nylon mesh walls and the floor was covered in black landscape fabric (Agway, Inc.). Nine model plants were arranged in a three-by-three array on the floor, and one nectar plant (*Pentas lanceolata* (Rubiaceae)) was placed in each of the four corners. Model plants were the same as first used in Chapter 3, four paper leaves were attached to each model plant, and a vial of volatiles could be inserted in the center of the leaf attachment points. Model leaves were cut from green card stock (Hammermill, Bright Hue® Cover) in the same shape as Chapter 3. Additional stability was added to the model leaves by stapling an additional 10.2cm long x 1.6cm wide strip of the same paper that had been folded in half lengthwise to the back side of the stem. Model leaves were scored on the top side with a blade, to add dimensionality. The total top surface area of the model leaves was 215cm<sup>2</sup>. Contact chemical extracts were painted onto the paper leaves as described below. Model leaves were attached to the model plants immediately prior to a bioassay, and no model leaves were used for more than one trial.

Extracts were arranged in the array as in Figure 5.2. There are eight possible arrangements of the array, keeping the *V. faba* model plants in place and rotating the model host plants in each of two permutations. Prior to the start of each trial, model plants were rotated. In each array, four model plants had contact and volatile chemical

extracts of *V. faba*. Two model plants each had the contact chemical extract of *D. carota* and *C. maculatum*. Of these four model plants, one of each with the *D. carota* and *C. maculatum* contact chemical extracts held *D. carota* volatile chemical extracts, while the remaining two model plants held *C. maculatum* volatile chemical extract. Volatile chemical extracts were removed from the freezer just prior to the beginning of a trial and 1ml of mineral oil was added, to slow the evaporation of the extract. Vials were uncapped immediately before butterflies were released into the array.

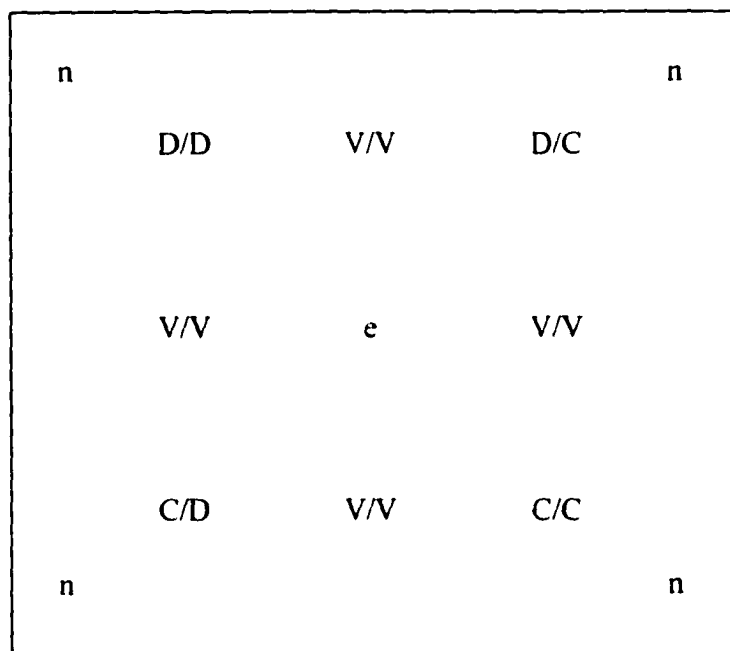


Figure 5.2 The arrangement of model and nectar plants in the experimental array. For each model plant, the first letter specifies the volatile chemical cues, while the second specifies the contact chemical cues. *D*'s are *D. carota* cues, *C*'s are *C. maculatum* cues, *V*'s are *V. faba* cues, *e*'s are empty (no extracts), and *n*'s are nectar plants (*Pentas lanceolata*). Eight arrangements are possible by rotating the extracts in this and one other permutation (exchanging the "hybrid" cued plants).

Butterflies were transported to the array from the growth chamber in butterfly envelopes (BioQuip) and released onto nectar plants. At least one but no more than 12 butterflies were tested at any one time. Activity was sporadic, such that it was possible to watch as many butterflies at one time. A total of 36 trials were used to test 33 butterflies, with a mean of 5.23 butterflies per trial. Trials lasted for 90 minutes and there was no interference during a trial. All behaviors towards the model plants, as well as transitional behaviors and the identities of the behaving butterflies, were recorded using a tape recorder and transcribed the day of the trial. For analysis, behaviors were classified into ten discrete categories, as shown in Table 5.1. The greenhouse temperature was set at 25.5 °C, and lights (Sylvania Lumalux LU400, sodium high intensity discharge) were used to supplement daylight on cloudy days.

*Extractions: Contact chemicals*

*D. carota*, *C. maculatum*, and *V. faba* leaves were collected from greenhouse plants. Leaves were weighed, and 100g batches were blended in 500ml boiling 95% ethanol. The slurry was filtered and the ethanol removed by evaporation under reduced pressure. The remaining aqueous solution was centrifuged and the supernatant was extracted three times each with diethyl ether, chloroform, and ethyl acetate. This aqueous extract contained virtually all of the stimulant activity of the parent ethanolic extract (Brooks, et al. 1996, Feeny, et al. 1988). The extract was evaporated to a concentration of 5 gram leaf equivalents ('gle') per ml and frozen in 0.5ml aliquots.

To prepare extracts for application to leaf shape models, the extracts were diluted to a concentration equivalent to 215cm<sup>2</sup> / 4 ml with HPLC-grade water. The equivalent weights per ml are 0.45g *V. faba* (Chapter 4), 0.75g for *D. carota*, and 0.64g *C. maculatum* (Chapter 3). One ml of extract was painted evenly on the top surface of each leaf, with eight leaves each of *D. carota* and *C. maculatum*, and 16

Table 5.1 Categories of behaviors observed during trials.

<b>Category of Behavior</b>	<b>Abbreviation</b>	<b>Examples of Behavior</b>
Non-plant directed behaviors	NP	Fly high (>1m over model plants) around cage. Bother other females on side of cage, often transitional between array activity and inactivity
Nectaring behaviors	Nectar	Nectar, Land on nectar plant
Fly mid over plants	Fly mop	Fly mid-level (<1m, >10cm) over model plants, may be directed to particular model plants
Fly low over plants	Fly lop	Fly low (<10cm) over model plants, may be directed to particular model plants
Approach	App	Approach a model plant
Inspect	Insp	Flutter low (<5cm) over a model plant
Land	Land	Land on a model plant
Drum	Drum	Drumming behavior on a part of a model plant
Curl	Curl	Abdomen curl while on a model plant
Oviposit	Ovip	Lay an egg on a model plant

leaves of *V. faba* per trial. The extract was allowed to nearly dry and leaves were flattened, as the moisture caused them to curl. Leaves were prepared ahead of trials and frozen until needed due to the time needed to apply extracts and dry leaves.

*Extractions: Volatile chemicals*

Volatile extracts were collected as in Chapter 3. *D. carota*, *C. maculatum*, and *V. faba* leaves were collected from greenhouse-grown plants. Leaves were weighed with the petioles removed, and 20–30g from a single species were placed in a large (2000ml) Erlenmeyer flask. Hexane was added to cover all of the leaves (1000ml), and allowed to soak. After five minutes, the hexane was decanted into a clean flask and frozen at -10°C. After all of the samples of a single plant species were extracted, the combined extract was reduced to a concentration equivalent to 215cm<sup>2</sup>/ml. The equivalent concentrations are 1.81 g/ml of *V. faba* (Chapter 3), 2.99g/ml of *D. carota*, and 2.57g/ml of *C. maculatum* (Chapter 4). The extract was stored in a -10°C freezer in 1ml aliquots in 2ml vials until needed.

*Extractions: Spectral properties of contact chemical extracts*

An S1000 fiber optic spectrophotometer (Ocean Optics, Inc.) with CSPEC software was used to sample the reflectance of extract-coated model leaves. Reflectance at wavelengths from 274nm to 821nm was measured at every 0.535nm. A model leaf with no extract was sampled as a reference standard.

*Analysis*

SAS software version 6.12 (SAS Institute, Inc.) was used for all analyses.

Non-parametric statistics were used for behavioral analyses due to the non-normality of the data (Conover 1980). Approaches and landings were primarily used for analyses due to the large number of both types of behavior, as well as the accuracy for

identifying the model plant at which the behavior was directed. Only females displaying a behavior at least once were included in analyses of such behavior.

Responses of naïve females to host and non-host extracts and the effect of host-plant extract on the responses of experienced females were both analyzed by the sign test. The null hypothesis was that there was no effect of plant extract or plant experience on behavior. For naïve females, approaches and landings on model plants containing the non-host, *V. faba*, extracts and on model plants containing extracts of either host, *D. carota* or *C. maculatum*, were each summed for each female. For each approaches and landings, the sum for the non-hosts was subtracted from the sum for the hosts and the sign of the result was scored. The same procedure was used for experienced females, except that comparisons were made between the extracts of the two host plants, using separate analyses for contact and volatile extracts.

The behaviors of experienced females toward host-plant volatile and contact extracts with respect to host-plant experience and host-plant extracts were analyzed using Fisher's exact test. Approaches and landings were each tested in a 2 x 2 table of experience type versus the host-plant extract of the model plant receiving the behaviors, with separate analyses for contact and volatile extracts. To test the effect of host-plant experience on experienced females, a median test was used, again with a null hypothesis of no effect. The numbers of approaches and landings on model plants containing host-plant extracts were compared between females who had experienced each of the two host-plant species.

Fisher's exact test was used to look for a relationship between the first host-plant extract approached or landed on during a trial and the extract most often approached or landed on during the same trial for each female for each of the four

trials. Chi-square tests were used to examine the data taking the sequence of behaviors into account. 2 x 2 tables were used to examine the relationship between approaches and landings for hosts and non-hosts for naïve females, and for the two different hosts for experienced females. The Mantel-Haenszel test was used to simultaneously examine the effects of experience, behavior following an approach or landing, and host-plant cues (Stokes, et al. 1995).

## RESULTS

The behavior of female *Papilio polyxenes* in this bioassay was similar to that in a bioassay using whole plants instead of model plants (Chapter 6). Females flew over the array, approached and inspected the model plants. They also landed frequently, drummed as they would on a natural leaf (e.g. Ilse 1955), curled their abdomens in preparation to lay an egg, and, on a few occasions, laid eggs on the model plants.

### *Responses of naïve females*

Naïve females did respond preferentially to host-plant cues, though not as strongly as the experiment of Chapter 3. Naïve females were equally likely to approach model plants with host (*D. carota* or *C. maculatum*) and non-host (*V. faba*) extracts, but they were slightly more likely to land on model plants with host extracts than non-host extracts (Figure 5.3; approaches: sign test, N=7, T=5.5, p=0.25; landings: sign test, N=7, T=9, p=0.1406). Once a model plant was approached, a naïve female was slightly more likely to land if host-plant extracts were present (Figure 5.4A: Fisher's exact test, N=83,  $\chi^2_1=3.217$ , p=0.091). Once a model plant was landed on, a female was significantly more likely to land, drum, or curl on the plant if it contained host-plant extracts (Figure 5.4B: Fisher's exact test, N=134,  $\chi^2_1=5.35$ , p=0.032).



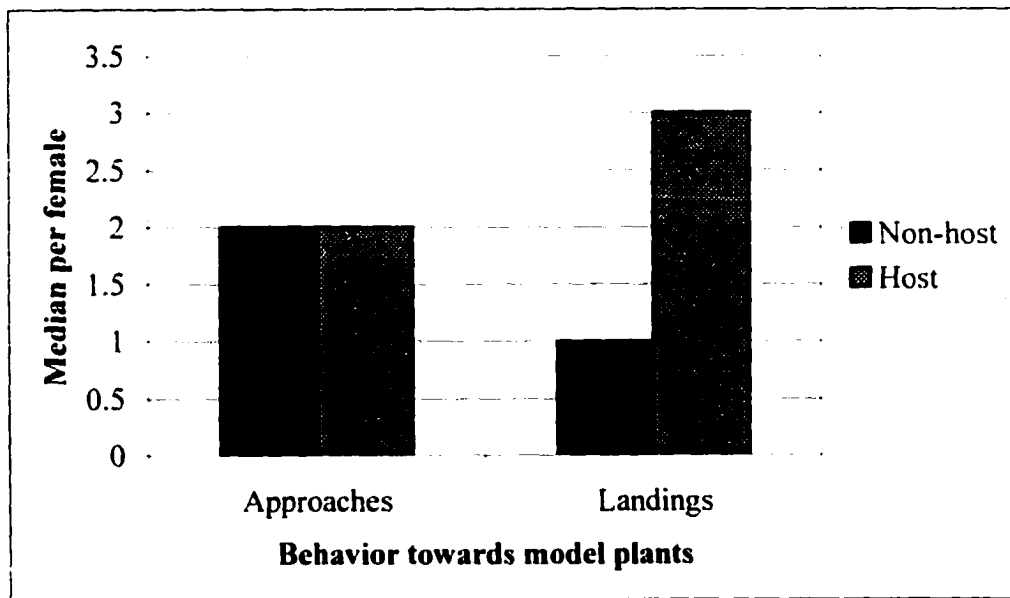


Figure 5.3 Median number of approaches and landings per female in naïve trials (trials 1 and 2). Host extracts are *C. maculatum* + *D. carota*, the non-host is *V. faba*.

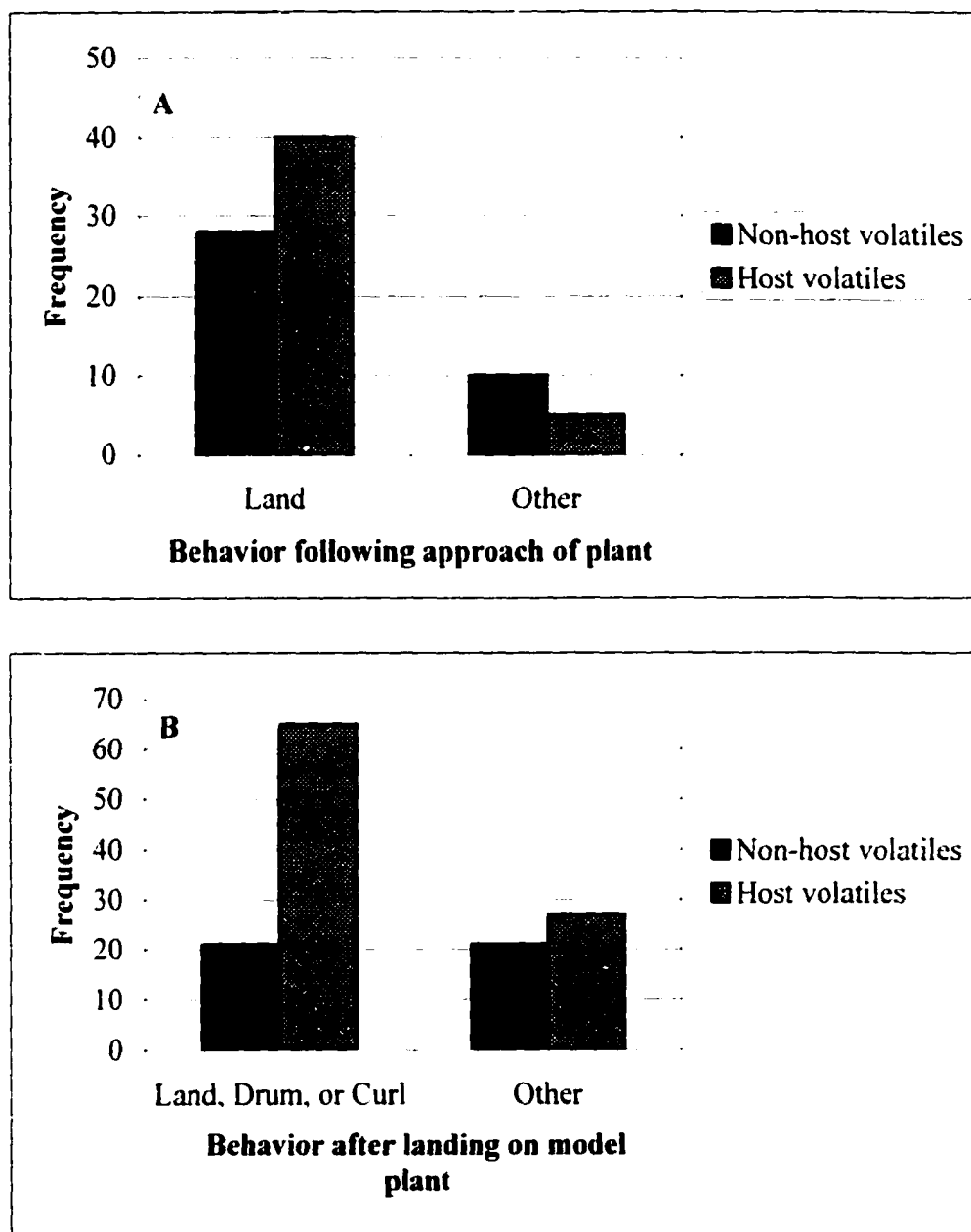


Figure 5.4 Frequency of landings or other behaviors immediately following approaches (A) and landings (B) to model plants with non-host or host-plant cues by naïve females.

*Responses of experienced females*

Host-plant experience did not increase the approaches on model plants with the volatile extract of the host-plant experienced (Figure 5.5A; Fisher's exact test:  $N=142$ ,  $\chi^2_1=0.425$ ,  $p=0.609$ ). The number of landings was correlated with host-plant experience for *C. maculatum*-experienced females landing significantly more often on model plants with *C. maculatum* volatiles (Figure 5.5B: Fisher's exact test:  $N=101$ ,  $\chi^2_1=11.462$ ,  $p<0.001$ ). However, the number of landings on model plants with *C. maculatum* volatiles was also increased for females with *D. carota* experience, suggesting that this is an effect of preference rather than learning. There was a significant effect of experience on the response to contact chemical cues. Females were significantly more likely to approach and land on model plants bearing the same contact chemical extract as the host-plant they had previously laid eggs on (Figure 5.6: Fisher's exact test: approaches:  $N=142$ ,  $\chi^2_1=17.841$ ,  $p=0.003$ ; landings:  $N=101$ ,  $\chi^2_1=11.558$ ,  $p<0.001$ ).

Females were more likely to approach and land on model plants with *C. maculatum* volatiles than with *D. carota* volatiles (Figure 5.7A; Sign test: approaches:  $N=11$ ,  $T=21$ ,  $p=0.0615$ ; landings:  $N=9$ ,  $T=18.5$ ,  $p=0.0273$ ). In contrast, females were only slightly and not significantly more likely to approach and land on model plants with *D. carota* contact extract than *C. maculatum* contact extract (Figure 5.7B: approaches:  $N=11$ ,  $T=-8$ ,  $p=0.4414$ ; landings:  $N=9$ ,  $T=-9.5$ ,  $p=0.2852$ ). Females experienced on *D. carota* did not differ from *C. maculatum*-experienced females in the number of approaches or landings on model plants with host-plant extracts (Figure 5.8: Median test: approaches:  $N=11$ ,  $\chi^2_1=0.96429$ ,  $p=0.3261$ ; landings:  $N=9$ ,  $\chi^2_1=0.2$ ,  $p=0.6547$ ).

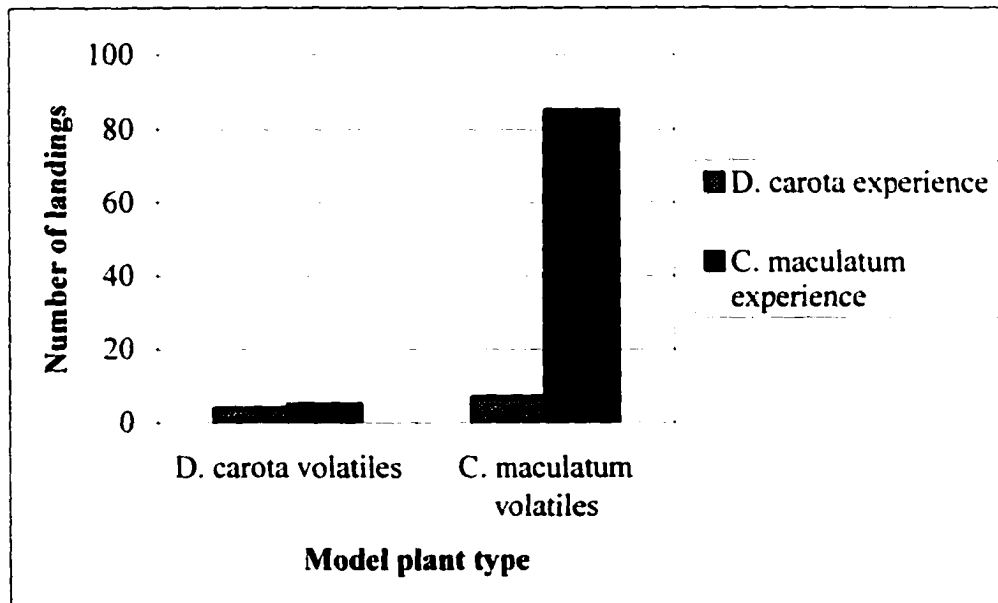
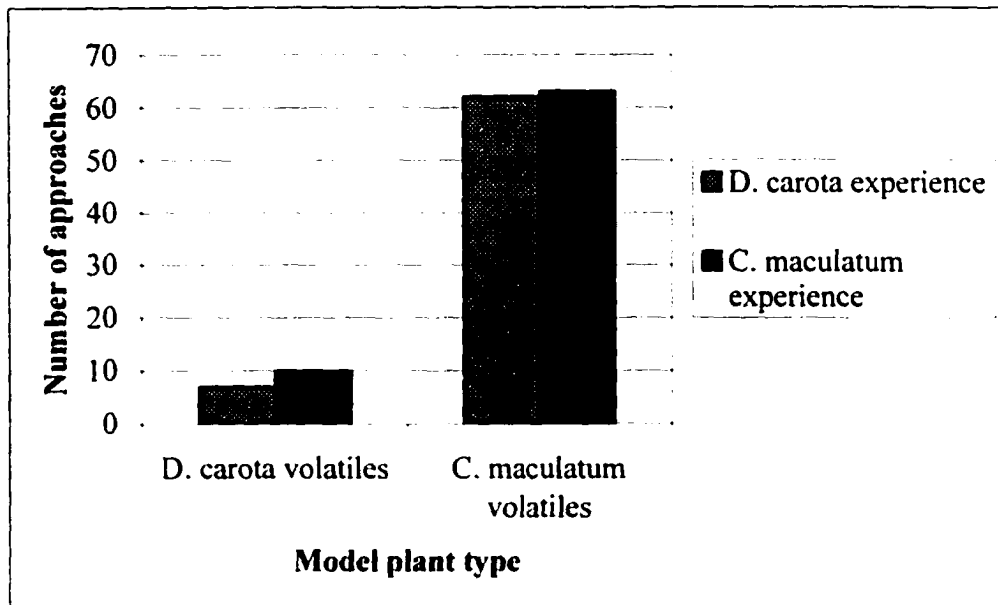


Figure 5.5 Number of approaches (A) and landings (B) by females in experienced trials on model plants with host plant cues with respect to the two host-plant volatile chemical cues.

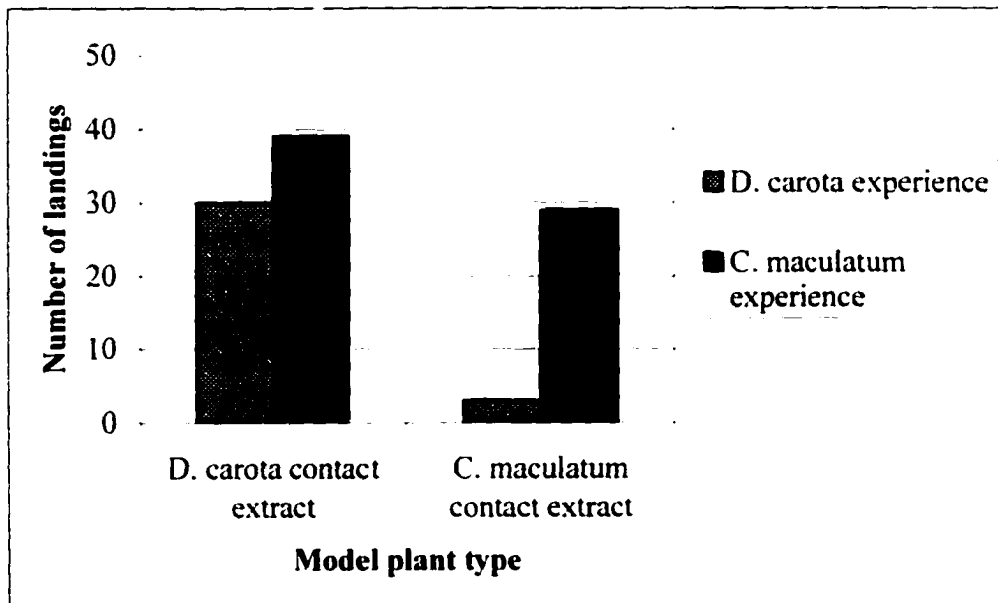
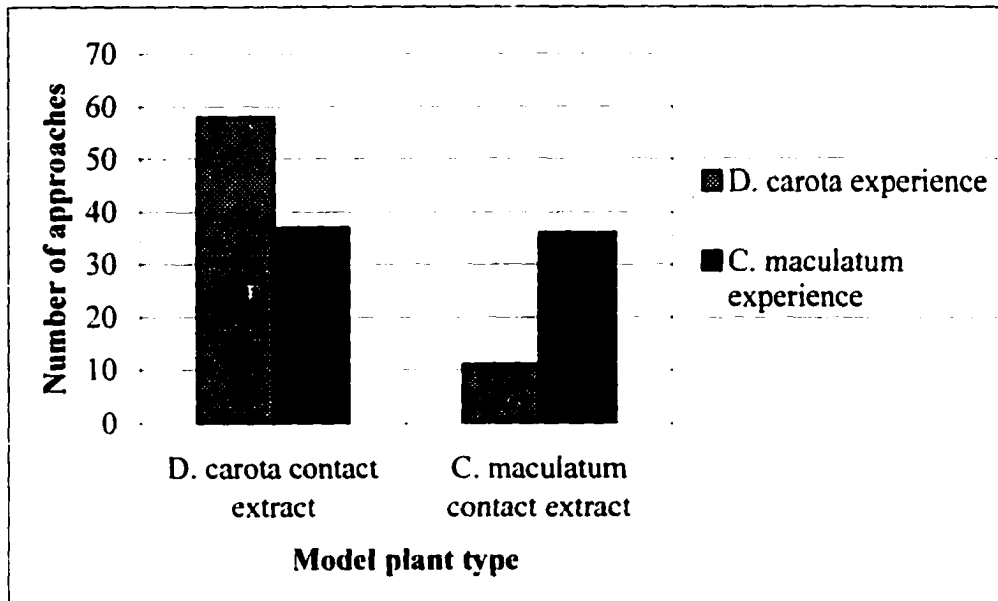


Figure 5.6 Number of approaches (A) and landings (B) by females in experienced trials on model plants with host plant cues with respect to the two host-plant contact chemical extracts.

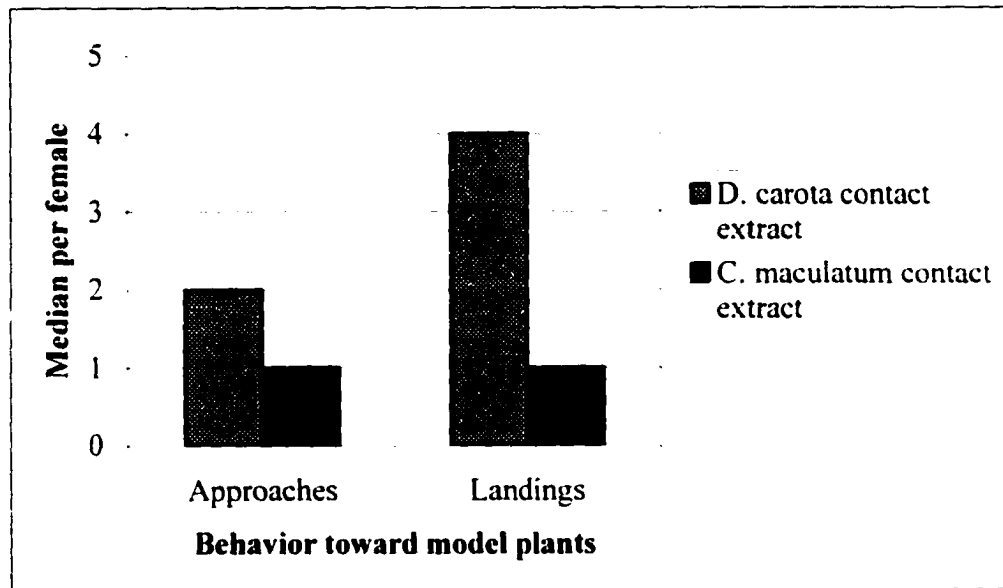
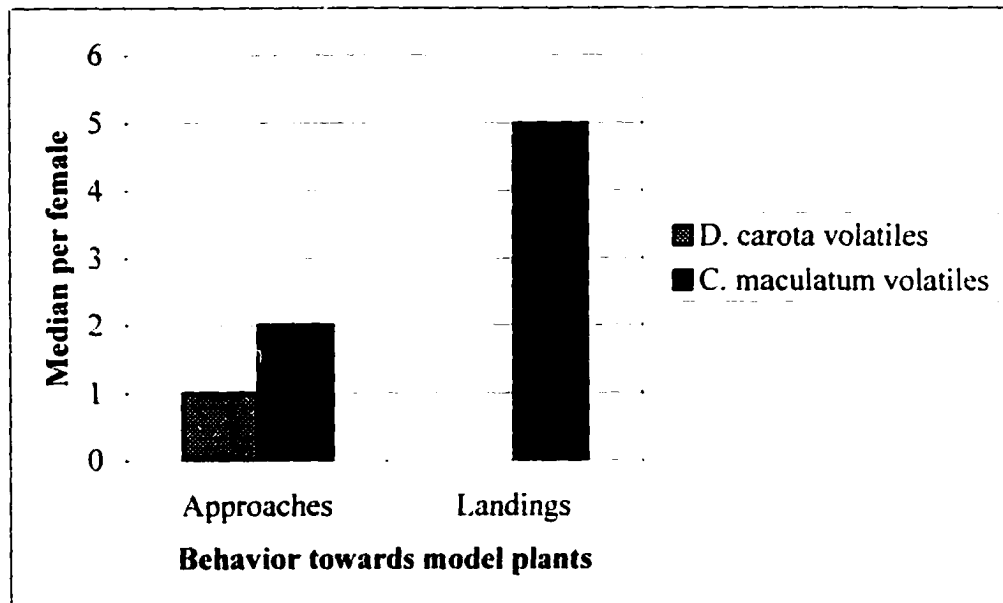


Figure 5.7 Median number of approaches and landings by experienced females on model plants with host-plant cues. Data for volatile chemical cues are shown in A; for contact chemical extracts, in B. (The median number of landings per female on model plants with *D. carota* volatiles is zero.)

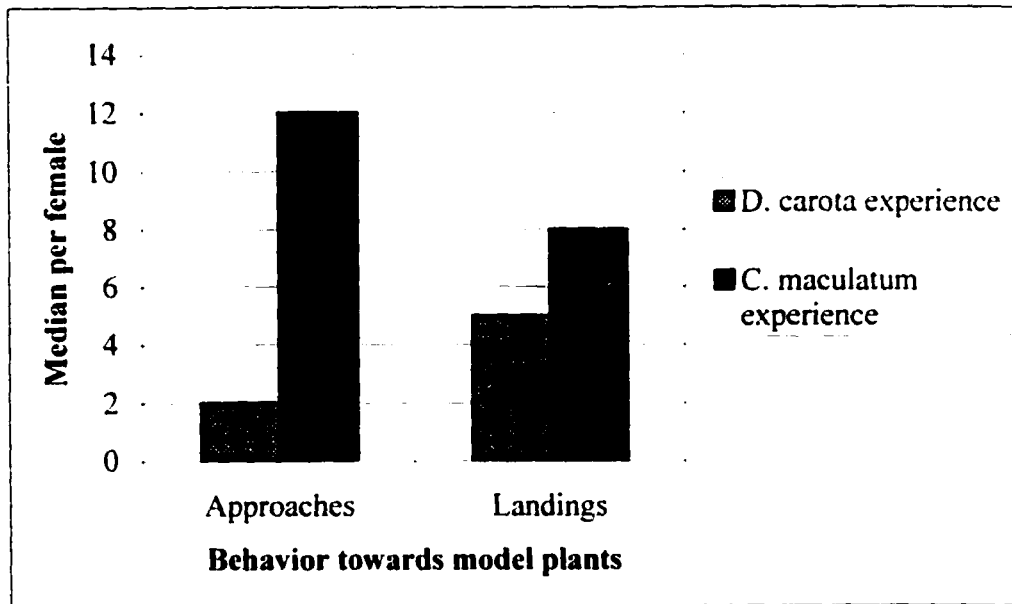


Figure 5.8 Median number of approaches and landings by experienced females on model plants with host-plant cues (*C. maculatum* + *D. carota*) with respect to the host plant experienced.

*Within-trial experience effects*

Fisher's exact test was used to examine the possibility of within-trial learning. There was a strong relationship between the first model plant with host-plant cues approached and the model plant with host-plant cues most often approached by an individual female within a single trial (Table 5.2; Fisher's exact test:  $N=19$ ,  $\chi^2_9=43.816$ ,  $p<0.001$ ). Females were most likely to approach most often the same model plant, with the same cues, as the first model plant with host-plant cues approached. The same is true for landings (Table 5.3;  $N=18$ ,  $\chi^2_9=41.625$ ,  $p<0.001$ ), with only two females landing most often on a different model plant than the first model plant landed on. One of the two females landed most often on the model plant with the same volatile chemical cues (but not contact chemicals) as the first model plant landed on, the other female landed most often on the model plant with the same contact chemical cues, but different volatile chemicals. To verify these results, the percent of landings on each type of host-plant cue by each female within each trial was graphed, giving a distribution of female "preference" within a trial (Figure 5.9, Figure 5.10). For approaches to model plants with host-plant volatiles, the distribution is clearly bimodal, with females mostly approaching only those model plants with either *C. maculatum* or *D. carota* volatiles (Figure 5.9A). For landings by volatiles cues, the distribution is heavily skewed towards most females landing on model plants with *C. maculatum* volatile cues, with only six of 19 females landing more often on *D. carota* than *C. maculatum* (Figure 5.9B). The distribution of females was clearly bimodal for their responses to contact chemical cues for both approaches and landings (Figure 5.10), with most females landing either wholly on model plants with *D. carota* or *C. maculatum* contact chemical extracts.



Table 5.2 First model plant approached and model plant most often approached within a trial. Numbers are number of females for each combination of volatile and contact chemical cues.

First approached		Most approached			
Volatiles	Contact	<i>D. carota</i> volatiles		<i>C. maculatum</i> volatiles	
		<i>C. maculatum</i>	<i>D. carota</i>	<i>D. carota</i>	<i>C. maculatum</i>
<i>D. carota</i>	<i>C. maculatum</i>	2	0	0	0
	<i>D. carota</i>	0	5	0	1
<i>C. maculatum</i>	<i>D. carota</i>	0	0	4	0
	<i>C. maculatum</i>	1	0	0	6

Table 5.3 First model plant landed on and model plant most often landed on within a trial. Numbers are number of females for each combination of volatile and contact chemical cues.

First landed on		Most landed on			
Volatiles	Contact	<i>D. carota</i> volatiles		<i>C. maculatum</i> volatiles	
		<i>C. maculatum</i>	<i>D. carota</i>	<i>D. carota</i>	<i>C. maculatum</i>
<i>D. carota</i>	<i>C. maculatum</i>	2	0	0	0
	<i>D. carota</i>	0	3	0	0
<i>C. maculatum</i>	<i>D. carota</i>	0	0	5	0
	<i>C. maculatum</i>	1	0	1	6

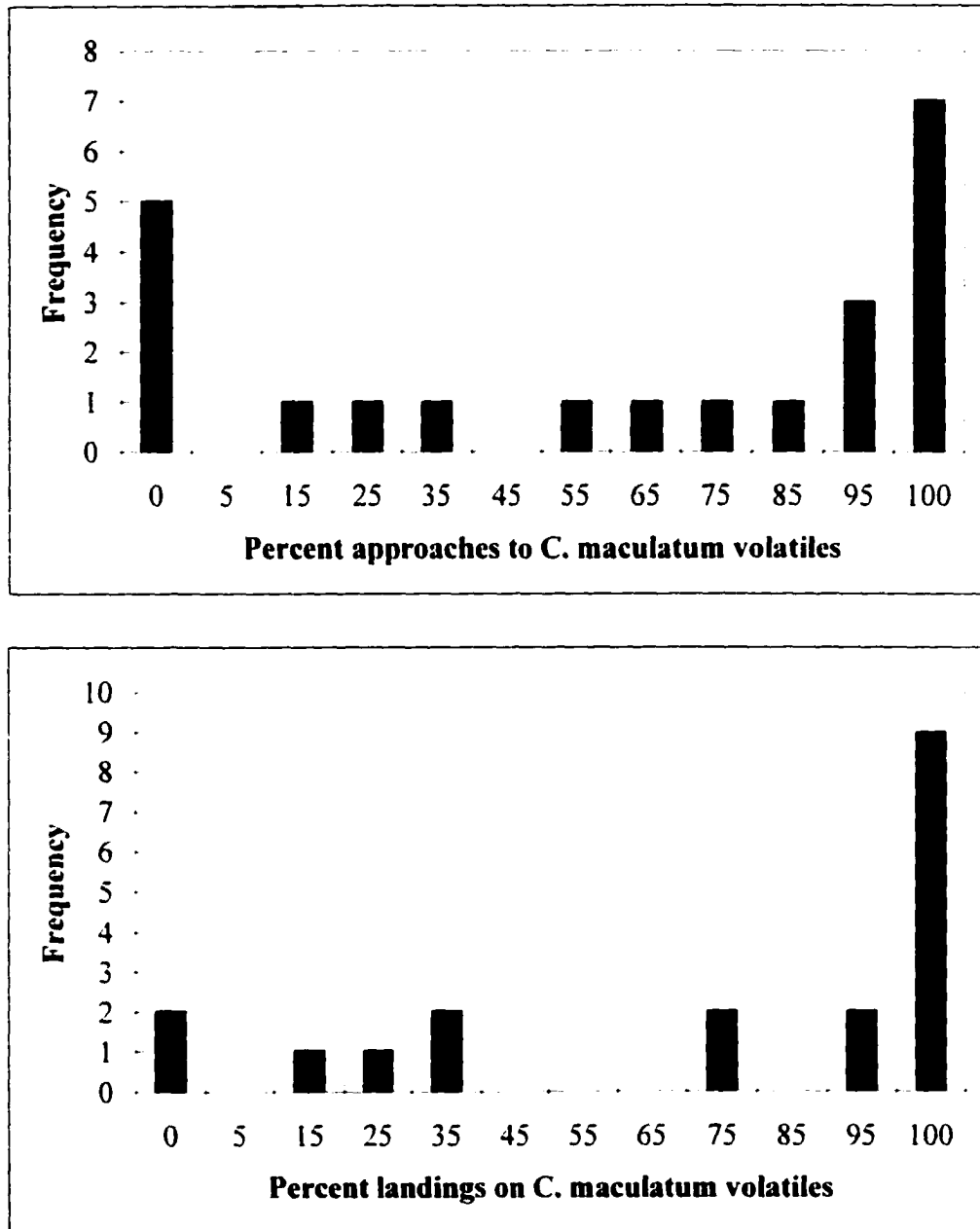


Figure 5.9 Distribution of approaches (A) and landings (B) of individual females within single trials on model plants with host-plant volatiles.

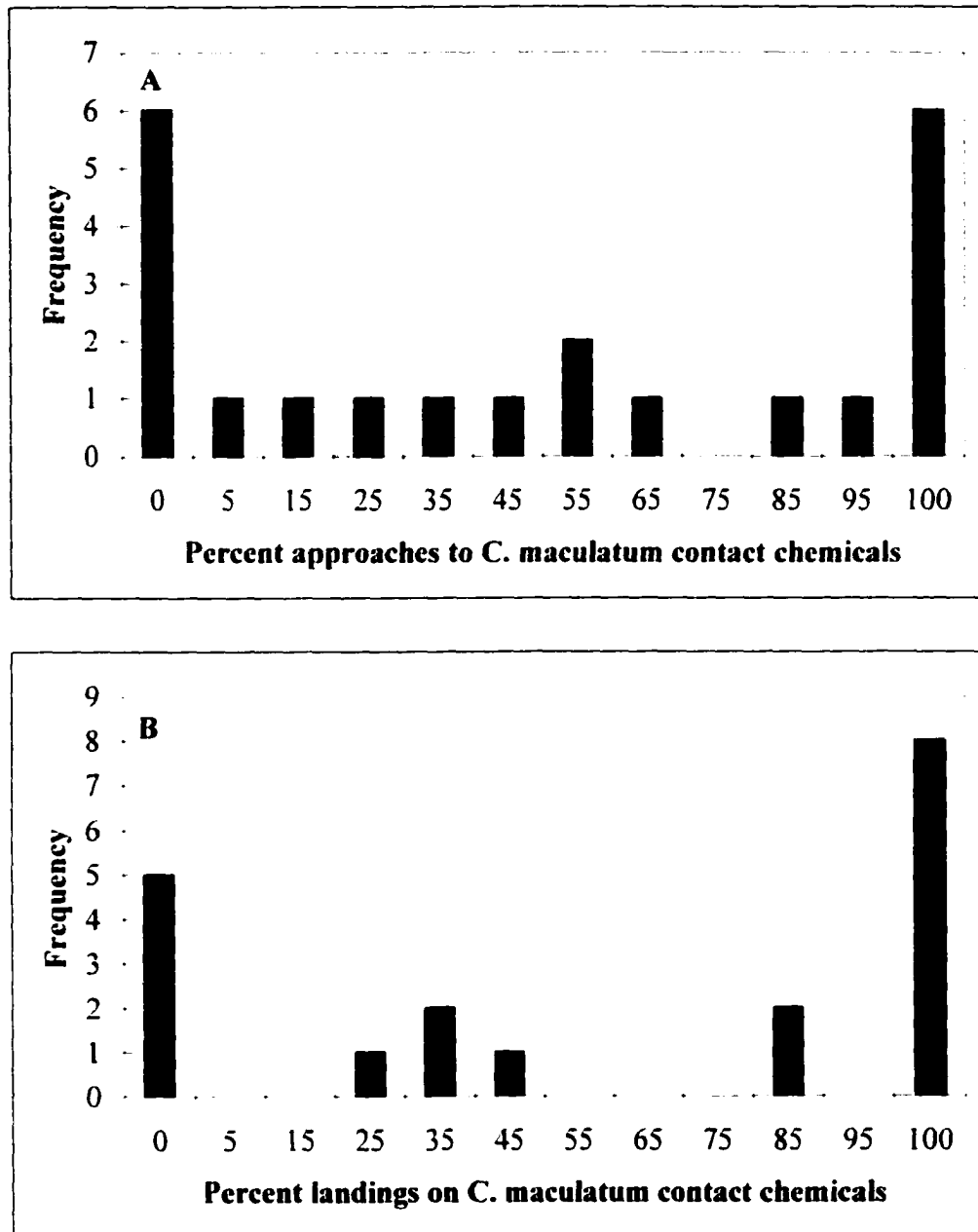


Figure 5.10 Distribution of approaches (A) and landings (B) by individual females within single trials on model plants with host-plant contact chemical extracts.

There was no effect of female experience on the behavior following an approach on a model plant with host–plant cues (Table 5.4; Mantel–Haenszel test for general association:  $N=130$ ,  $T=3.755$ ,  $p=0.289$ ). There was a slightly significant effect for the behavior following a landing on a model plant with host–plant cues (Table 5.5; Mantel–Haenszel test for general association:  $N=97$ ,  $T=7.121$ ,  $p=0.068$ ). This is likely due to the tendency of *C. maculatum*–experienced females to land, curl, or drum more frequently on plants with *D. carota* contact chemical extracts and less frequently on plants with *C. maculatum* contact chemical extracts.

#### *Spectral properties of contact chemical extract on model leaves*

The reflectances of extracts of *C. maculatum* and *D. carota* on model leaves are shown in Figure 5.11. (The reflectance of an untreated leaf was subtracted at each data point, so that differences between the extracts would be visible.) Reflectance was examined to determine whether there were any visual color cues inherent in the extracts, although none were visible to the human eye.

## DISCUSSION

Despite the absence of learning observed when contact and volatile chemical cues were examined independently (Chapters 2 and 3), there are several suggestions of learning in the host–finding behavior of *Papilio polyxenes* females when these two types of chemical cue are combined. Experience with a host plant led to an increase in approaches to and landings on model plants with the contact chemical extract of the same plant (Figure 5.6). The experience of a female within a single trial also led to apparent learning, of extracts or positions, as nearly all females approached and landed most often on the same model plant that they had first approached or landed upon, respectively (Tables 5.3 and 5.3).

Table 5.4 Within-trial experience effects of experience and model plant on post-approach behaviors. ("Other" behaviors include all pre-landing behaviors (see Table 5.1).)

Experience	Volatiles	Contact Chemicals	Observed		Expected	
			Land	Other	Land	Other
<i>D. carota</i>	<i>D. carota</i>	<i>C. maculatum</i>	0	1	0.303	0.697
		<i>D. carota</i>	3	3	1.818	4.182
	<i>C. maculatum</i>	<i>D. carota</i>	15	36	15.46	35.55
		<i>C. maculatum</i>	2	6	2.424	5.576
<i>C. maculatum</i>	<i>D. carota</i>	<i>C. maculatum</i>	2	1	1.172	1.828
		<i>D. carota</i>	1	3	1.563	2.438
	<i>C. maculatum</i>	<i>D. carota</i>	7	22	11.33	17.67
		<i>C. maculatum</i>	15	13	10.94	17.06

Table 5.5 Within-trial experience effects of experience and model plant on post-approach behaviors. ("Land +" refers to landing again, drumming, or curling, while "other" behaviors include all pre-landing behaviors (see Table 5.1).)

Experience	Volatiles	Contact Chemicals	Observed		Expected	
			Land+	Other	Land+	Other
<i>D. carota</i>	<i>D. carota</i>	<i>C. maculatum</i>	0	0	0	0
		<i>D. carota</i>	1	3	0.625	3.38
	<i>C. maculatum</i>	<i>D. carota</i>	4	22	4.063	21.94
		<i>C. maculatum</i>	0	2	0.313	1.688
<i>C. maculatum</i>	<i>D. carota</i>	<i>C. maculatum</i>	0	1	0.446	0.554
		<i>D. carota</i>	1	1	0.892	1.108
	<i>C. maculatum</i>	<i>D. carota</i>	21	15	16.06	19.94
		<i>C. maculatum</i>	7	19	11.60	14.40

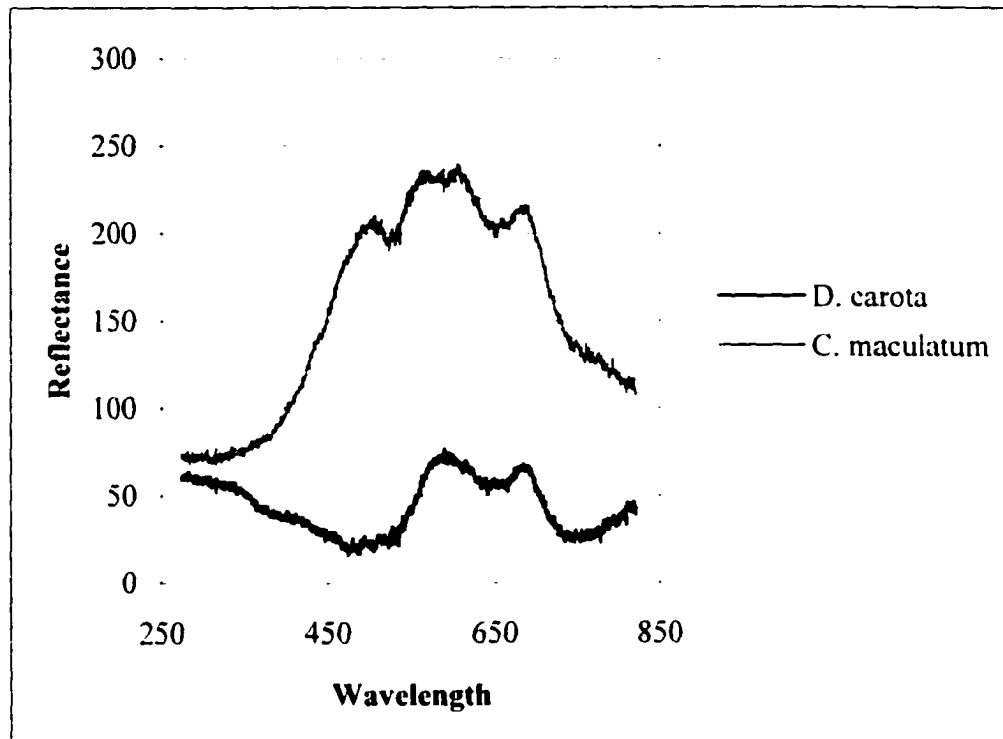


Figure 5.11 Reflectance spectra of *D. carota* and *C. maculatum* extracts on model leaf surfaces.

These responses are not entirely learned, however. Females preferentially responded to the extracts from host plants prior to adult experience with a host plant (Figure 5.3 and Figure 5.4). Although the effects of age make direct comparison between the “naïve” and “experienced” tests impossible in this case, it is likely that females do have a naïve response to this set of cues, as well as an ability to learn specific aspects of the cues with experience. This experience can either be with the host plant, as between the “naïve” and “experienced” trials, or with the model plants, as during a single trial. This suggests that the learning is of the sensitization type, with an increase in the response after experience, as opposed to associative learning, as there are no unlearned cues to act as unconditional stimuli (Papaj and Prokopy 1989). However, this depends heavily on the assumption that each type of cue presents only a single stimulus to a female, when this is not at all likely to be the case. Synergism is extremely important in the chemical cues stimulating oviposition in this butterfly (e.g. Feeny, et al. 1988), and the extracts are relatively crude, with very little separation of compounds. Therefore, it is possible that the females are initially recognizing one or more specific compounds as cues and then learning to associate them with other, innately neutral compounds in the extracts. Unfortunately, more detailed knowledge of the exact chemical cues that the butterfly can respond to (both before and after host-plant experience) is needed before this can be determined with any certainty.

Within the context of a single trial, individual females landed mostly on the same model plant, that is, the one model plant with the same contact and volatile chemical cues. This is seen not only in Tables 5.2 and 5.3, but also in Figures 5.9 and 5.10, in that the distribution of approaching and landing behaviors tended to be clustered at either 0% (=100% on *D. carota*-cued) or 100% on *C. maculatum*-cued model plants. If females were learning only one cue type, the distribution would not



have been bimodal for the non-learned cue, as there are two model plants that share a single specific cue type (species and type), but no model plants shared both specific cue types. It is possible that the females are learning the position of the model plant, and not the cues themselves, and the current experiment does not allow for this to be decided directly. However, circumstantial evidence suggests that the cues themselves are being learned. There is evidence in Chapter 4 of females learning leaf shapes within a trial without regard to position, and by extension, learning pairings of leaf shape and chemical cues within a trial. In addition, the hours of observation for this experiment did not suggest this result: in fact, this result was not suspected until the data were analyzed.

Females were therefore learning both the contact chemical cues as well as the volatile chemical cues for a single model plant in the context of approaches as well as that of landing. In contrast, after host-plant experience, the females have only learned the contact chemical cues. The lack of learning of volatile cues from host-plant experience may be due to a difference between the volatiles emitted by the plants and those collected by the extraction method (e.g. Heath and Manukian 1992). Females also appear to be more attracted to the volatiles of the *C. maculatum* extract than that of *D. carota*, as was also seen in Chapter 3, which may be stronger than any possible effects of learning for this cue.

The ability of females to identify contact chemical extracts prior to landing is likely due to visual cues resulting from differences in the chemistry of the two plants. The reflectances of the two extracts, as seen on the model leaf surface, are shown in Figure 5.11, and there are clearly differences between the extracts. As discussed previously in Chapter 4, a closely related swallowtail butterfly, *Papilio xuthus*, has visual receptor with peaks at 360, 400, 460, 520, and 600nm (Arikawa, et al. 1987),

covering the range of the divergence thoroughly. This result is worthy of further study, both to identify the chemicals responsible for the divergence in reflectances and to verify that the butterflies are able to see this difference.

Another parallel with Chapter 4 is the apparent linking of cues in the learning behavior of *P. polyxenes*. In those experiments, when each of the chemical cues was paired with leaf shapes, females learned the pair of cues within independent trials, apparently linking the visual cue with the chemical cue. In the current experiment, females learned pairs of chemical cues, approaching or landing most frequently on the same model plant as the first model plant approached or landed upon. In Chapter 3, volatile chemical cues were not observed to be learned when presented without relevant visual or contact chemical cues. If the contact chemical cue is perceived by the females as a visual cue, then it may be that the females are only able to learn volatile chemical cues in conjunction with a visual cue. Although there is evidence for the linking of cues in the learning of honey bees (Bogdany 1978), there is no prior evidence of a butterfly or moth being able to learn a cue only when it is liked with another.

*Papilio polyxenes* females do not touch the surface of a leaf with their antennae. The distinction between volatile and contact chemical cues is thus clearer than it may be in other systems where the antenna does contact the leaf surface, as is the case for *Danaus plexippus* (Nymphalidae) with several of its host plants (Haribal and Renwick 1998). *D. plexippus* appears to use different cues, though certainly different combinations of appendages, to recognize different host plants. For example, antennae were used most often on the host *Asclepias curassavica*, while forelegs were used most often on *A. incarnata*, the most preferred host. A similar situation is seen in *Agraulis vanillae incarnata* (Nymphalidae), where the responses to a single cue do not

explain the preference rankings of the hosts. Some host plants (*Passiflora manicata* and *P. sp.*) release volatiles that are apparently deterrent to searching females (Copp and Davenport 1978). However, when contact with the plants is allowed, *P. manicata* and *P. sp.* receive more eggs than two other species (*P. mollissima* and *P. edulis*) that have neutral (neither stimulatory nor deterrent) volatiles.

This appears to be similar to the situation for *P. polyxenes*, as females are most strongly attracted to *C. maculatum* volatiles (present study and Chapter 3), but respond more strongly to *D. carota* contact chemicals in post-landing assays (Chapter 2), while whole plant assays show a normal distribution of eggs between the two species (Chapter 3). When the two cues are combined, as in the present experiment, the most attractive model plant was that with *C. maculatum* volatile chemical cues and *D. carota* contact chemical cues, regardless of a female's prior experience (e.g. Tables 5.4 and 5.5). This suggests that the more important cues for finding *C. maculatum* are in the volatiles extract, while the more important cues for finding *D. carota* are in the contact chemical extract. Identification of the specific compounds important in *P. polyxenes* oviposition behavior is currently underway (M. Haribal and P. Feeny, pers. comm.), and should elucidate this situation further.

Although it is clear that both volatile and contact chemical cues are important in at least two other swallowtail species, *Papilio demoleus* and *Eurytides marcellus* (Haribal and Feeny 1998, Saxena and Goyal 1978), the current experiment is the first to specifically test both cues simultaneously with the goal of determining the relative roles of each. It has generally been thought that volatile chemicals would play a larger role prior to landing on a plant than after, and that contact chemicals could only play a role after landing on the leaf surface. Ramaswamy (1988), in his review of host finding by moths, determined exactly this pattern from the somewhat limited

information known about moth host location. He postulated that highly polyphagous species would not use olfaction, while oligophagous and monophagous species would, with little to no evidence of odor use after landing upon the leaf surface. Contact chemistry is, by his reasoning, the most important and most common determinant of host acceptance. In fact, this pattern holds for the lepidopteran species tested.

Ramaswamy based some of his ideas on his work with *Heliothis virescens*, a Noctuid generalist. He (and colleagues) had previously found no role for close-range olfaction in this species, and a large role for contact chemoreception once on the leaf surface (Ramaswamy, et al. 1987). Similarly, Foster and Howard (1998) report the same pattern in the generalist *Epiphyas postvittana* (Tortricidae), with little or no pre-landing effect of volatile cues, but a large role for post-landing cues such as contact chemistry and surface texture. Another generalist, *Helicoverpa armigera* (Noctuidae), uses contact and volatile cues, but only at short range, with the long-range use of volatiles unknown, and contact required for oviposition (Jallow, et al. 1999). In contrast, the relative specialist *Agrius convolvuli* (Tortricidae), uses both contact and volatile cues at close range (Shimoda and Kiuchi 1998). Volatiles are used in the selection of the oviposition site, while contact chemical cues stimulate oviposition. Volatile cues alone can stimulate oviposition, though the pairing of cues leads to more response from moths than the sum of either alone, thus demonstrating a synergism between the two cue types.

Unfortunately, due to the apparent linking of volatile and contact chemical cues during learning of these cues, it is not possible to determine a relative importance for each of these cues for the host-finding behavior of *P. polyxenes*. It appears that both types of cue play an important role in both attracting a female to the immediate vicinity of the plant (e.g. approaches) and to actually land on the leaves of a plant. The

ability of a female to recognize contact chemical cues prior to landing is likely due to a visual analog, which is, of itself, worthy of further study. Volatile chemical cues alone (Chapter 3) and contact chemical cues alone (Chapter 2) are both able to stimulate females to lay eggs, though, in each case, the results are skewed towards the cues of one of the two plant species (*D. carota* or *C. maculatum*). Combining the cues in a free-flight assay leads to more natural behavior than the tests of contact chemicals alone (Chapter 2), and more activity than presenting contact chemical cues with visual (leaf shape) cues (Chapter 4).

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CHAPTER SIX  
RESPONSES TO ALL THREE CUES TOGETHER,  
AND RESPONSES TO WHOLE PLANTS

In the previous chapters, the host plants of *Papilio polyxenes* have been broken down into several component parts (contact chemical extracts, volatile chemical extracts, and leaf shapes). The responses of female butterflies to these parts were examined, as were the roles of experience in these responses. In these two final experiments, the sum of those parts is considered. Searching female butterflies were tested for their responses to model plants with all three component cues (contact and volatile extracts and leaf shapes), both before and after host-plant experience. In a second experiment, female responses were again tested, but this time the responses to whole, real plants were tested.

Despite the amount of research that has been performed on the host relations of swallowtail butterflies (see Scriber, et al. 1995), relatively few experiments have been published involving the responses of butterflies to whole host plants with behavioral observations. Preference studies involving relatively small cages (<2m<sup>3</sup>) have been completed for several species, including *Papilio machaon* (Wiklund 1974), several species in the *P. machaon* complex (including *P. polyxenes*) (reviewed in Thompson 1995; Thompson 1998), *P. glaucus* (Scriber 1993), and *P. polyxenes* (P. Feeny and L. Rosenberry, unpublished data). Blau (1981) completed life tables, including data on oviposition rates, for *P. polyxenes* females from central New York and Costa Rica. None of these studies attempted to account for a role of experience in the behaviors, and all used egg counts as opposed to detailed behavioral observations.

An unpublished study by Inger Åhman did attempt to examine the role of experience in the oviposition behavior of *P. polyxenes* using behavioral observations. In one of several experiments, two groups of females were given three days worth of oviposition experience with a host plant, one group with *Daucus carota* (carrot) and one group with *Petroselinum crispum* (parsley). A third group had received no oviposition experience, and was several days younger as well. When released into a flight cage (5 x 3.5 x 2m) with six of each of the two host plants, *D. carota*-trained females were more likely to land on *D. carota* than *P. crispum* (72% versus 28%), while *P. crispum*-trained females were only slightly more likely to land on *P. crispum* than *D. carota* (56% to 44%). Naïve females were nearly equally likely to land on either plant (51% on *D. carota* and 49% on *P. crispum*). Åhman attributes these results to females experienced with *D. carota* learning plant cues, while females experienced with *P. crispum* were deprived and thus less discriminating in their host finding, though eggs were not counted during the three days of host-plant experience to verify this claim.

The current study expands Åhman's, again looking at the role of host-plant experience on the host finding behavior of *P. polyxenes*, but this time using more comparable host plants (*Pastinaca sativa* (wild parsnip) and *Conium maculatum* (poison hemlock)). Trials were controlled for length, and the behavior of individual females was followed during the course of each trial. This chapter also contains the final experiment in the series of model plant experiments, in which the model plants are treated with all three previously tested host-plant cues.

## GENERAL METHODS

*Plants*

The apiaceous host plants, *Pastinaca sativa* (wild parsnip) and *Conium maculatum* (poison hemlock) were grown in a greenhouse atop Seeley Mudd Hall at Cornell University. Seed was collected from wild sites near Ithaca, New York. A non-deterrent non-host, *Vicia faba* (fava bean, Fabaceae), was grown from commercial seed (var. Broad Windsor, Agway). Nectar plants, *Pentas lanceolata* were bought from a nursery and kept in the greenhouse with the bioassay arena.

*Butterflies*

Butterflies were diapausing, first generation offspring of wild females caught during 1999 in southeastern Canada. Butterflies were kept in an environmental growth chamber (Environmental Growth Chambers, Chagrin Falls, OH) with 16:8 LD, 27°C daytime and 15.5°C nighttime temperatures, at 75±5% relative humidity. The growth chamber was devoid of host plant material at all times. Butterflies were fed and numbered as described in Carter and Feeny (1985), with two exceptions. Butterflies were fed a 20% (increased from 10%) by volume solution of honey in water and several colors of Sharpie<sup>®</sup> permanent markers (Sanford, Inc.) were used for numbering to aid in individually identifying females in flight. Females were mated by hand pairing (Carter and Feeny 1985) more than 24 hours after emergence, on the second day after eclosion.

*Analyses*

SAS software version 6.12 (SAS Institute, Inc.) was used for all analyses. Non-parametric statistics were used for behavioral analyses due to the non-normality of the data (Conover 1980). Approaches and landings were primarily used for analyses

due to the large number of both types of behavior, as well as the accuracy for identifying the model plant at which the behavior was directed. Only females displaying a behavior at least once were included in analyses of such behavior.

Responses of naïve females to host and non-host cues or plants and the effect of host-plant experience on the responses of experienced females were both analyzed by the sign test. The null hypothesis was that there was no effect of plant cues or plant experience on behavior. For naïve females, approaches and landings on non-host plants (or model plants with non-host cues) and on host plants (or model plants with host-plant cues), were each summed for each female. For each approaches and landings, the sum for the non-hosts was subtracted from the sum for the hosts and the sign of the result was scored. The same procedure was used for experienced females, except that comparisons were made between the two host plants (or extracts), rather than between host and non-hosts.

The behaviors of experienced females towards host plants (or cues) with respect to host-plant experience and were analyzed using Fisher's exact test. Approaches and landings were each tested in a 2 x 2 table of experience type versus the host plants (or cues of the model plant) receiving the behaviors. To test the effect of host-plant experience on experienced females, a median test was used, again with a null hypothesis of no effect. The numbers of approaches and landings on host plants (or model plants with host-plant cues) were compared between females who had experienced each of the two host-plant species.

Fisher's exact test was used to look for a relationship between the first host plant (or model plant with host extracts) approached or landed upon during each trial

and the host plants (or model plants with host extracts) most often approached or landed upon during the same trial for each female. Fisher's exact test was also used to examine the sequence of some behaviors, with 2 x 2 tables used to examine the relationship between approaches and landings. The Mantel–Haenszel test was used to simultaneously examine the effects of experience, host–plant cues, and the behavior following an approach or landing (Stokes, et al. 1995).

### EXPERIMENT 1: ALL THREE CUES

#### *Specific methods: Leaf shapes*

The leaf shapes used in this experiment were the same used in Chapter 4 (Figure 4.1). The surface area of one side of the leaf shape (not including any stem) was 53.75cm<sup>2</sup> (= 215cm<sup>2</sup>/4). The *C. maculatum* leaf shape was the same as that used in Chapter 3, and was originally based on a shape found attractive by female carrot root flies, *Psila rosae* (Degen and Städler 1997). The shapes used for *P. sativa* and *V. faba* were based on tracings of actual leaves; this was not done for *C. maculatum* due to the high complexity of the leaf shape and the need for more than one hundred leaves of this shape for this experiment alone.

Leaves were cut from green card stock (Hammermill, Bright Hue<sup>®</sup> Cover). Additional stability was added by stapling an additional 10.2cm long by 1.6cm wide strip (of the same paper) that had been folded in half lengthwise to the back side of the stem of each leaf. Leaves were scored with a blade to add dimensionality. Scores of *V. faba* leaves were on the top side of the leaf, so that the leaf was convex; all other leaves were scored on the bottom side.

*Extractions: Contact chemicals*

*C. maculatum*, *P. sativa*, and *V. faba* leaves were collected from the greenhouse plants as in Chapter 2. Leaves were weighed, and 100g batches were blended in 500ml boiling 95% ethanol. The slurry was filtered and the ethanol removed by evaporation under reduced pressure. The remaining aqueous solution was centrifuged and the supernatant was extracted three times each with diethyl ether, chloroform, and ethyl acetate. This aqueous extract contained virtually all of the stimulant activity of the parent extract (Feeny, et al. 1988; Brooks, et al. 1996). The extract was evaporated to a concentration of 5gram leaf equivalents ('gle') per ml and frozen in 0.5ml aliquots.

To prepare extracts for application to model plant leaves, the extracts were diluted to a concentration equivalent to 215cm<sup>2</sup>/4ml with HPLC-grade water. One ml of extract was painted evenly on the top surface of each leaf, with eight leaves each of *C. maculatum* and *P. sativa* and 16 leaves of *V. faba* per trial. The extract was allowed to nearly dry and leaves were flattened, as the moisture caused them to curl. Leaves were prepared several days ahead of time and frozen until needed due to the time needed to apply extracts and dry leaves.

*Extractions: Volatile chemicals*

Volatile chemical extracts were collected as in Chapter 3. *C. maculatum*, *P. sativa*, and *V. faba* leaves were collected from the greenhouse-grown plants. Leaves were weighed with the petioles removed, and 20–30g from a single species were placed in a 2000ml Erlenmeyer flask. Hexane was added to cover all of the leaves (1000ml), and allowed to soak. After five minutes, the hexane was decanted into a clean flask and frozen at –10°C. After all of the samples of a single plant species was extracted, the combined extract was reduced to the desired concentration, as determined in Chapters

4 and 5: *C. maculatum*, 2.99g; *P. sativa*, 1.65g. *V. faba*, 2.48g. The extract was stored in a  $-10^{\circ}\text{C}$  freezer in 1ml aliquots in 2ml vials until needed.

### *Bioassay*

Bioassays took place in a 3.66 x 3.66 x 1.83m cage placed in a large greenhouse module atop Seeley Mudd Hall at Cornell University. The greenhouse module, and all adjacent modules, contained no apiaceous plants at any time during the experiment. The cage had nylon mesh walls and the floor was covered in black landscape fabric (Agway, Inc.). Eight model plants were arranged in a three-by-three array on the floor (with no center plant), and one nectar plant was placed (*Pentas lanceolata* (Rubiaceae)) was placed in each of the four corners. Model plants were the same as first used in Chapter 3, four leaves were attached to each model plant, and a vial of volatiles could be inserted in the center of the model plants. Two model plants received cues (leaf shapes, contact and volatile chemical extracts) of *C. maculatum*, two model plants received cues of *P. sativa*, and four model plants received cues of *V. faba*. The plants were arranged within the cage in an array as in Figure 6.1.

Butterflies were each subjected to four trials, two "naïve" and two "experienced," as shown in Figure 6.2. The first two trials were combined into a single "naïve" category for some analyses. After the second trial, each female was placed in an individual 16 x 31 x 9cm cage with a sprig of one of the two host species (*C. maculatum* or *P. sativa*) in a water-filled aquapic, with the plant species alternating by female so that an equal number of females would receive each plant species. Cages were kept in a greenhouse chamber away from that of the bioassays, and females were fed at least once per day. Females were allowed to lay eggs *ad libitum* until the morning of the third day after mating, at which point they were returned to the growth



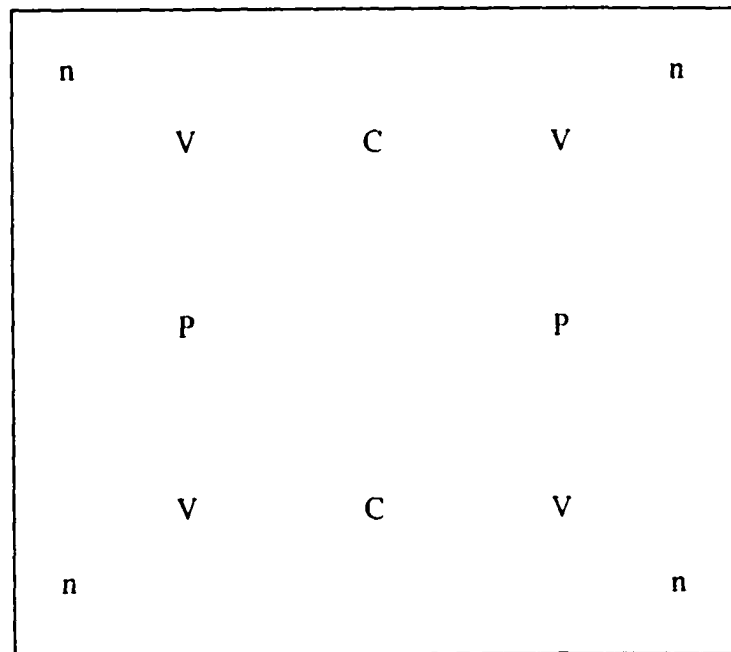


Figure 6.1 The arrangement of model and nectar plants in the first experiment, with all three plant cues. *V*: *Vicia faba* cues; *C*: *C. maculatum* cues; *P*: *P. sativa* cues; *V*: *V. faba* cues; *n*: nectar plants (*Pentstemon lanceolatus*). Two arrangements are possible by rotating the host-plant cued model plants.

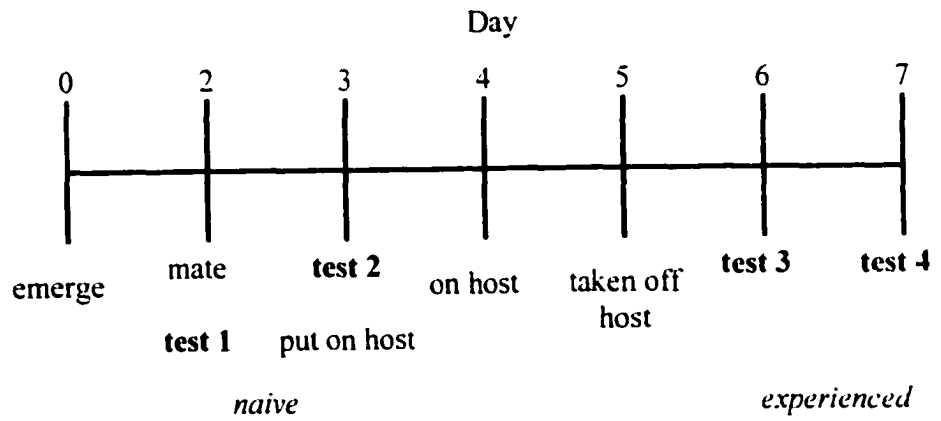


Figure 6.2 Timeline for females in Experiment 1.

chamber devoid of host–plant material. The following day, females were given their third test, their first “experienced” test. The fourth and final test occurred on the fifth day after mating. Again, the two trials after host–plant experienced were combined into a single “experienced” category for some analyses.

Butterflies were transported to the array from the growth chamber in butterfly envelopes (BioQuip) and released onto nectar plants. At least 1 but no more than 23 butterflies were tested at any one time. Activity was sporadic, such that it was possible to watch as many butterflies at one time. A total of 15 trials were used to test 32 butterflies, with a mean of 8.53 butterflies per trial. Trials lasted for 90 minutes and there was no interference during a trial. All behaviors towards the model plants, as well as transitional behaviors and the identities of the behaving butterflies, were recorded using a tape recorder and transcribed the day of the trial. For analysis, behaviors were classified into ten discrete categories, as shown in Table 6.1. The greenhouse temperature was set at 25.5°C, and lights (Sylvania Lumalux LU400, sodium high density discharge) were used to supplement daylight on cloudy days.

## *Results*

### *Responses of naïve females*

Naïve females approached and landed more often on model plants with host–plant cues than non–host cues, but these differences were not significant due to very small sample sizes (Figure 6.3; Sign tests: approaches:  $N=6$ ,  $T=6$ ,  $p=0.1875$ ; landings:  $N=3$ ,  $T=3$ ,  $p=0.25$ ). Naïve females were significantly more likely to follow an approach to a model plant with host–plant cues with a landing than a model plant with non–host cues (Figure 6.4; Fisher’s exact test:  $N=48$ ,  $\chi^2_1=11.859$ ,  $p<0.001$ ). In fact, no females landed on a model plant with the non–host *V. faba* cues.

Table 6.1 Categories of behaviors observed during trials.

<b>Category of Behavior</b>	<b>Examples of Behavior</b>
Non-plant directed behaviors	Fly high (>1m over plants) around cage, Bother other females on side of cage, often transitional between array activity and inactivity
Nectaring behaviors	Nectar, Land on nectar plant
Fly mid over plants	Fly mid-level (<1m, >10cm) over model or host plants, may be directed to particular model or host plants
Fly low over plants	Fly low (<10cm) over model or host plants, may be directed to particular model or host plants
Approach	Approach a model or host plant
Inspect	Flutter low (<5cm) over a model or host plant
Land	Land on a model or host plant
Drum	Drumming behavior on a part of a model or host plant
Curl	Abdomen curl while on a model or host plant
Oviposit	Lay an egg on a model or host plant

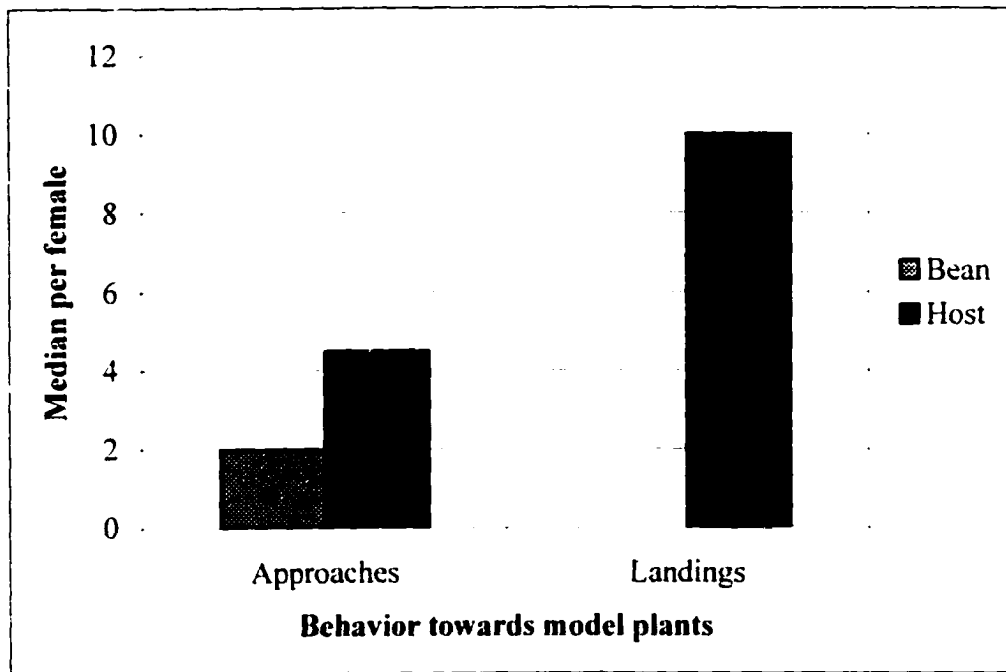


Figure 6.3 Median number of approaches and landings per female in naïve trials in Experiment 1 (all three cues). Host cues are *C. maculatum* + *P. sativa*, the non-host is *V. faba* (bean). (The median number of landings on the non-host is zero.)

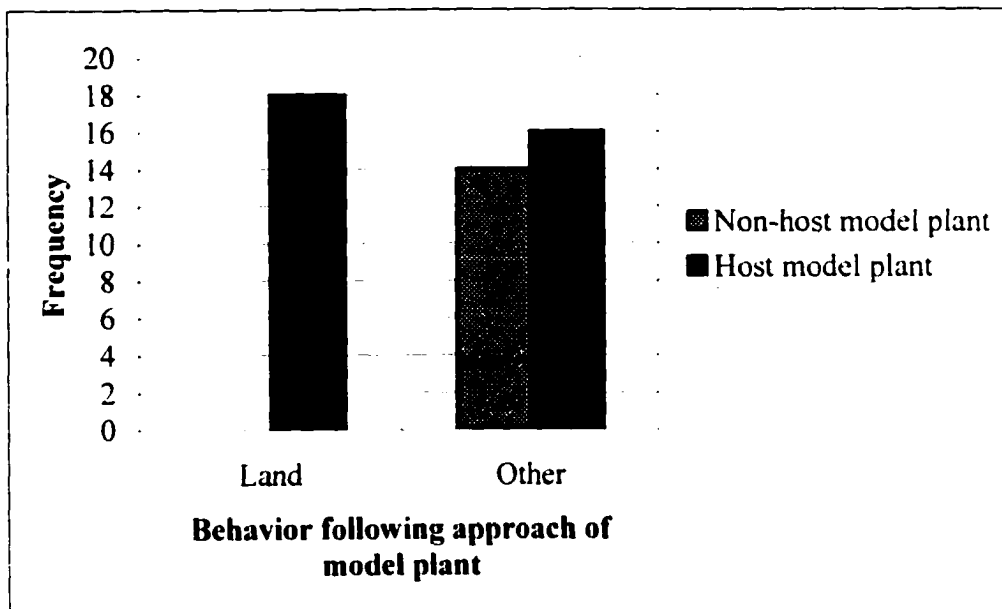


Figure 6.4 Frequency of landings or other behaviors (see Table 6.1) immediately following approaches on model plants with non-host or host cues by naïve females. (There were no landings on non-host model plants by naïve females.)

*Responses of experienced females*

There was not a significant effect of host-plant experience on the tendency of females to approach or land on model plants with the same host-plant cues (Figure 6.5; Fisher's exact test: approaches:  $N=19$ ,  $\chi^2_1=0.891$ ,  $p=0.603$ ; landings:  $N=11$ ,  $\chi^2_1=2.037$ ,  $p=0.455$ ). Females were more likely to approach or land on the model plants with *P. sativa* cues, regardless of host-plant experience, although this was not a statistically significant trend (Figure 6.6; Sign test: approaches:  $N=8$ ,  $T=4$ ,  $p=0.6729$ ; landings:  $N=6$ ,  $T=-1.5$ ,  $p=1.0$ ). Females with *C. maculatum* experience tended to approach and land more often on model plants with host-plant cues, without regard to the specific host-plant, but, again, this was not a significant trend (Figure 6.7; Median test: approaches:  $N=8$ ,  $\chi^2_1=0.4667$ ,  $p=0.4945$ ; landings:  $N=6$ ,  $\chi^2_1=1.25$ ,  $p=0.2636$ ).

*Within-trial experience effects*

There was a trend for females to approach most often the model plants with the same host-plant cues as the first such model plant approached (Figure 6.8A; Fisher's exact test:  $N=13$ ,  $\chi^2_1=3.745$ ,  $p=0.103$ ). This trend was not seen for landings: although females first landing on a model plant with *P. sativa* cues all landed mostly on such model plants, females landing first on a model plant with *C. maculatum* cues were equally likely to land most often on model plants with either set of host-plant cues (Figure 6.8B; Fisher's exact test:  $N=8$ ,  $\chi^2_1=2.667$ ,  $p=0.429$ ). There was no effect of female experience and the behavior following an approach or landing while taking the host-plant cues also into account (Table 6.2 and Table 6.3; Mantel-Haenszel test: approaches:  $N=17$ ,  $T=1.123$ ,  $p=0.289$ ; landings:  $N=8$ ,  $T=0.5$ ,  $p=0.823$ ).

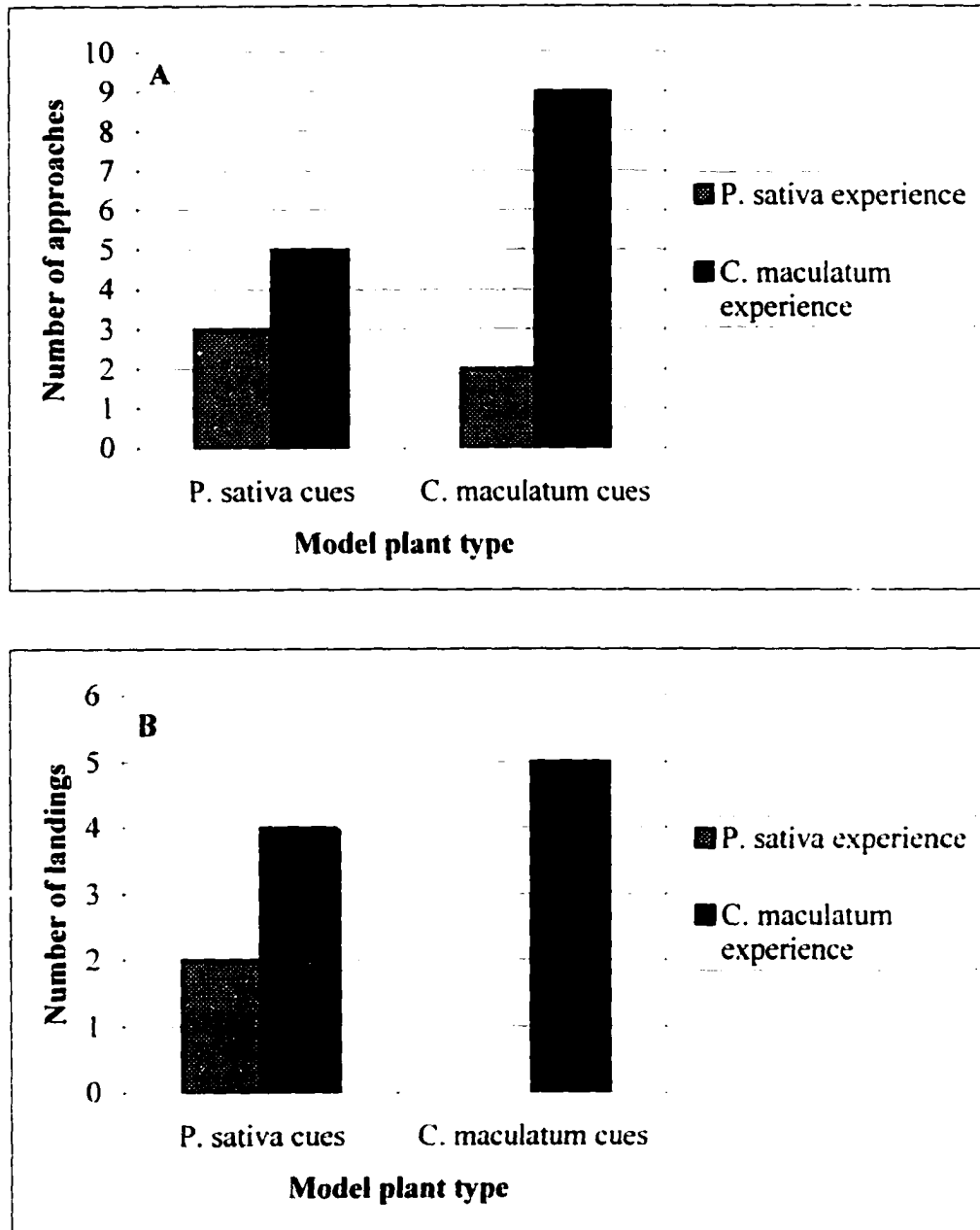


Figure 6.5 Number of approaches (A) or landings (B) by females in experienced trials on model plants with host-plant cues with respect to host-plant experience. (No females with *P. sativa* experience landed on a model plant with *C. maculatum* cues.)



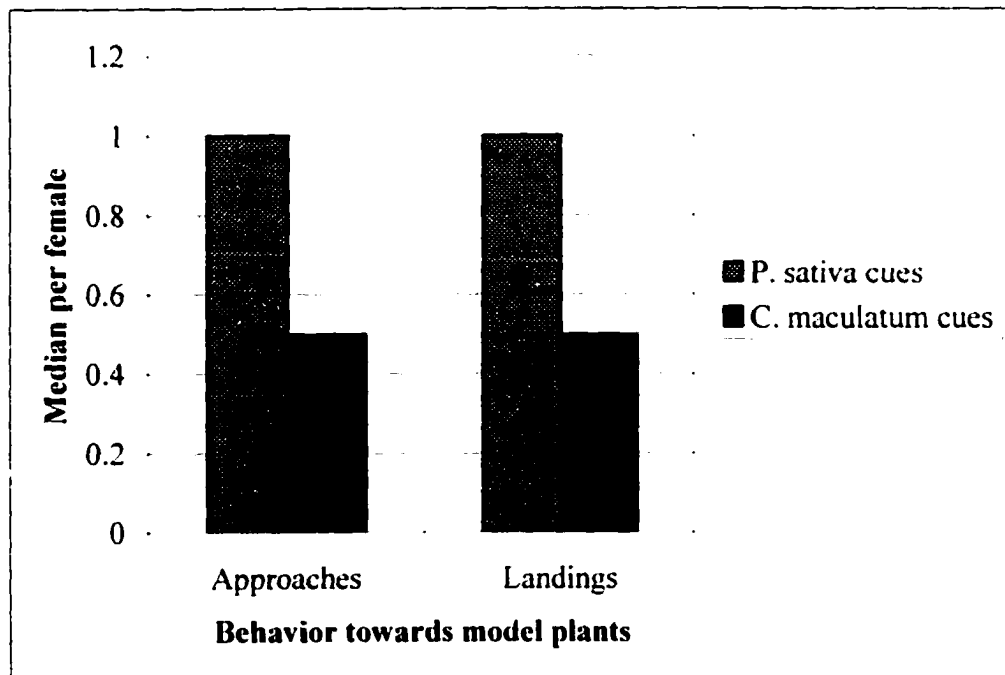


Figure 6.6 Median number of approaches and landings by females in experienced trials on model plants with host-plant cues with respect to the two types of host-plant cues.

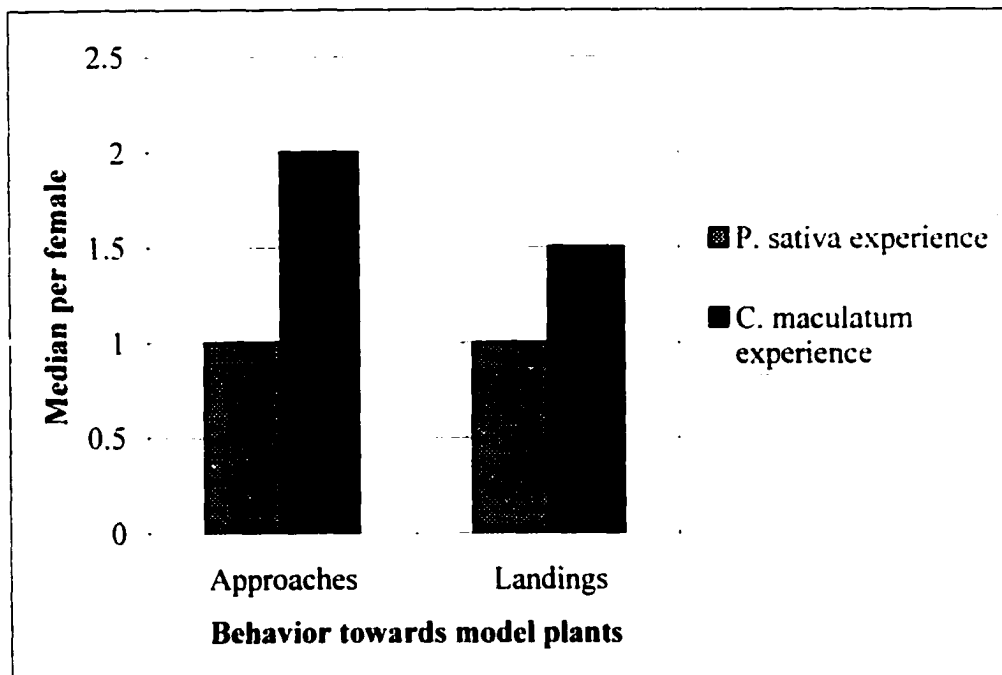


Figure 6.7 Median number of approaches and landings by experienced females on model plants with host-plant cues (*C. maculatum* + *P. sativa*) with respect to the host plant experienced.

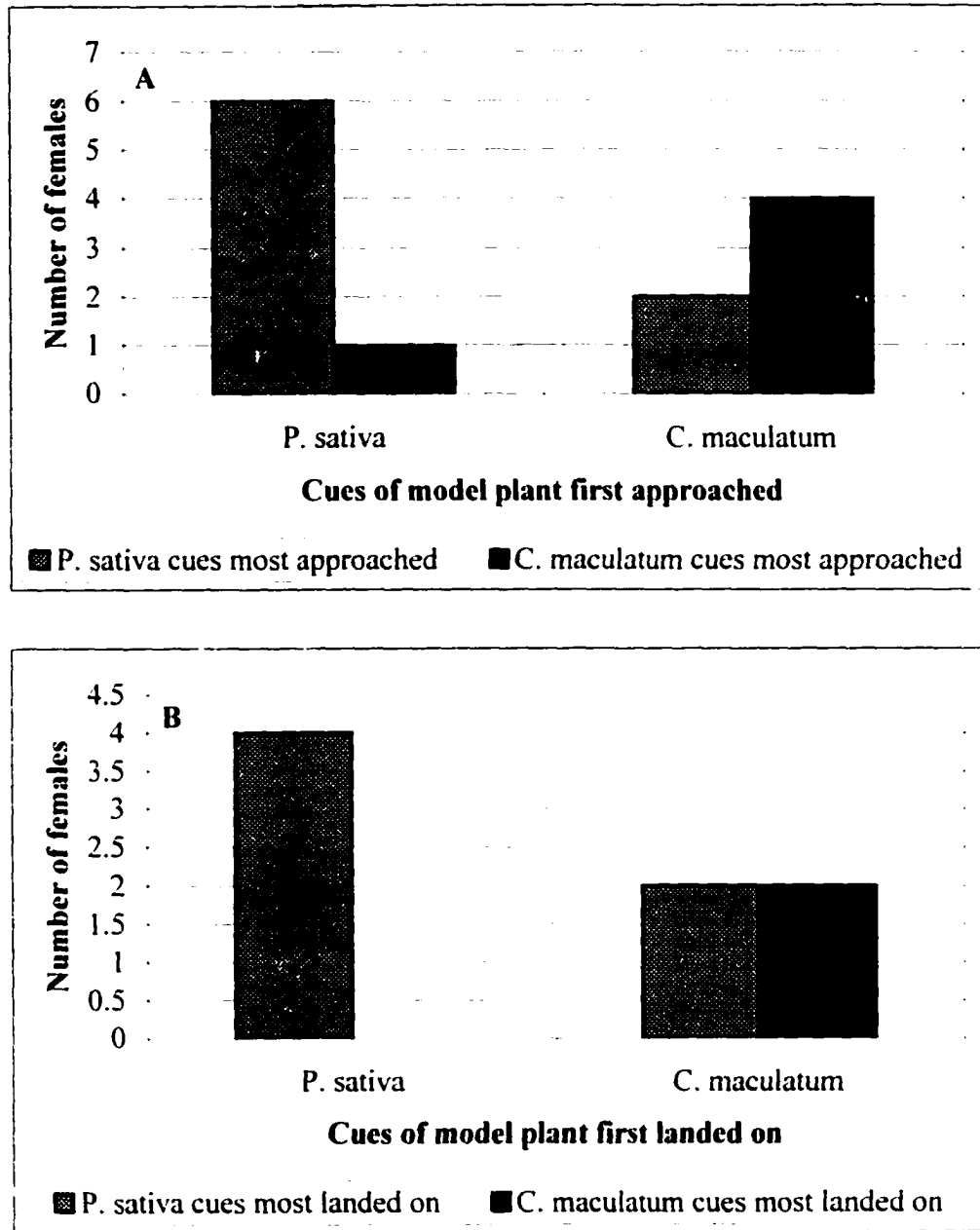


Figure 6.8 Number of females approaching (A) and landing (B) most often on model plants bearing host-plant (*C. maculatum* or *P. sativa*) cues with respect to the type of model host plant first landed upon during a single trial. (No females landed first on a model plant with *P. sativa* cues and most on a model plant with *C. maculatum* cues.)

Table 6.2 Within-trial experience effects of experience and model host-plant cues on post-approach behaviors in Experiment 1.

Experience	Host-plant Cues	Observed		Expected	
		Land	Other	Land	Other
<i>P. sativa</i>	<i>P. sativa</i>	2	1	1.5	1.5
	<i>C. maculatum</i>	0	1	0.5	0.5
<i>C. maculatum</i>	<i>P. sativa</i>	4	1	3.462	1.539
	<i>C. maculatum</i>	5	3	5.539	2.462

Table 6.3 Within-trial experience effects of experience and model plant on post-landing behaviors in Experiment 1. ("Land+" refers to landing, drumming, or curling.)

Experience	Host-plant Cues	Observed		Expected	
		Land+	Other	Land+	Other
<i>P. sativa</i>	<i>P. sativa</i>	1	0	1	0
	<i>C. maculatum</i>	0	0	0	0
<i>C. maculatum</i>	<i>P. sativa</i>	2	1	2.143	0.857
	<i>C. maculatum</i>	3	1	2.857	1.143

## EXPERIMENT 2: WHOLE PLANTS

*Specific methods: Bioassay*

Bioassays took place in the same large cage set up in the same greenhouse module as in Experiment 1. Whole plants were used for all trials. Two *C. maculatum* plants, two *Pastinaca sativa* plants, and four *Pentas lanceolata* plants were arranged as shown in Figure 6.9. Plants were grown in two-gallon (7.57l) pots and were approximately seven months old, having been started from seed. Plants were placed on inverted two-gallon pots in order to raise them above the level of the floor. The same plants were used for every trial; eggs were removed from the plants after each trial, though *P. polyxenes* has never been shown to recognize conspecific eggs.

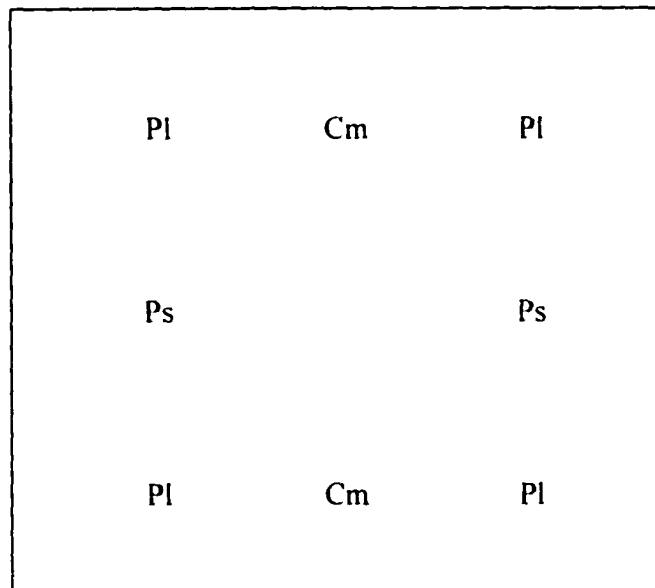


Figure 6.9 The arrangement of model and nectar plants in the second experiment. with all real plants. *Cm*: *C. maculatum* plant; *Ps*: *P. sativa* plant; *Pl*: *Pentas lanceolata* nectar plant. Two arrangements are possible by rotating the host plants.

Each female butterfly was subject to two trials, one “naïve” and one “experienced,” as seen in Figure 6.10. After the first trial, each female was placed in an individual 16 x 31 x 9cm cage with a sprig of one of the two host species (*C. maculatum* or *P. sativa*) in a water-filled aquapic, with the plant species alternating by female so that an equal number of females would receive each plant species. Cages were kept in a greenhouse chamber away from that of the bioassays, and females were fed at least once per day. Females were allowed to lay eggs *ad libitum* until the morning of the third day after mating, at which point they were returned to the growth chamber devoid of host-plant material. The following day, females were given their second and final test, their “experienced” test.

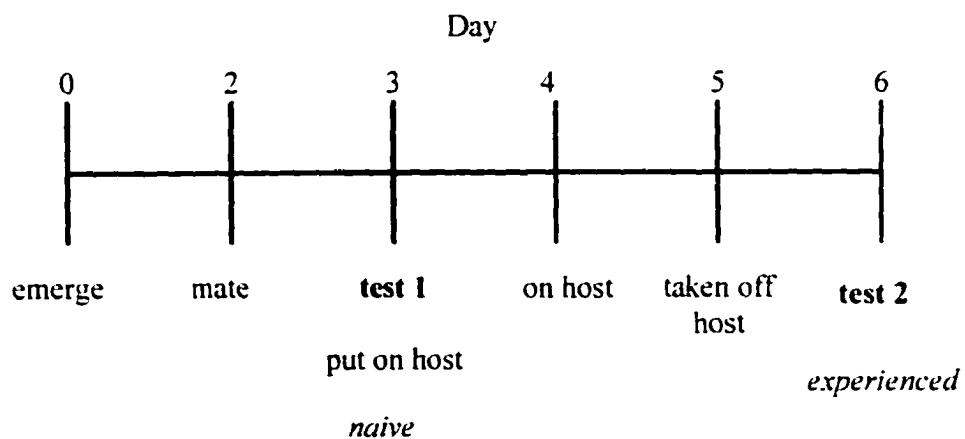


Figure 6.10 Timeline for females in Experiment 2.

Butterflies were transported to the array from the growth chamber in butterfly envelopes (BioQuip) and released onto a *Pentas lanceolata* nectar plant. At least one

but no more than six butterflies were tested at any one time. Activity was sporadic, such that it was possible to watch as many butterflies at one time. A total of 12 trials was used to test 21 butterflies, with a mean of 3.58 butterflies per trial. Trials lasted for 30 minutes and there was no interference during a trial. All behaviors towards the plants, as well as transitional behaviors and the identities of the behaving butterflies, were recorded using a tape recorder and transcribed the day of the trial. For analysis, behaviors were classified into ten discrete categories, as shown in Table 6.1. The greenhouse temperature was set at 25.5°C, and lights (Sylvania Lumalux LU400, sodium high intensity discharge) were used to supplement daylight on cloudy days.

## *Results*

### *Responses of naïve females*

Naïve females were significantly more likely to approach a plant if it was a host plant, even when the non-hosts were nectar plants (Figure 6.11A; Sign test:  $N=10$ ,  $T=19.5$ ,  $p=0.0469$ ). The effect for landings was similar, but the result was less significant (Figure 6.11B; Sign test:  $N=10$ ,  $T=17.5$ ,  $p=0.0742$ ). Females were slightly more likely to land on a host plant following an approach, as opposed to the nectar plants (Figure 6.12A; Fisher's exact test:  $N=177$ ,  $\chi^2_1=3.454$ ,  $p=0.071$ ). Once a female had landed on a plant, she was much more likely to either land again, drum on the surface, or curl her abdomen if the plant was a host plant, with no females showing such behaviors on the nectar plants (Figure 6.12B;  $N=139$ ,  $\chi^2_1=62.632$ ;  $p<0.001$ ).

### *Responses of experienced females*

While there was no effect of the host-plant experienced on the likelihood of a female to approach the same plant during an "experienced" trial (Figure 6.13A; Fisher's exact test:  $N=110$ ,  $\chi^2_1=2.203$ ,  $p=0.176$ ), there was a significant effect on the likelihood of a



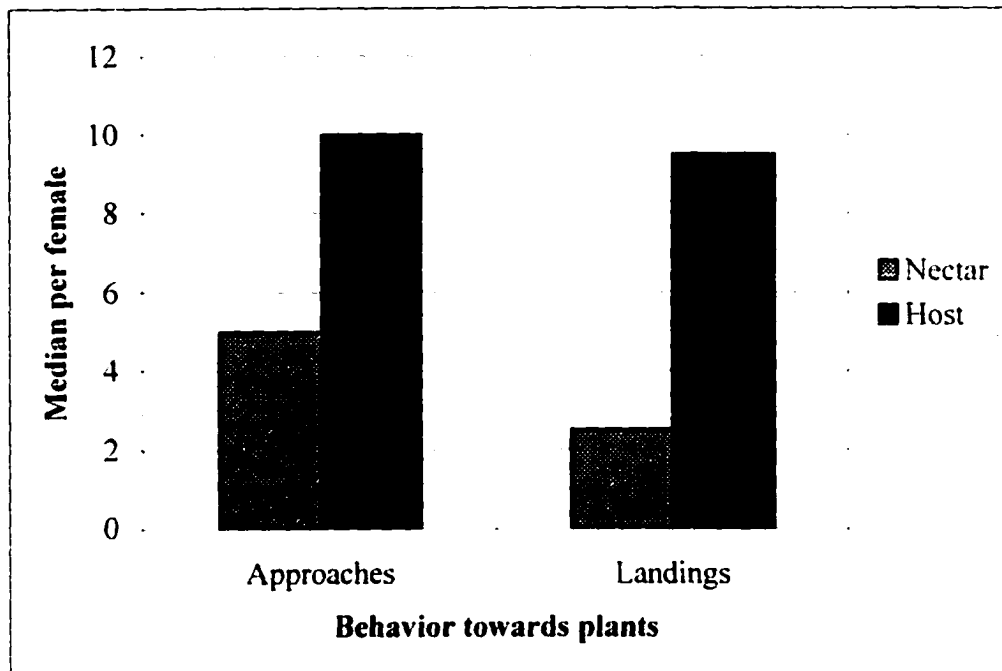


Figure 6.11 Median number of approaches and landings per female in naïve trials in Experiment 2, with whole plants. Host plants are *C. maculatum* + *Pastinaca sativa*. Nectar plants are *Pentas lanceolata*.

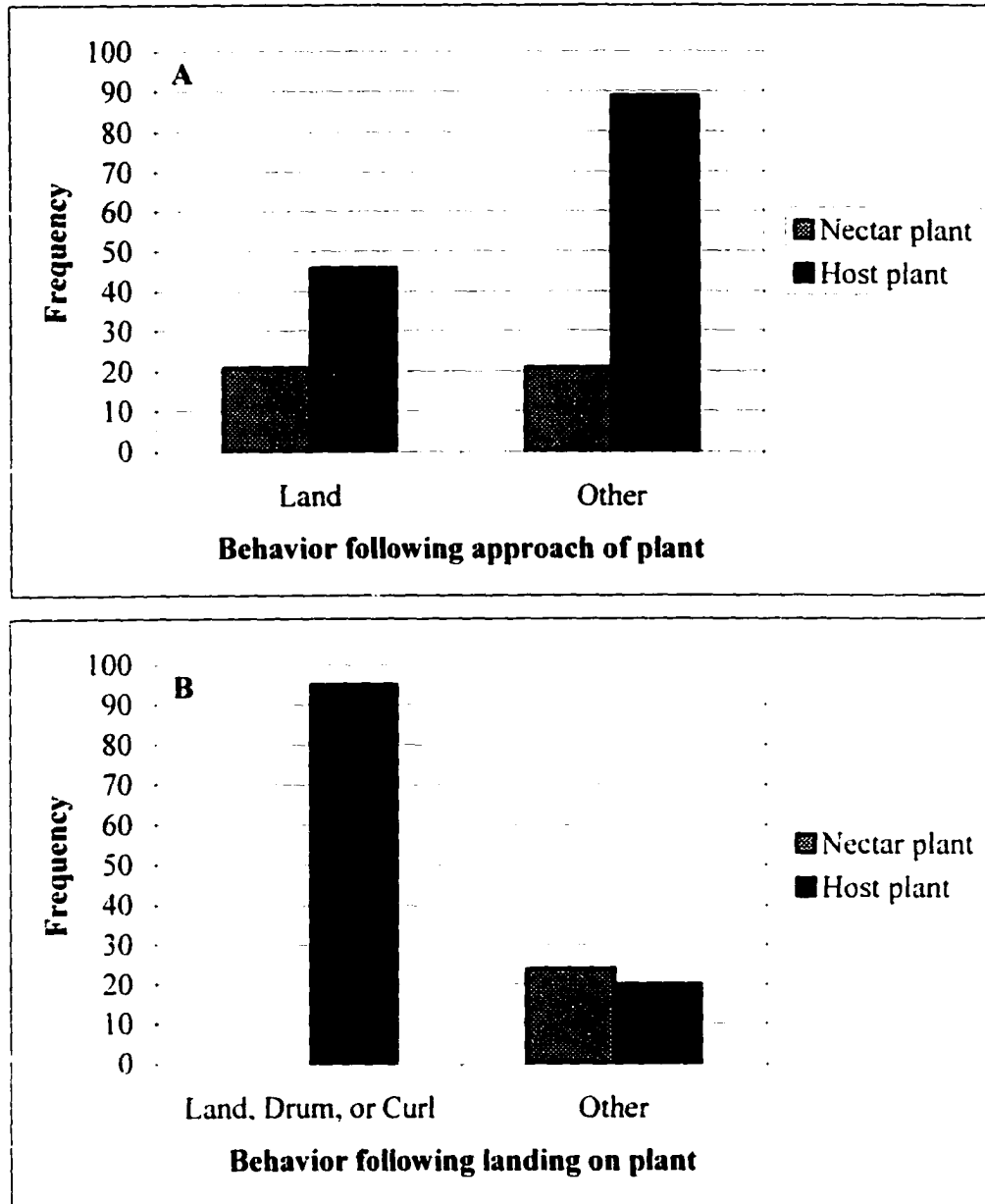


Figure 6.12 Frequency of behaviors following approaches (A) and landings (B) by naïve females in Experiment 2. Behaviors after approaches are split into landings or other, pre-landing behaviors (see Table 6.1), while behaviors after landings are split into post-landing behaviors (landing again, drumming, or curling) or other, pre-landing behaviors. (All females landing on nectar plants performed a pre-landing behavior following the landing.)

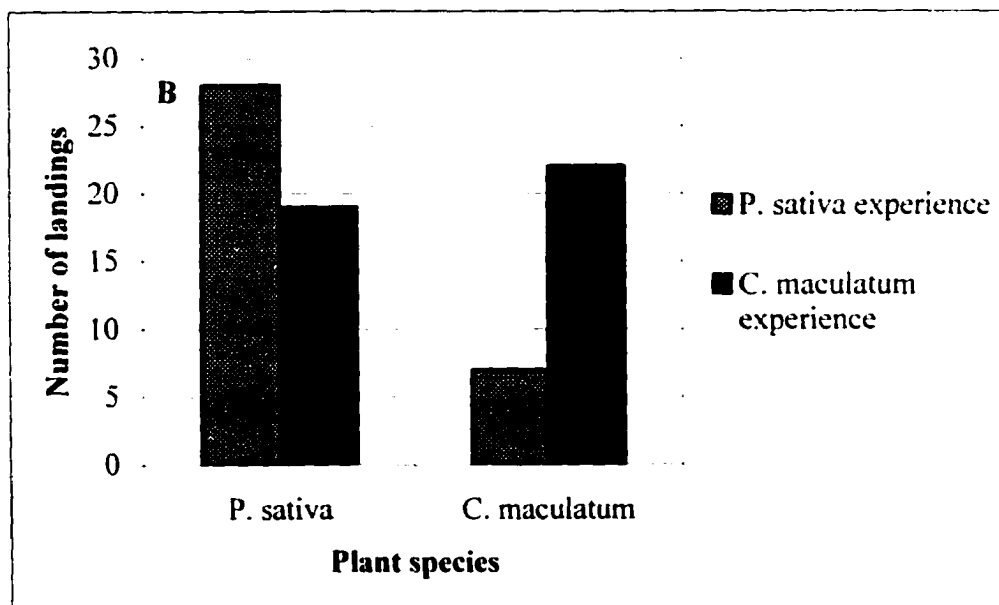
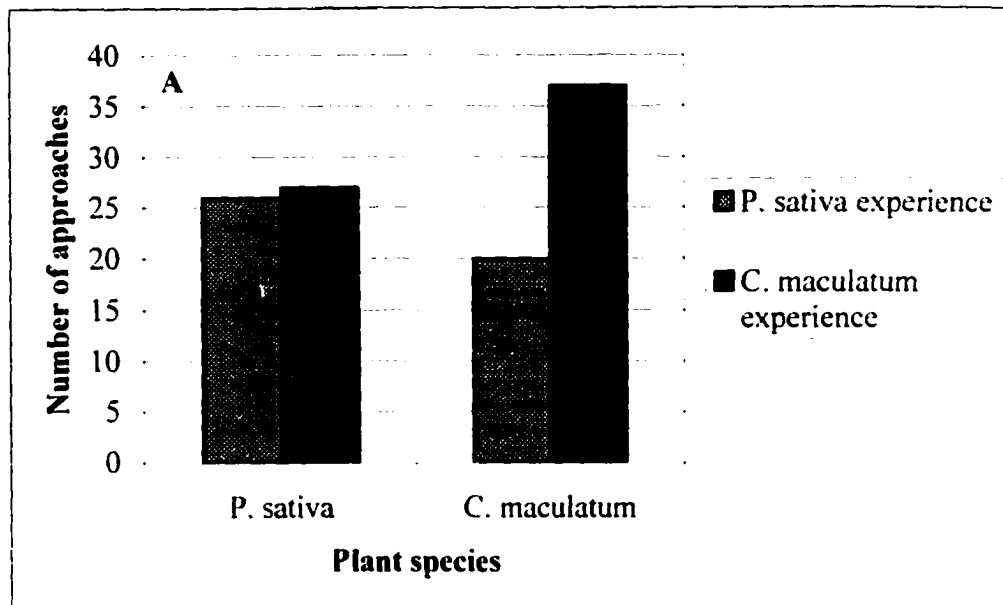


Figure 6.13 Number of approaches (A) and landings (B) by females in experienced trials on host plants with respect to the host plant experienced and the plant species.

female to land on the same plant during an “experienced” trial (Figure 6.13B; Fisher’s exact test:  $N=76$ ,  $\chi^2_1=9.065$ ,  $p=0.004$ ). There were no significant differences in the number of approaches or landings on the two species of host plant, regardless of a female’s experience (Figure 6.14; Sign test: approaches:  $N=7$ ,  $T=1.5$ ,  $p=0.8906$ ; landings:  $N=5$ ,  $T=-2$ ,  $p=0.625$ ). There was also no significant effect of the number of approaches or landings to the host plants, regardless of species, during the “experienced” trials (Figure 6.15; Median test: approaches:  $N=7$ ,  $\chi^2_1=0.05$ ,  $p=0.8231$ ; landings:  $N=5$ ,  $\chi^2_1=0.111$ ,  $p=0.7389$ ).

#### *Within-trial experience effects*

There was no significant relationship between the first host plant approached or landed on during a trial and the host plant most frequently approached or landed on during a trial (Figure 6.16; Fisher’s exact test: approaches:  $N=14$ ,  $\chi^2_1=0.311$ ,  $p=1$ ; landings:  $N=9$ ,  $\chi^2_1=0.225$ ,  $p=1$ ). There were also no effects of female host–plant experience and the behavior following an approach or landing while taking the host plant targeted by the approach or landing into account (Table 6.4 and Table 6.5; Mantel–Haenszel test: approaches:  $N=108$ ,  $T=0.001$ ,  $p=0.972$ ; landings:  $N=75$ ,  $T=0.845$ ,  $p=0.358$ ).

## DISCUSSION

Overall, the responses in these trials were somewhat disappointing, as female response rates were relatively low both in terms of the number of responses per female (with model plants only) and number of females responding (both experiments). The reasons for this are not known, but are probably due in part to the fact that these experiments took place in the fall with diapausing females from the previous fall. The butterflies were not entirely unresponsive, but were less so than their counterparts in earlier

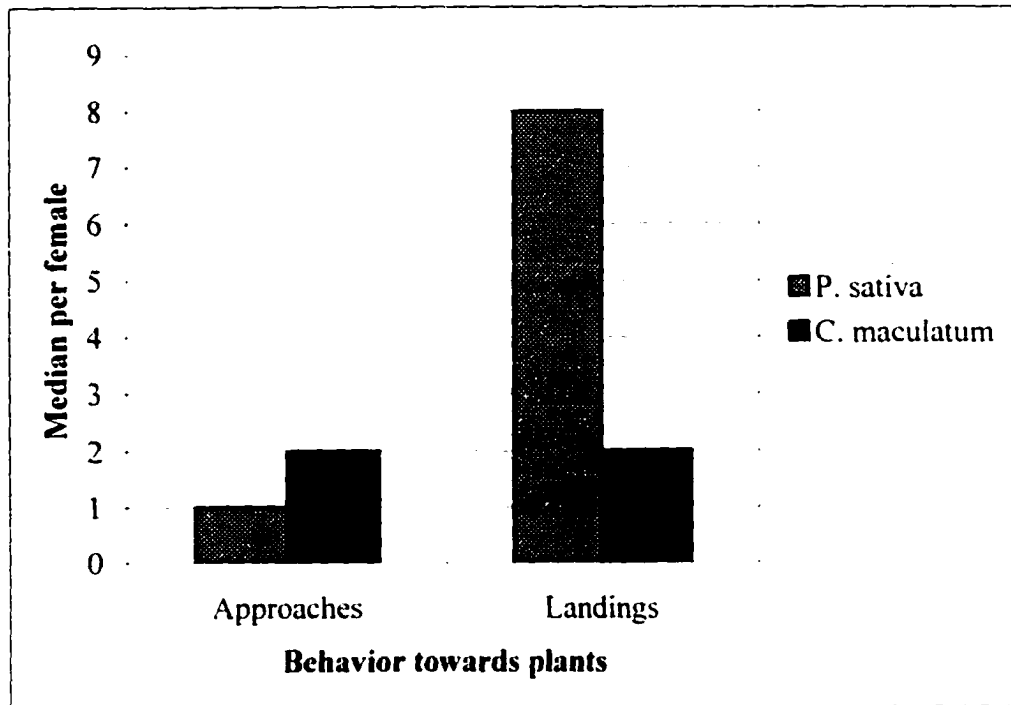


Figure 6.14 Median number of approaches and landings by females in experienced trials on host plants, with respect to only the plant species.

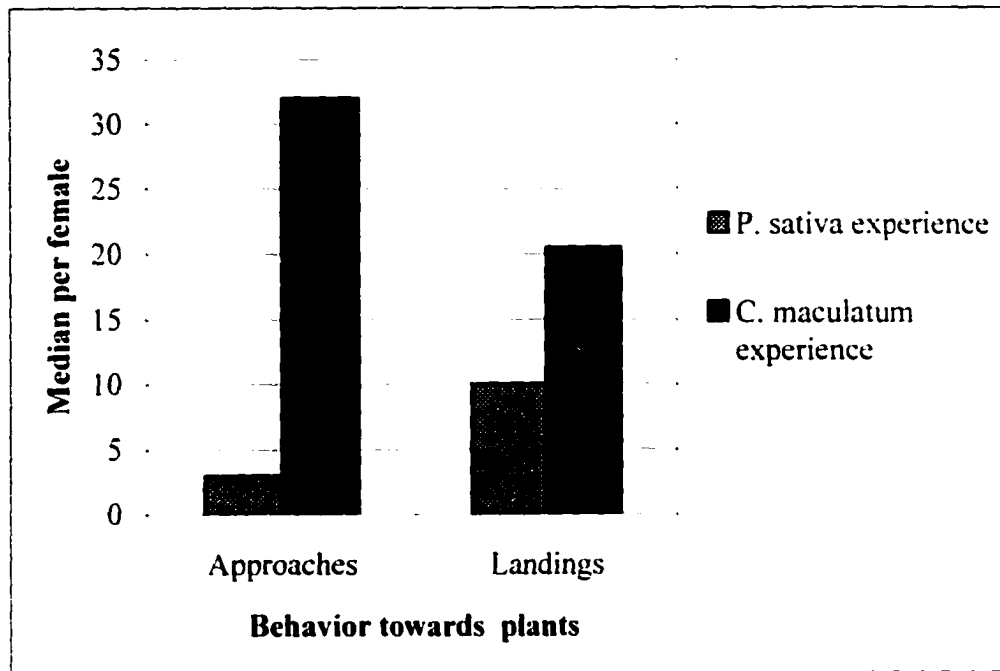


Figure 6.15 Median number of approaches and landings by experienced females on host plants (*C. maculatum* + *Pastinaca sativa*) with respect to the host plant experienced.

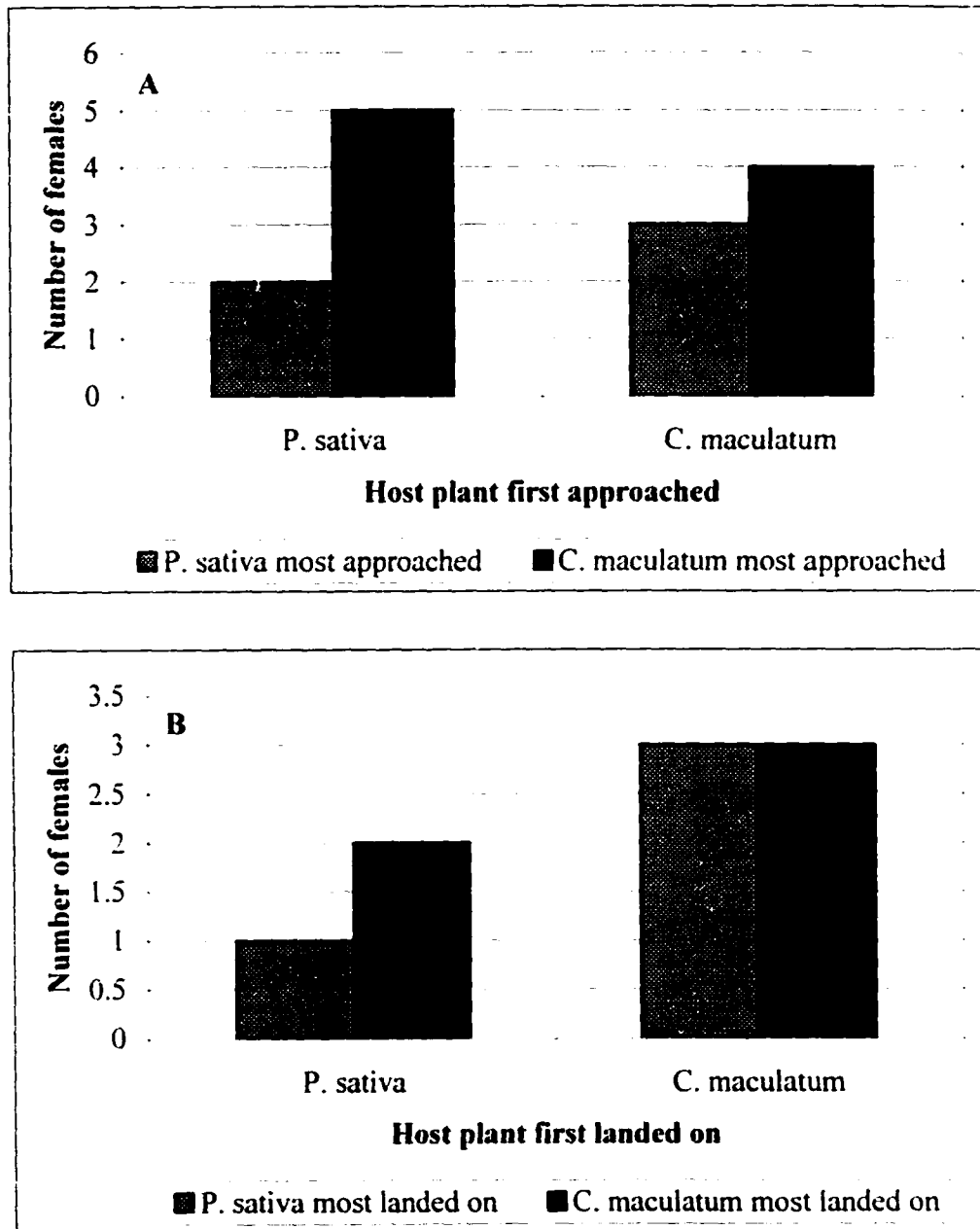


Figure 6.16 Number of females approaching (A) and landing (B) most often on host plants (*C. maculatum* or *P. sativa*) with respect to the species of host plant first landed upon during a single trial.

Table 6.4 Within-trial experience effects of host-plant experience and host-plant species on post-approach behaviors in Experiment 2 (whole plants).

Experience	Host-plant	Observed		Expected	
		Land	Other	Land	Other
<i>P. sativa</i>	<i>P. sativa</i>	6	20	6.356	19.64
	<i>C. maculatum</i>	5	14	4.644	14.36
<i>C. maculatum</i>	<i>P. sativa</i>	4	22	3.714	22.29
	<i>C. maculatum</i>	5	32	5.286	31.71



Table 6.5 Within-trial experience effects of host-plant experience and host-plant species on post-landing behaviors in Experiment 2. ("Land+" refers to landing again, drumming, or curling.)

Experience	Host-plant	Observed		Expected	
		Land+	Other	Land+	Other
<i>P. sativa</i>	<i>P. sativa</i>	24	4	24	4
	<i>C. maculatum</i>	6	1	6	1
<i>C. maculatum</i>	<i>P. sativa</i>	14	4	15.3	2.7
	<i>C. maculatum</i>	20	2	18.7	3.3

experiments (Chapters 3, 4, and 5). As a result, many of the sample sizes are too small for statistically significant outcomes.

Nonetheless, some trends were still evident. In the trials with model plants, females responded preferentially to host-plant cues prior to host-plant experience, with no females landing on model plants with non-host cues (Figure 6.3). There appears to be a trend for females to approach most often the model plants with the same cues as the first model plant approached (Figure 6.8A), perhaps demonstrating a role of experience in the host finding behavior in the arena, but this is not significant.

Similarly, in the trials with whole plants, females were more likely to approach and land on host plants than non-hosts, even when the non-hosts were similarly-sized nectar plants (Figure 6.11). They were somewhat more likely to land on a host plant following an approach, but were much more likely to follow a landing on a host plant with further post-landing behaviors than to do so on the non-host nectar plants (Figure 6.12). No landings on the non-host plants resulted in drumming or curling. There was also evidence for females learning during the host-plant experience in between the two trials, although not within a single trial. Females were much more likely to land on the host-plant they had been given in between the two trials than would be expected by chance (Figure 6.13B), particularly for females who had experience with *C. maculatum*. This pattern was not seen for approaches, as females experienced with *P. sativa* were equally likely to approach either host species, although females experienced with *C. maculatum* approached *C. maculatum* most often. Interestingly, there were no significant within-trial experience effects (Figure 6.16), although this may be a result of either the relatively small sample size (14 or 9

female-trial pairs) or of the shorter trial length (30 minutes, as opposed to 90 in earlier experiments).

The ability of *P. polyxenes* females to learn their host plants with experience was previously suggested by Åhman's results, though they were not conclusive, and learning was only shown for one host plant in the pair (*Daucus carota*, but not *Petroselinum crispum*). In the present case, using two host plants that females regularly encounter in the field (*P. crispum* is not commonly found outside gardens) (Scriber and Feeny 1979), females were found to land more often on the plant previously experienced (Figure 6.13). Females also landed on the host that had not been experienced, and this is not evidence against the learning ability of the females, but rather evidence that they do not exclusively and irreversibly learn.

Learning is most easily, but not necessarily best, described as "a change in behavior with experience" (e.g., Shettleworth 1984). Better is to judiciously add additional criteria, as suggested by Papaj and Prokopy (1989), including the repeatability of the phenomenon (to exclude chance), a gradual change in the behavior (to exclude motor programs, though it also, unfortunately, excludes single trial learning as well), and reversibility (to exclude maturational processes). The host finding behavior of *P. polyxenes* likely fits these criteria, with repeatability in the form of a statistically significant result from the sample of females tested. The learning curve has not been extracted from these data, but there is a possibility of single trial learning, as seen in the significant relationship between the first model plant approached and the model plants approached most frequently in Experiment 1. Finally, the effect is reversible, as not all females landed exclusively on the plants experienced nor the model plants first landed upon, thus demonstrating a

non-permanent change in the behavior. Additional proof of the reversibility is the lack of correspondence between the first plant approached or landed upon and the plant most frequently approached or landed upon in Experiment 2, as females were clearly not approaching or landing only upon a single plant species within individual trials.

The exact type of learning cannot be determined from these experiments. The two likely candidates are sensitization (an increase in the response after experience) or associative learning (or conditioning), with the association of innate cues, such as the contact chemistry (Chapter 2) with other, initially neutral cues, such as overall plant shape (Papaj and Prokopy 1989). Examination of each type of cue (both contact and volatile chemical cues and a visual cue, leaf shape) has not been able to elucidate the type of learning (Chapters 3, 4, and 5). Especially for the chemical cues, each type of cue is likely composed of several specific cues, and until the specific compounds capable of eliciting a host finding or oviposition response from *P. polyxenes* have been identified, this determination cannot be made. It is likely that each of the chemical cues consists of both compounds eliciting an innate response and compounds or characteristics (e.g. visual aspects of contact chemistry, see Chapters 4 and 5) that can be learned. Fortunately, work is in progress to identify all of the compounds eliciting a response from *P. polyxenes* in the two host plants studied in these two experiments, *Pastinaca sativa* and *Conium maculatum*, as well as a third host plant, *Daucus carota* (M. Haribal and P. Feeny, pers. comm.).

Learning in relation to oviposition behavior has been demonstrated in several species of phytophagous insects. Among the Lepidoptera, notable examples include the learning of colors in association with host contact chemistry in *Pieris rapae* (Traynier 1984) and *P. brassicae* (van Loon, et al. 1992), the learning of leaf shapes in

association with host contact chemistry in the pipevine swallowtail, *Battus philenor* (Rausher 1978; Papaj 1986), the learning of host odor by *Trichoplusia ni* (Landolt and Molina 1996), the learning of odor cues by *Helicoverpa armigera* (Cunningham, et al. 1998), and the short-term learning of general host-plant cues by three *Colias* species (Stanton and Cook 1983). In contrast, *Euphydryas editha* has been shown to be unable to learn in its host finding behavior (Parmesan, et al. 1995). Phytophagous flies are best represented by the apple maggot fly, *Rhagoletis pomonella*, which can even learn to differentiate between biotypes within a species of its host plants (apples) (Prokopy and Papaj 1987). The weevil *Diaprepes abbreviatus* is most likely to approach the host plant that it has most recently experienced (Harari and Landolt 1999), with odors implicated as the learned cue. Even generalist grasshoppers have been trained to associate colors or odors with a foodplant (Lee, et al. 1987).

Learning is predicted when the availability of a resource is predictable within a generation, but unpredictable from one generation to the next (Stephens 1993). The most common host plants for *P. polyxenes* throughout most of its range are introduced species living in disturbed environments (Scriber and Feeny 1979; Blau 1981). In addition, the suitability of these plants as hosts and the apparency of these plants for host finding is likely to vary throughout the year (pers. obs.). The specific location of the plants will vary greatly over longer timeframes as succession proceeds in a disturbed environment, although presently many host plant species are found in perpetually disturbed environments, such as along railroad tracks, roads, and landfills (pers. obs.). The ability of *Papilio polyxenes* females to learn in their host finding behavior is predictable from Stephens' (1993) hypothesis, as the host plants are predictable within a generation, but the apparency of the same plants will vary across generations. By learning cues of the available and apparent hosts, females can

decrease the time they spend searching for host plants and thus increase the number of eggs laid. Given a high mortality for the larval stages in this species (Feeny, et al. 1985), any increase in the number of eggs laid is likely to result in a higher fitness level for that female. Therefore, the ability of this butterfly to learn may be predictable, and though it has now been demonstrated, there are details yet to be worked out.

#### ACKNOWLEDGEMENTS

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## CHAPTER SEVEN

### EPILOGUE

In the previous chapters, the host plants of *Papilio polyxenes* have been broken down into component parts and then combinatorially put back together again. Throughout, the host-finding responses of mated female *Papilio polyxenes*, both before and after host-plant experience, were examined. Two main questions were asked at each step: 1) Do females respond to the cues presented prior to adult experience with a host plant? and 2) Is there any evidence of learning in the females' responses to each cue or set of cues? The first question relates to the presence of an innate response in the behavior of these butterflies. The second, to the flexibility inherent in these behaviors. What roles do "nature" and "nurture" play in the oviposition behavior of *P. polyxenes*?

The answer is nearly as complex as the questions are simple. In Chapters 2, 3, and 4, each component part (contact chemistry, volatile chemistry, and leaf shapes) was tested individually. The pairings of leaf shape with each type of chemical cue were also included in Chapter 4, while the pairing of the two types of chemical cues was the subject of Chapter 5. In Chapter 6, the host plants were put back together again, first as the sum of the components from the earlier chapters, and finally as the whole, real plants. Contact chemicals alone (Chapter 2) were tested using a filter paper bioassay, while the remaining cues or sets of cues (Chapters 3–6) were tested using free-flight bioassays. While the details of the answers to each question for each cue or set of cues can be found in the appropriate chapter, the main themes of each chapter can be woven together to create a more complete picture of the host-finding behavior of *P. polyxenes*.

*The naïve response*

Prior to any adult experience with a host plant, female *P. polyxenes* will preferentially respond to host plant extracts as opposed to extracts of a non-host. Both contact and volatile chemical cues generated a strong response from naïve females, either alone, paired together, or in combination with leaf shapes. In most cases, once a female had approached a model plant with chemical cues, it was more likely to land if the model plant had host cues than non-host cues.

The responses to model leaf shapes were more complex. The females did not appear to have a preference for any particular shape. When shapes were combined with chemical cues, though, the females were more likely to land on model host plants than non-hosts. They were also significantly more likely to land again, drum, or curl on the model plant if it contained host volatiles and leaf shapes.

The responses to chemical cues are specific to the hosts, in that most of the approaches and landings were on model host plants. The approaches and landing were not entirely on model host plants; this is likely due to both the non-deterrence of the non-host used in these experiments (*Vicia faba*) and the probability that the solvent alone was at least slightly attractive. Nonetheless, in every experiment containing chemical cues, at least one statistical test of naïve females was significant, whether it was the numbers of approaches or landings, or the type of behavior following an approach or landing. All significant results were in the direction of a preference for host cues, as opposed to the non-host cues.

In addition, naïve females also approached and landed more often on whole host plants than non-host nectar plants in the final experiment. Although the nectar

plants were also attractive to the females, as they readily fed on these plants, females spent most of their time during the trials with the whole plants in a host searching or ovipositing mode, as opposed to a nectaring mode.

*The role of experience: Host-plant experience*

For each cue or set of cues, females were tested after host-plant experience as well as before. Females were given experience by being individually caged with a sprig of one of the two host plants being used in the current experiment. They were allowed to lay eggs *ad libitum* for at least 36 hours, giving ample time for females to learn any host-plant cues.

In only three of the eight cases was an effect of host-plant experience seen. In two of these cases, contact chemical cues were implicated, while the third case is that of the whole plants. In the experiment with contact chemical cues and leaf shapes, there was a significant effect of host-plant experience on the model host plant approached. The direction of this effect, however, was not one to suggest a positive influence of the contact chemicals; rather, the females were more likely to approach a model host plant with the contact chemicals of the plant that was not experienced. The two host plants for this experiment were *Daucus carota* and *Pastinaca sativa*.

In contrast, in the experiment with contact and volatile chemical cues, females were significantly more likely to approach and land on the model host plants with the same contact chemical cues as the host plant experienced. The model plants used in this experiment were *Conium maculatum* and *P. sativa*. It is unclear why females would appear to be learning the contact chemical cues and avoiding them in one case, while being attracted to them in another. It is possible that the addition of volatile

chemical cues changes the perception of the contact chemical cues, where the lack of relevant odor would make the learned cues less attractive. Further experimentation, such as an array with the model plants with leaf shapes and contact chemicals, and with and without volatile cues, could allow this question to be directly addressed.

In the last experiment (Chapter 6, Experiment 2), which utilized whole plants, females also showed an effect of learning, but only in the landing behavior, not in approaches. Females were most likely to land on the same host plant that they had been given individually-caged experience with (*P. sativa* or *C. maculatum*). While earlier experiments by Åhman (unpublished) had shown a possibility of females learning after experience with *D. carota*, the results were not as clear. Also, the other host plant in this case was *Petroselinum crispum*, and females were no more likely to land on *P. crispum* after experience with the same host as females who had no prior oviposition experience. The results in the experiment presented here are much clearer, with learning taking place on both host plants.

*The role of experience: Within-trial effects*

In addition to looking at the effect of host-plant experience as carried from the individual cage to the tests and trials, the role of learning within a trial was also examined in experiments with free-flight bioassays. This was done by comparing the first model host plant approached or landed upon to the most often approached or landed upon model host plant. In nearly all cases, females showed an ability to learn the cues of the first model host plant approached or landed upon, approaching or landing upon that model plant (or model plants with the same cues) most frequently through the course of a trial. For volatiles alone, nearly all females approached and landed first and most frequently upon the model plants with *C. maculatum* cues. This

effect is most likely due to an overwhelming preference for the volatile cues of *C. maculatum* over those of *D. carota*, as opposed to learning. It is possible that females are still learning the volatile cues of *C. maculatum*, after first being attracted by an innate preference, but this cannot be determined from these data.

Model leaf shapes alone show a significant within-trial effect for approaches, with females most often approaching the model plants with the same leaf shapes as the first model plant approached. Combined with chemical cues, there is a stronger effect that is likely attributable to learning. Females almost exclusively approach and land most often on the same model host plant as the first model host plant approached or landed upon. As each model host plant in a trial had a unique pair of chemical and leaf shape cues, this demonstrates that females are learning pairs of cues, rather than one type of cue over another. The same pattern is seen when pairs of contact and volatile chemical cues were tested (Chapter 5). Females again approach and land most often on the same model host plant as the first model host plant approached or landed upon.

When all three cues are combined, there is a trend for females to approach the same kind of model host plant most often as the first model host plant approached. No such effect was seen for landings. This test gave no significant results in the trials with whole plants.

It is possible that the effects seen in the paired-cue trials are a result of position effects, as each unique pair of cues appeared only once in an array. However, females would still need to be learning the position of the model plants, as different females within a trial were behaving towards different model plants, making the likelihood of a favored position unlikely. Also, for those females who did behave in multiple trials,

females were likely to approach or land first and most frequently on the same type of model plant in more than one trial. As model plants were rotated between trials, this would remove the position effect across trials. Although rotating the model plants during a trial would have solved this problem, it was more important to allow the females to behave without interruption for the full length of the trial.

### *Concluding remarks*

Female *P. polyxenes* demonstrated the ability to learn all three types of cues, albeit in conjunction with other cues, in the paired cue trials. Females could also learn contact chemical cues with host-plant experience in the free-flight trials, despite no sign of an experience effect when these cues were tested independently. There was no effect of host-plant experience on the response to model leaf shapes or volatile chemical cues alone. One conclusion that can be drawn from this is that the model plants did not accurately represent the leaf shapes or volatile chemical cues as experienced by the females. This was largely the result of practical matters, such as the need for a simplified leaf shape for *D. carota* and *C. maculatum*, in order to generate the quantity required for all of the free-flight experiments. Despite this drawback, the within-trial results still allow the recognition of learning for all three cue types. In addition, an effect of host-plant experience was also seen for whole plants in the final experiment.

Experiments with paired cues were designed to determine the relative importance of each cue type in host finding by *P. polyxenes*. Unfortunately, the apparent pairing of cues during learning precludes this attempt. Nonetheless, it demonstrates the importance of each cue type in the host finding behavior of this butterfly. The importance of chemical cues in this species was already known (Feeny.

et al. 1988; Feeny, et al. 1989), but this is the first experimental demonstration of the use of visual cues by this species.

While both types of chemical cue elicited an innate response, there was also evidence of learning for the same cue types. For contact chemicals, at least, this is likely due to a non-chemical associated cue, such as a visual cue like reflectance (Chapters 4 and 5). For volatile chemical cues as well, it is likely that the specific compounds eliciting an innate response are not the same as those being learned. However, the current experiments could not differentiate between associative learning and sensitization, as would otherwise be the case.

The patterns of contact chemical cue use in the family Papilionidae has been a focus of several laboratory groups, including those of Paul Feeny, Ritsuo Nishida, and Keiichi Honda. Similar types of compounds, such as flavonoid glycosides and cyclitols, are required by species using host plants from a botanically diverse group of families: Annonaceae, Apiaceae, Aristolochiaceae, and Rutaceae (Feeny 1995; Nishida 1995; Carter, et al. 1998; Haribal and Feeny 1998). It has been hypothesized that the similarity in structure of these chemical cues is due to constraints on the evolution of responses (by either behavioral or receptor-level mechanisms) (Feeny 1991). Now that the innate nature of the post-landing response to contact chemical cues has been verified in at least one species, this type of work can proceed into looking at the evolution of the use of specific compounds or classes thereof. In addition, volatile chemical cues should also be examined, as they also elicit an innate response in *P. polyxenes*, and are likely to be an important cue for the recognition of at least one important host plant, *C. maculatum*.



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## APPENDIX ONE

### LEAF SURFACE AREA REGRESSIONS

When determining a method by which to standardize a concentration of volatile chemical cues from a leaf surface, the most meaningful measure is likely to be surface area. However, surface area is not a practical measure when hundreds of leaves are being extracted. Therefore, a more easily measured proxy for surface area is desired. A relationship between the total surface area and weight of a leaf for *Daucus carota* was previously reported by Brooks, et. al (1996). No such relationships had been reported for *Conium maculatum*, *Pastinaca sativa*, or *Vicia faba*. Therefore, a study of the relationships of weight, width, and length to total surface area for leaves of each of these species was performed.

#### MATERIALS AND METHODS

Fifty leaves each of *D. carota*, *C. maculatum*, *P. sativa*, and *V. faba* were individually weighed, and measurements for length, width, and surface area were taken. Two different seed sets of *V. faba* were tested, the first was an unknown variety and the second var. Broad Windsor (Agway, Inc.). Terminal leaflets of *P. sativa* were considered "leaves" for the purpose of this study. Petioles were not included in any measurements. Surface area was measured using a Li-Cor Model 3100 area meter (Li-Cor, Inc., Lincoln, NE) set at low resolution (1mm) to allow for measurement of large leaves. Surface area of each leaf was measured three times and the largest measurement of the three was used for further calculations. To find a practical proxy for surface area, weight, length, and width were regressed onto twice the surface area (top and bottom surfaces) using JMP statistical exploration software (SAS Institute, Inc.).

## RESULTS

Regressions for each of the five sets of leaves are shown in Figures A1.1–A1.15. Table A1.1 shows the regression parameters for each species or variety. For *D. carota*, *C. maculatum*, and *P. sativa*, weight was the best predictor of surface area. For both varieties of *V. faba*, width was the best predictor, though the parameters did vary among varieties.

## DISCUSSION

Weight is an acceptable proxy for surface area for each of the species tested. It is the best predictor for the apiaceous species (*D. carota*, *C. maculatum*, and *P. sativa*), while surface areas of the two *V. faba* varieties were best predicted by width.

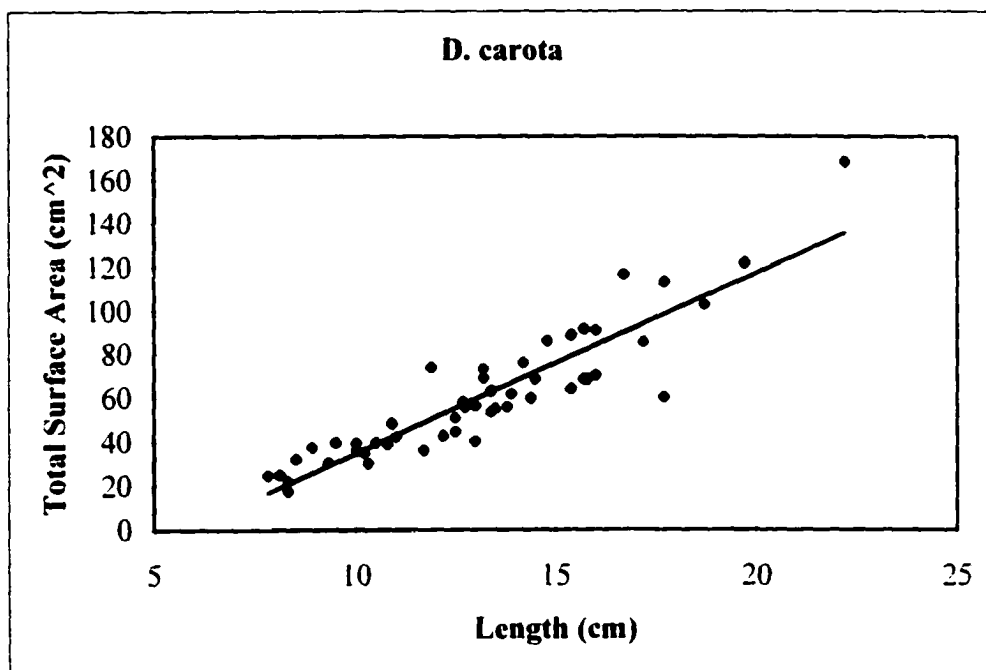


Figure A1.1 Regression of leaf length on total surface area for *D. carota*.

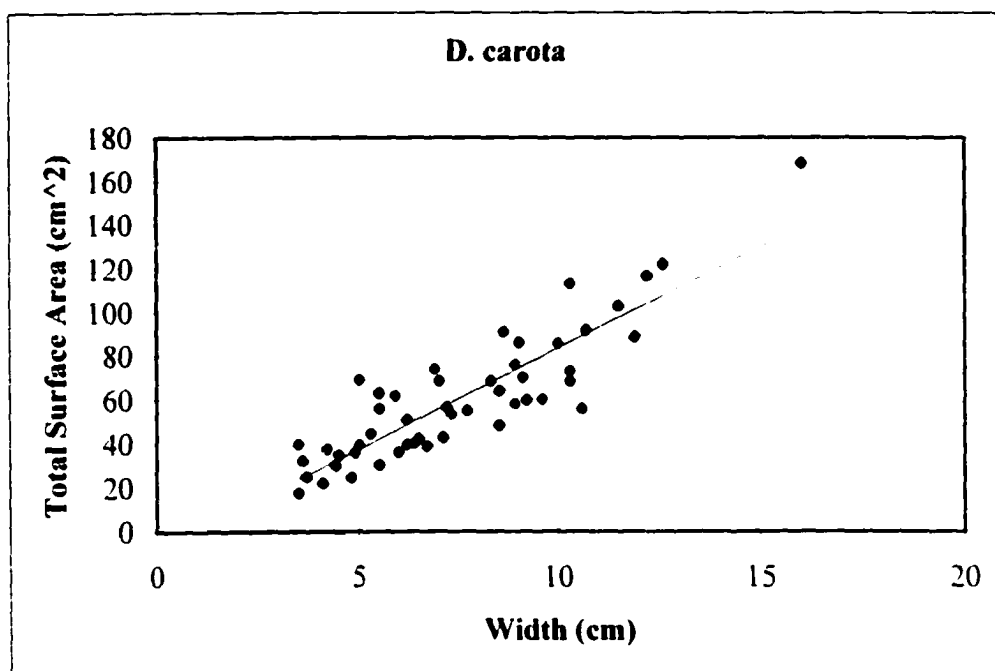


Figure A1.2 Regression of leaf width on total surface area for *D. carota*.

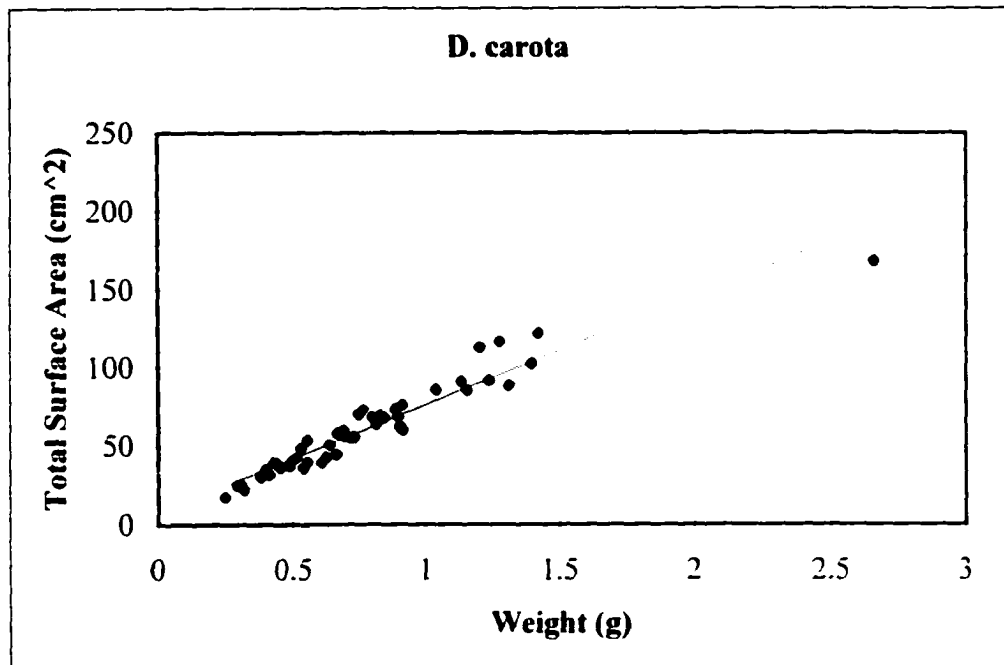


Figure A1.3 Regression of leaf weight on total surface area for *D. carota*.

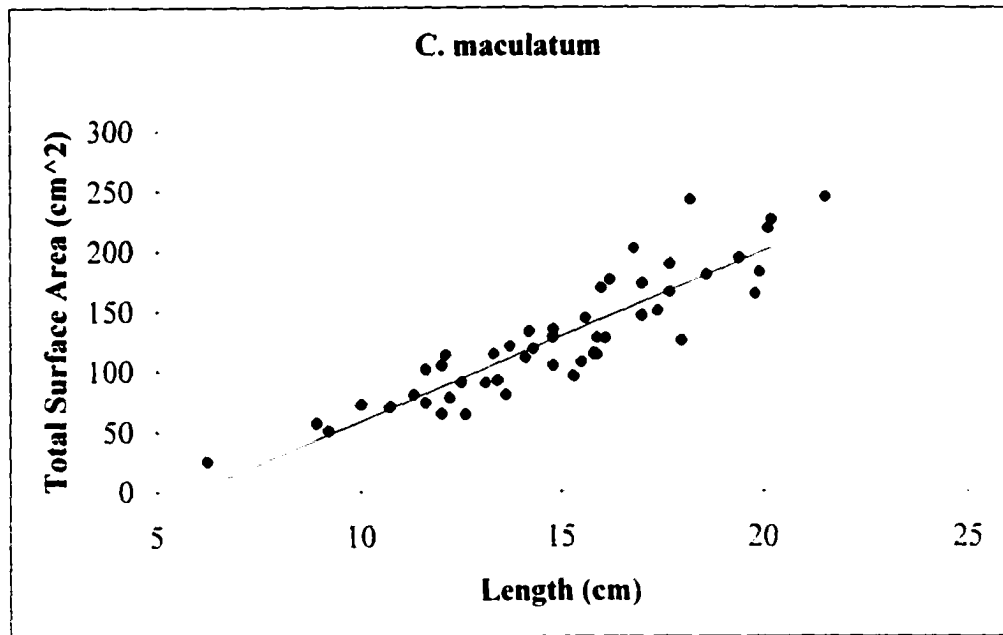


Figure A1.4 Regression of leaf length on total surface area for *C. maculatum*.

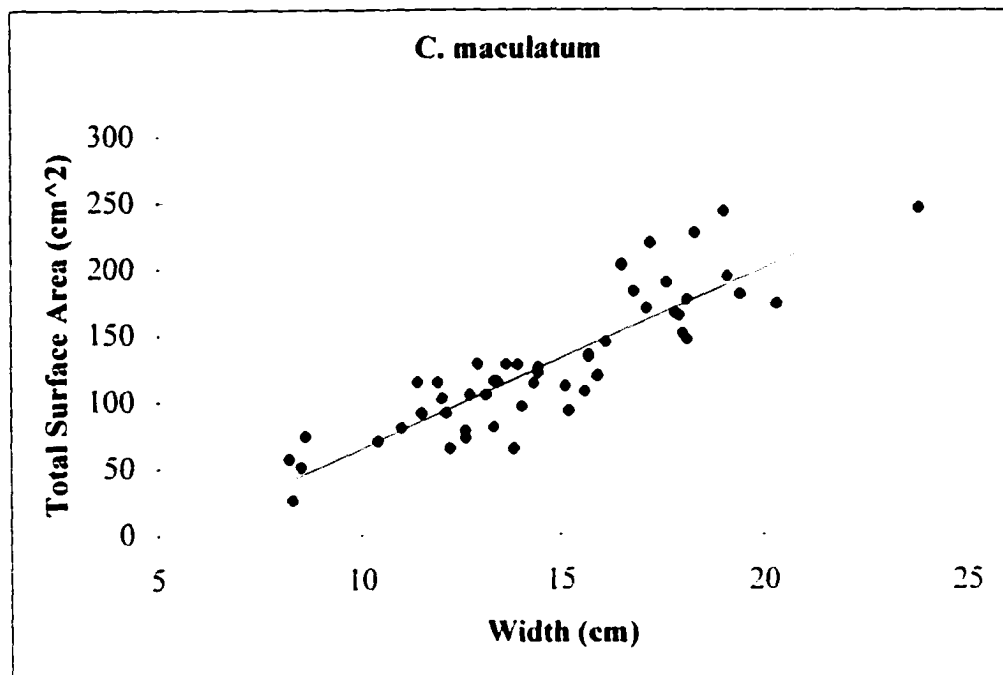


Figure A1.5 Regression of leaf width on total surface area for *C. maculatum*.



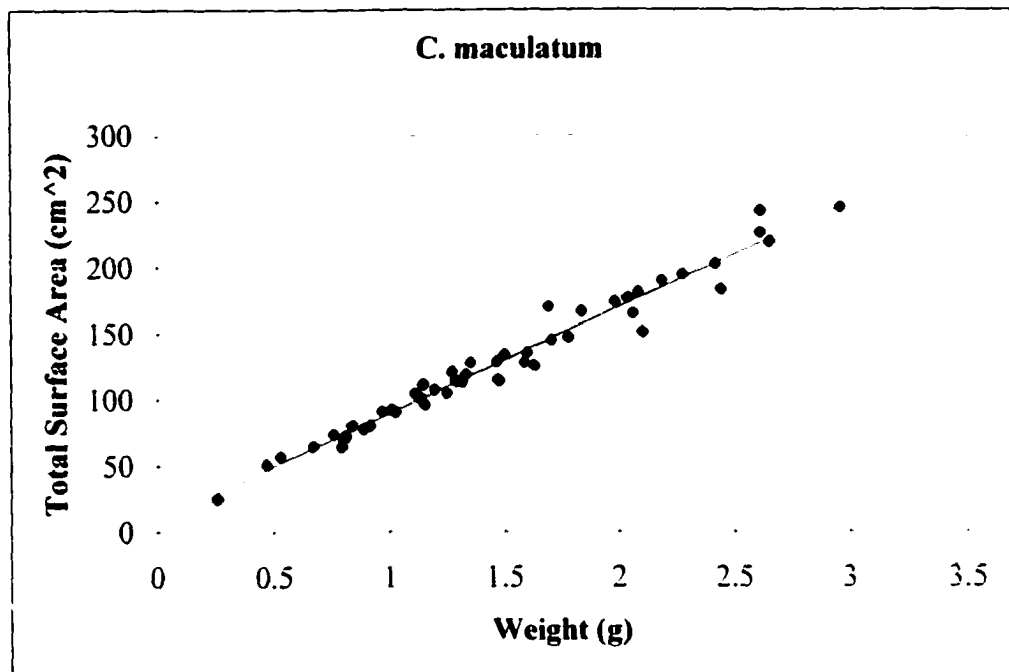


Figure A1.6 Regression of leaf weight on total surface area for *C. maculatum*.

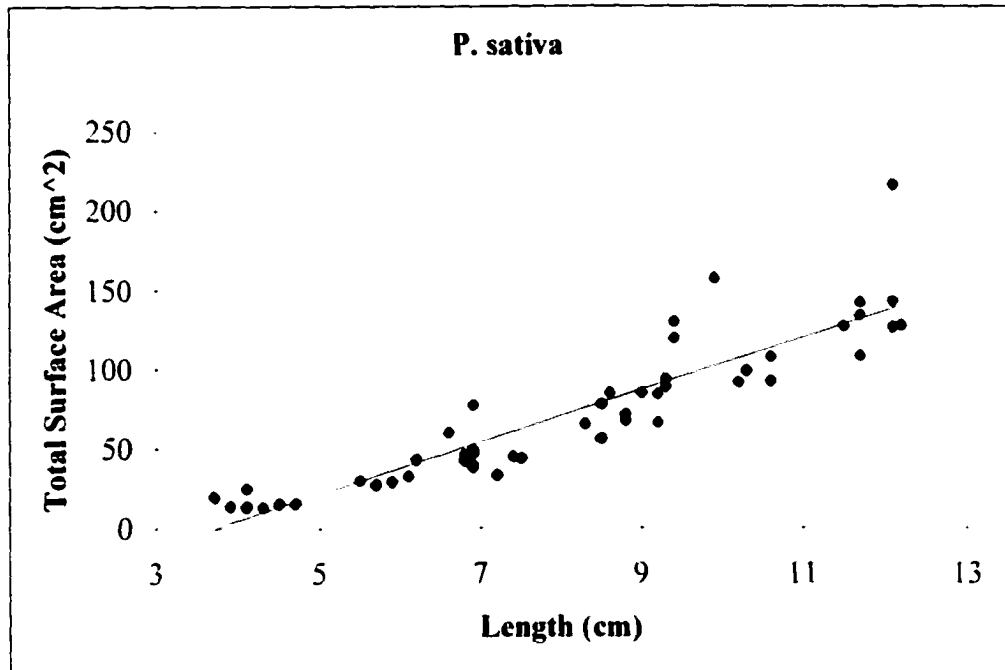


Figure A1.7 Regression of leaf length on total surface area for *P. sativa*.

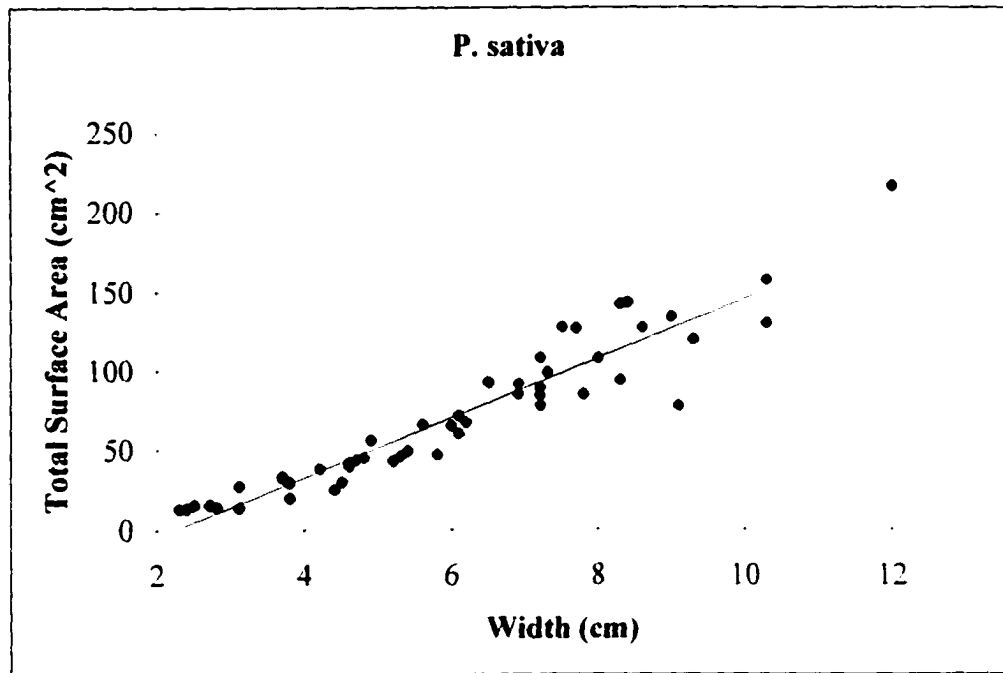


Figure A1.8 Regression of leaf width on total surface area for *P. sativa*.

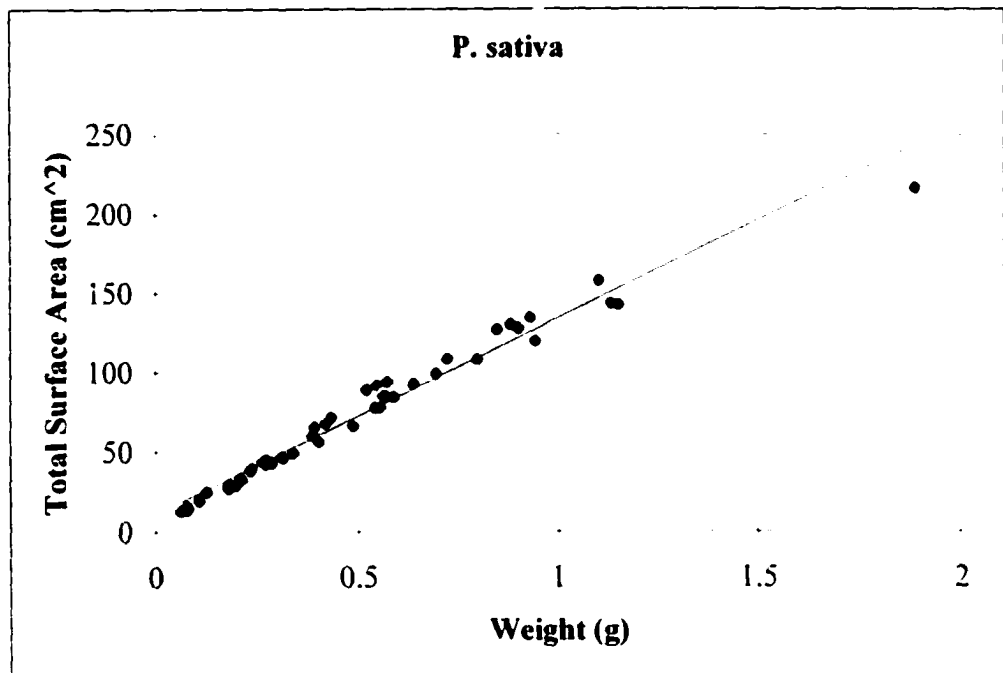


Figure A1.9 Regression of leaf weight on total surface area for *P. sativa*.

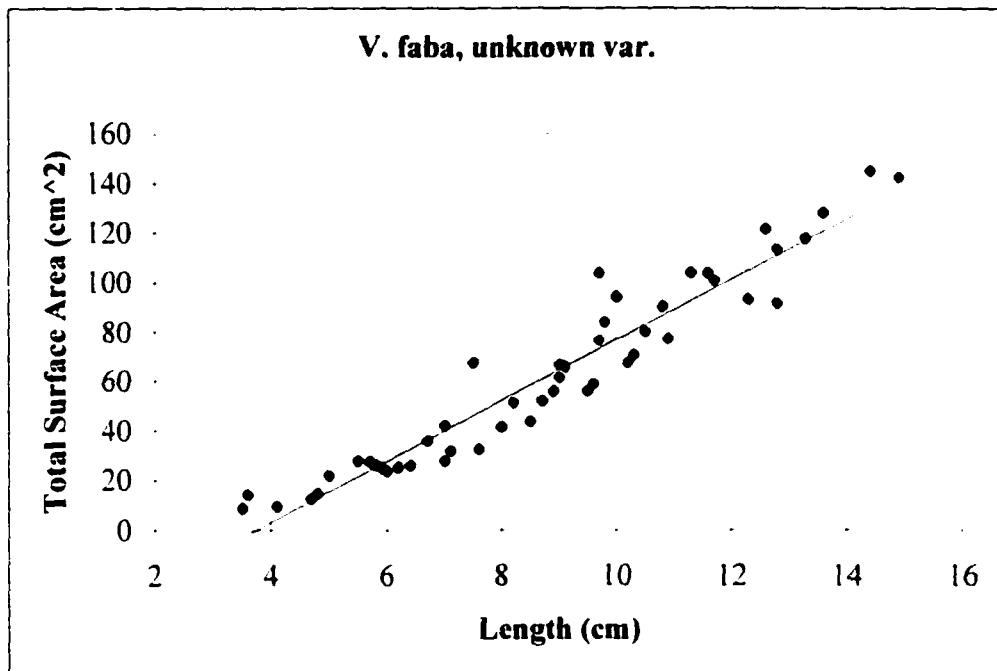


Figure A1.10 Regression of leaf length on total surface area for *V. faba*, var. unknown.

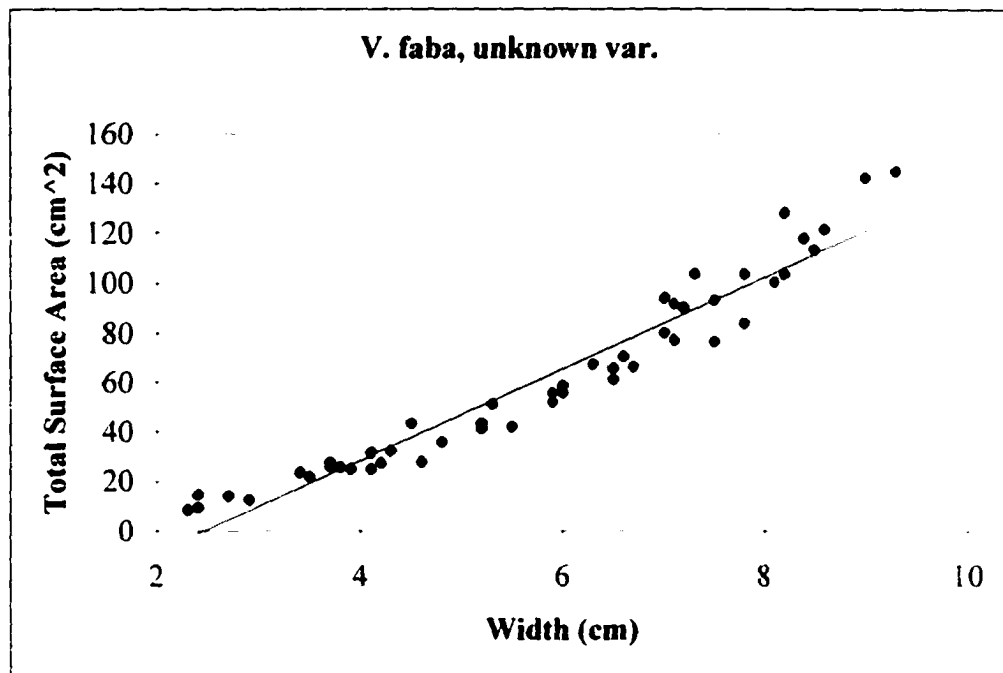


Figure A1.11 Regression of leaf width on total surface area for *V. faba*, var. unknown.

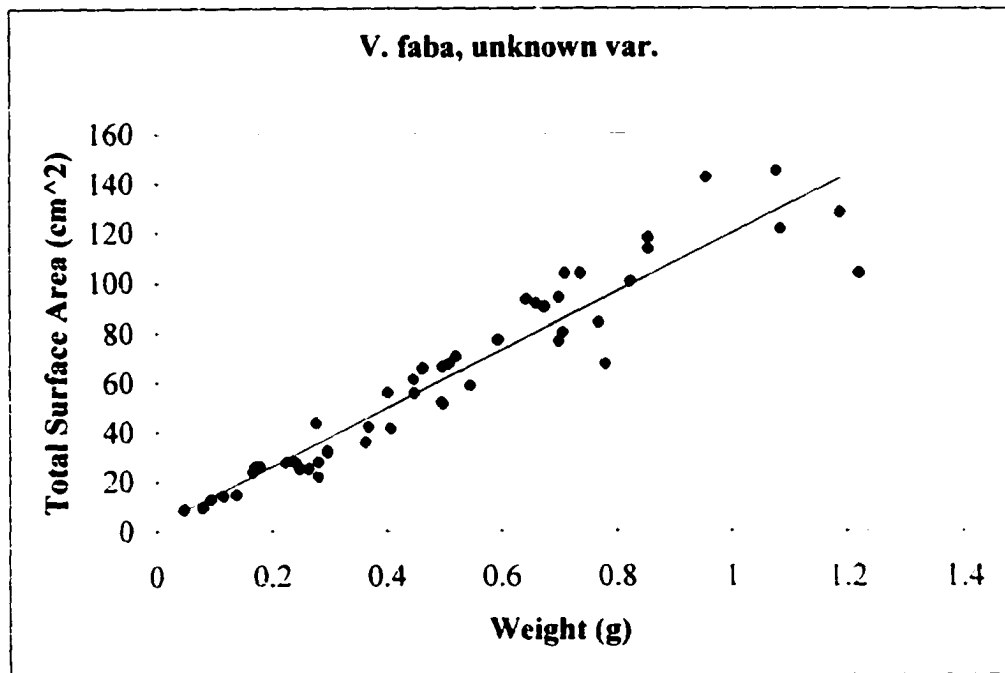


Figure A1.12 Regression of leaf weight on total surface area for *V. faba*, var. unknown.

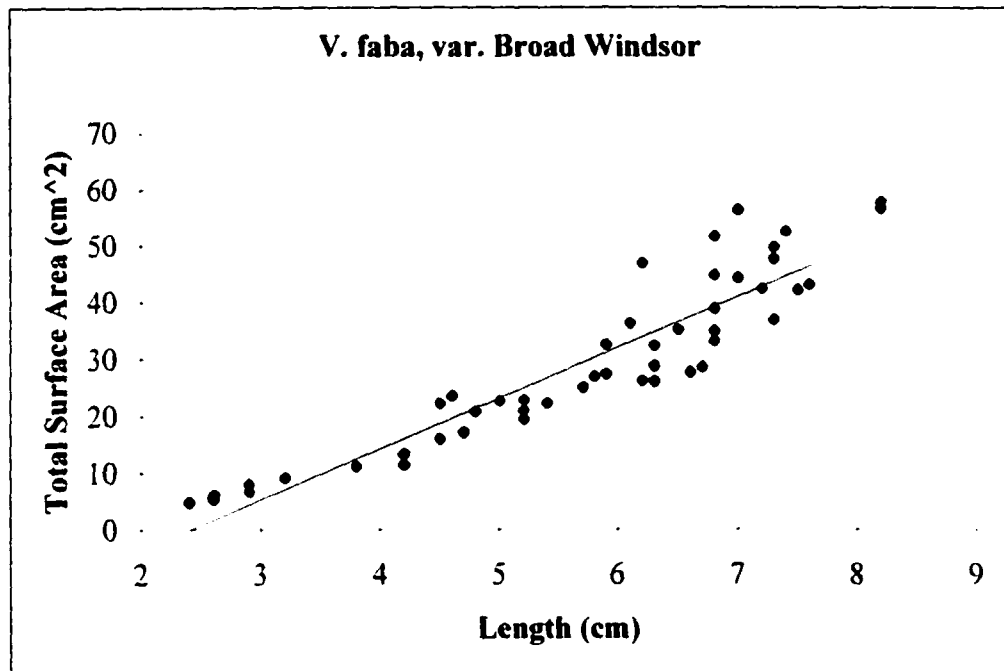


Figure A1.13 Regression of leaf length on total surface area for *V. faba*, var. Broad Windsor.



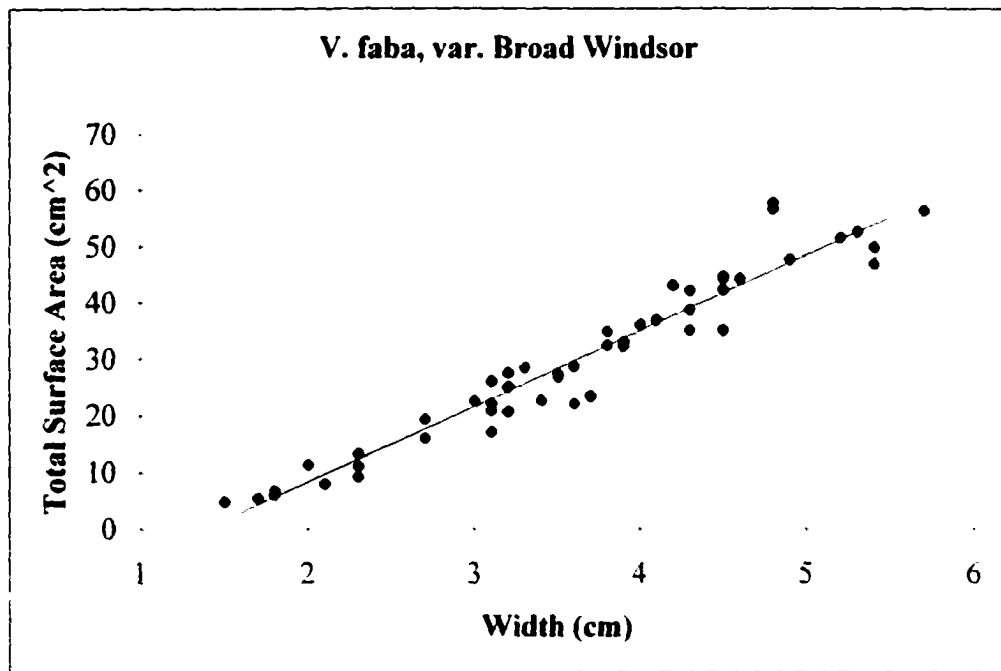


Figure A1.14 Regression of leaf width on total surface area for *V. faba*, var. Broad Windsor.

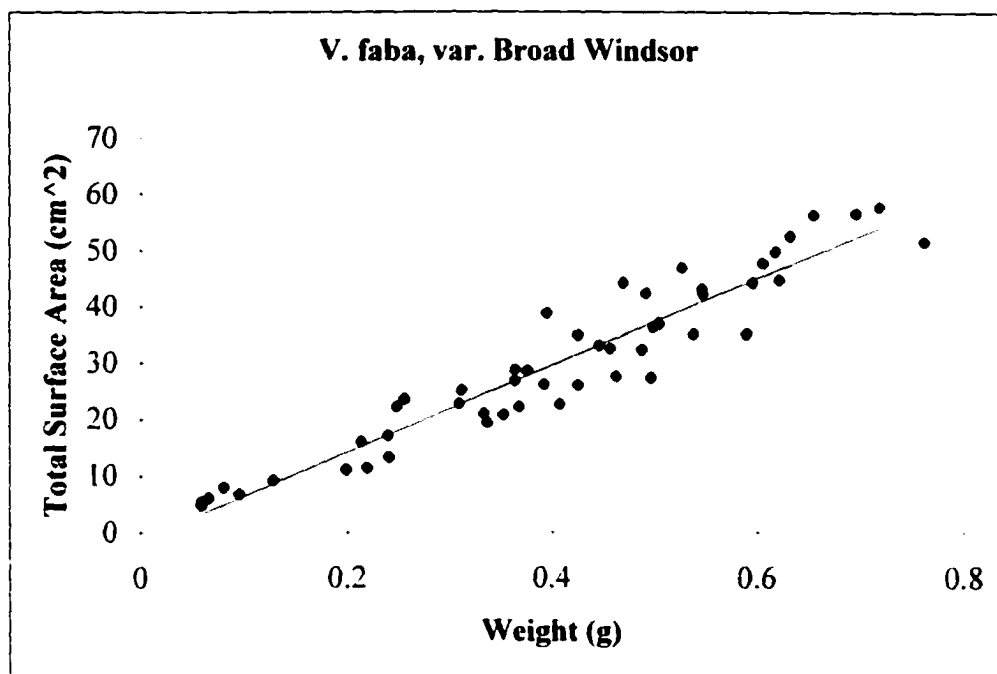


Figure A1.15 Regression of leaf weight on total surface area for *V. faba*, var. Broad Windsor.

Table A1.1 Leaf surface area regression parameters for each species.

Species	Measure	Intercept	Slope	$r^2$
<i>D. carota</i>	Length	-47.503	8.25401	0.828302
	Width	-8.3019	9.2284	0.771244
	Weight	7.61246	69.3676	0.922384
<i>C. maculatum</i>	Length	-81.788	14.0726	0.80295
	Width	-70.372	13.5182	0.771912
	Weight	10.218	79.7505	0.968334
<i>P. sativa</i>	Length	-60.973	16.4829	0.824758
	Width	-42.180	18.7812	0.892483
	Weight	11.0141	123.394	0.973947
<i>V. faba</i> , var. unknown	Length	-45.600	12.2275	0.918486
	Width	-45.219	18.4481	0.93308
	Weight	2.71806	117.129	0.901326
<i>V. faba</i> , var. Broad Windsor	Length	-21.385	8.90838	0.85415
	Width	-18.405	13.3759	0.932509
	Weight	-1.1823	76.9117	0.904548

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