THE BIOLOGY OF *HELICONIUS* NIGHT-ROOSTING: A FOUNDATION

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2010

To my parents, Fernando and Laura, who let me wander freely into an unknown world for them: the study of life

A mis padres Fernando y Laura, que me permitieron navegar libremente en un mundo desconocido para ellos: el estudio de la vida

ACKNOWLEDGMENTS

I wish to thank Dr. Thomas Emmel for his hospitality, generosity, and for giving me the opportunity to come to the University of Florida Entomology and Nematology Department and to the McGuire Center for Lepidoptera and Biodiversity. To Mirian Hay-Roe for introducing me to the world of *Heliconius* chemical ecology, for infusing me with interest for Passion vines, for many afternoons of scientific discussions, and for her friendship. To Peter Teal and his research team for opening the doors of his laboratory at the USDA chemistry unit with no reservations and letting me discover, little by little, many useful tools to study chemical ecology. Drs. Sandy Allan and Erin Vrzal from USDA-ARS for training on behavioral software use and for loaning video equipment. To Dr. David Evans for letting me use his microscope for pollen analysis. To Drs. Jackie Miller, Annette Aiello, Keith Willmott, Chris Jiggins, Marta Wayne, Harris Marion, Luana Maroja, and several anonymous reviewers for useful comments and corrections in my proposals, manuscripts, and experimental designs. I also thank Drs. Chris Jiggins and Owen McMillan for providing rearing facilities in Gamboa, Panama. Doctoral student Alejandro Merchán for help with *Heliconius erato* rearing, and student assistants Chava Weitzman, Susan Finkbeiner, Fazle Hasnain, Edwin James, Edwin Johnson, Aamer Shareef, and Lyndall Brezina for help in rearing, field collections and observations, data processing, and in-cage experiments. I also want to thank Christine Eliazar and Jim Schlachta for their prompt assistance on many occasions through these years and to Court Whelan for IR video editing. To many friends and fellow scientists that made my stay the best it could have been in La Selva field station, Costa Rica and Gamboa, Panama.

I wish to thank the Smithsonian Tropical Research Institute, Organization for Tropical Studies, Panama Canal Authority (ACP), and Panama Environmental Authorities (ANAM) for processing and facilitating permits to access field sites. Funding for this work came from the McGuire Center for Lepidoptera and Biodiversity, two Smithsonian Tropical Research Station short-term Fellowships, American Museum of Natural History grant, Organization for Tropical Studies Research Fellowship, Explorer's Club grant, IDEA WILD grant, Journal of Experimental Biology grant, and a Sigma Xi Research Fellowship.

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By

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August 2010

Chair: Thomas C. Emmel
Major: Entomology and Nematology

Aggregative behaviors are usually adaptively important, not only in insects, but in almost every animal group. Understanding the importance of such behavior in a megadiverse tropical group can open windows towards the better understanding of key speciation and diversification mechanisms. *Heliconius* (L.) butterflies are a successful tropical group widely used in evolution and genetic studies. Some species within the genus express nocturnal roosting. Roosts are usually formed under relatively dense vegetation mats where dry vines or branches provide a perch for the night. These sites may last for months. Despite the lack of understanding of the importance of *Heliconius* roosting, only a few studies have been made. In order to understand the importance of factors related to the expression of *Heliconius* roosting, an ethological study was made, and data on light, temperature, relative humidity, wind, use of wing color cues, predation and disturbance events, and use of foraging resources were recorded at several *Heliconius erato* and *H. sara* roost sites in Honduras, Costa Rica, and Panama. Chemical compounds from the cuticle of *H. erato* males and females were analyzed and its importance in roosting interactions was tested in bioassays. Additionally, an already identified chemical compound found in *H. melpomene*, (*E*)-β-ocimene, was tested for

attraction in bioassays. The results show that roost sites offer reduced light conditions at dusk, provide a drier environment compared to its vicinity, offer protection from wind and rain, and are almost predation-free. Individuals from different broods are able to roost together. Wing color recognition under reduced light conditions at dusk is essential to successfully assemble aggregations. *Heliconius* do not learn pollen preferences at roost sites and females digest pollen overnight. In addition, a variety of volatile and non-volatile compounds from the cuticle were identified. The roles of such compounds in chemical communication remain unknown, except for (*E*)-β-ocimene, which was found to attract males and females in diurnal interactions. These findings provide the first in-depth study of *Heliconius* roosting behavior and set a landmark for future studies on this particular behavior and on similar behaviors in other insect groups.

CHAPTER 1
INTRODUCTION

Heliconius butterflies belong to the family Nymphalidae of the insect order Lepidoptera (Penz, 1999). The genus is widespread over the tropical and subtropical regions of the New World and many species have been used broadly as evolutionary models in biology (Brown, 1981, Emsley, 1965, Turner, 1981). One of the most remarkable features of these butterflies is their toxicity. *Heliconius* butterflies sequester and biosynthesize toxic compounds from their host plants, passion vines (*Passifloraceae*) (Engler-Chaouat & Gilbert, 2007, Hay-Roe, 2004, Hay-Roe & Nation, 2007). These compounds are stored in their bodies and protect them from predators. Toxicity in this genus presumably is the basis of a phenomenon called Müllerian mimicry (Müller, 1879) in which two toxic species (co-mimics) converge upon the same color pattern enhancing the power of both species to educate their predators not to eat them (Mallet & Gilbert, 1995b). Groups of Müllerian species often belong to mimicry 'rings' (groups of unpalatable species, together with some palatable species that have converged in the same warning color pattern) (Mallet & Gilbert, 1995b). Birds are among the most common predators of butterflies butterfly (Chai, 1986, Chai, 1990). Typically when a naïve bird attempts to eat one of these butterflies, it regurgitates the bad tasting meal butterfly (Chai, 1986, Chai, 1990). Afterwards, it will associate the bad taste with the specific wing pattern of the bad-tasting butterfly (Chai, 1986, Chai, 1990).

Behavior and mimicry seem to be correlated in *Heliconius* butterflies. Co-mimics fly in similar habitats, and non-mimics fly in different habitats that overlap the habitats of other non-mimics (Mallet and Gilbert, 1995b). Co-mimics roost at night in similar habitats and at similar heights above the ground, but in habitats and at heights different

from those of species in other mimicry rings (Mallet & Gilbert, 1995b). *Heliconius*., especially species belonging to the *erato* taxonomic group (*H. hewitsonii, H. sara, H. charithonia*), are renowned for roosting gregariously and for the unique trait of pupal mating (Mallet & Gilbert, 1995b). Co-mimics roost gregariously with each other more often than with non-mimics (Mallet & Gilbert, 1995b). Gregarious roosting is therefore common among species, as well as within species (Mallet & Gilbert, 1995b). Aggregative behaviors are widespread in the animal kingdom. By forming aggregations many species of vertebrates and invertebrates reduce the probability, through the selfish herd effect, that any one individual will be killed (Hamilton, 1971, Gamberale & Tullberg, 1998). In Lepidoptera there are few reports on adult aggregative behaviors. In most cases in involves distasteful species and include a variety of contexts including lekking aggregations for mating, predator defense, and thermoregulatory strategies. Some of these include *Celaenorrhinus fritzgaertneri* (Hesperidae) (DeVries et al., 1987), *Marpesia berania* (Nymphalidae) (Benson & Emmel, 1973), *Eumaeus atala* (Lycaenidae) (Hall and Butler, 2000), *Estigmene acrea* (Arctiidae) (Davenport & Conner, 2003), Ithomiinae (Nymphalidae) (Pinheiro et al. 2008), and *Danaus plexippus* (Nymphalidae) (Brower et al., 2008).

In *Heliconius* butterflies, typically several individuals (males and females) begin to land usually on twigs, tendrils, and dry leaves under the shade of a tree 1-2 h before sunset. About 45 min before sunset a series of behavioral interactions take place before perching. After sunset a group has usually been formed, and most members remain together until sunrise (Crane, 1955, Crane, 1957a, Jones, 1930, pers. obs). Although most of the published information on *Heliconius* roosting only outlines anecdotal

observations and speculations (Turner, 1975, Crane, 1955, Crane, 1957a, Jones, 1930, Gilbert, 1972, Young & Carolan, 1976, Young, 1978, Young & Thomason, 1975), there are some studies that reveal key ecological information on *Heliconius* communal roosting. Home ranges (area that an individual uses where all the resources it needs to survive are present, normally 3-5 km^2) of individuals are independent of those of their roostmates (Mallet 1986), mimicry rings roost at specific heights (Mallet and Gilbert 1995), and foraging sites are apparently used as recruiting centers, where young individuals meet and follow older butterflies to roost sites (Waller & Gilbert, 1982). These studies reveal some information that may be essential to determine the adaptive importance of gregarious *Heliconius* roosts (i.e. why they roost gregariously); however, there is no data to explain the proximate causes and mechanisms involved in the expression of the behavior (i.e. how they do it). Understanding how *Heliconius* butterflies roost is important not only because it will reveal information that can be used to further understand previous studies, but also because it will set a stronger basis for more complex hypotheses that can lead to a full understanding of the adaptive importance of this behavior.

The work described here establishes a foundation on the proximate causes and mechanisms involved in the expression of gregarious roosting behavior in *Heliconius* butterflies and tests hypotheses on the adaptive importance of the behavior. In order to understand how *Heliconius* butterflies are able to express roosting, behavioral interactions were identified, environmental factors at roosting sites were analyzed, visual cues used in roosting interactions were studied, and chemical cues potentially used at roosting interactions were identified. Hypotheses about the adaptive importance

of *Heliconius* roosting behavior converned the role of roosting aggregations as a predator defense strategy, as a resource information center for foraging purposes, and as a potential kin selection strategy.

CHAPTER 2
BEHAVIORAL TRAITS INVOLVED IN *HELICONIUS* BUTTERFLY ROOSTING BEHAVIOR

Introduction

Several species from the butterfly genus *Heliconius* display communal nocturnal roosting. The most gregarious species are in the erato-sara-sapho clade (*Heliconius erato*, *H. sara*, *H. hecale*, *H. charithonia*, and *H. hewitsoni*) (Mallet & Gilbert, 1995a). This group is also characterized for pupal mating, a behavior in which female pupae are pierced and mated by males just moments before eclosion (Beltran et. al 2007). Every day, a few h before sunset, the butterflies start navigating towards the roost sites. When arriving at the roost sites and meeting with conspecifics, the butterflies display a series of behavioral interactions until they are all finally perched. Butterflies usually return to the same sites night after night (Mallet, 1986b), unless the site is subject to physical alteration. Roosting sites are usually under vegetation mats, in man-made living fences, or forest edges, sometimes near streams, and in areas with almost no wind (Mallet 1986b, pers. obs.). Butterflies perch gregariously from dead hanging vines, leaves or twigs (Mallet 1986b).

Most of the work on *Heliconius* roosting behavior has been based on field notes or on observations of the behavior without appropriate statistical support (Young, 1978, Young & Thomason, 1975, Jones, 1930, Crane, 1955, Crane, 1957a, Young & Carolan, 1976, Gilbert, 1972, Turner, 1975), and in-depth studies are lacking. Behavioral traits of *Heliconius* roosting behavior were first addressed by Mallet (1986). Using *H. erato* from Costa Rica and Colombia, Mallet (1986) used the term “fanning” to describe behavioral interactions that involved various levels of interest in roosted conspecifics ranging from brief approaches to hovering closely with probable antennal and wing contact; he also

used the term “clutching” to describe grasping at the perch or the wings of a roosted butterfly with the tarsal claws. Using natural and in-cage roosting aggregations of *H. sara*, *H. erato*, and *H. charithonia*, the present work identified and described several other behavioral interactions.

Methods

Ethogram Production

Behavioral interactions were recorded and observed at roost sites of wild and captive *Heliconius charithonia*, *H. erato* and *H. sara* at dusk. In order to identify the interactions, aggregations sites were observed for at least 10 days for each species at dusk for approximately 60 min. To identify behavioral traits (i.e. generate an ethogram) and their frequency, recordings were made with a SONY DCR-SR220 handheld camera. In addition, high-speed photography (Pentax® K100D Super SLR digital camera) was used to identify specific contact surfaces during contact interactions between individuals. A total of 160 min of recordings were obtained for *H. erato* over the course of 7 days and 140 min for *H. sara* over the course of 7 days.

Video Recordings and Video Analysis

In order to determine if a behavioral interaction was consistently expressed, field and in-cage recordings were made. Following a particular individual with a video camera was difficult, and to detect accurately behavioral interactions zooming in and out was done frequently, hence, whenever an interaction was detected by the observer, video was recorded. The videos were analyzed in slow motion using specialized software (Observer XT by Noldus).

Several species were used in the wild and in cages. In-cage observations included a group of 10 males and 10 females of *Heliconius erato favorinus* from Peru, and a

group of 4 males and 4 females of *H. erato petiverana* from Costa Rica. Field observations included a group of 5 males and 3 females of *H. sara* from Costa Rica and a captive colony of *H. charithonia*. About 30 days of recordings produced 460 min of observations.

Results and Discussion

General Description of *Heliconius* Roosting Behavior

In general, *Heliconius* home ranges are determined by a network formed by foraging patches, and roost sites are usually located near foraging patches (Mallet, 1986b). *Heliconius* butterflies perch gregariously in selected roost sites night after night (Brown 1981, Cook et. al 1976, Mallet & Jackson, 1980, Turner, 1981). These sites are fairly stable (Mallet, 1986b) unless the perch gets destroyed by a natural disturbance or by human intervention. When the perch is physically altered, the butterflies do not come back and instead they spread out, and perch in nearby sites until a new perch is found (pers. obs.). Once a new perch is found, they gradually start perching gregariously again (pers. obs.). The recruiting mechanisms are unknown.

In the typical situation, the butterflies start navigating towards their roost sites as early as 3 h before sunset (Mallet, 1986b, pers. obs.). Individuals start navigating along forest edges and clearings (pers. obs.). It is likely that they use these navigational cues together with other landmarks to locate the sites. Once they arrive at the sites, they express a pre-roosting behavior, which is composed of sitting in nearby areas (3-5 m) to the roost sites, usually between 2-5 m above the ground, brief air-chases, and occasional basking. Once light levels start dropping rapidly, approximately 30-45 min before sunset, the butterflies fly towards the perch and start expressing a set of behavioral interactions. These interactions seem to be more frequent when they are in

captivity and also when the number of butterflies is larger (pers. obs). During the course of these interactions, where physical contact between individuals is frequent, individuals start to perch until they are all perched gregariously at sunset. Occasionally, there are individuals that do not perch with the group but a meter or so from it. In the case of *Heliconius erato* aggregations, it is common to see one or two individuals of the co-mimic *H. melpomene* roosting within 0.5-1.5m of the aggregation (Mallet & Gilbert, 1995a, pers. obs.). In fact, 46% of melpomene group species roosts with the commonest co-mimics, which means they are as likely to roost with conspecifics or with co-mimics (Mallet & Gilbert, 1995a, pers. obs.). Similarly there is evidence from other mimicry rings (*H. pachinus-H. hewitsonii* and *H. hecale- H. ismenius*) where co-mimics roost together (Mallet & Gilbert, 1995a). At night, the butterflies remain motionless and conserve their position until the next day (Salcedo, 2006). In the morning, they disband at sunrise, usually one by one, unless one of them disturbs closely perched neighbors or a physical disturbance of the perch occurs (Mallet 1986b, pers. obs.).

Behavioral Interactions

Five separate behavioral traits were identified: approach, fanning, clutching, antenna-wing, and fending-off (Table 2-1).

Approach involves direct flight towards an individual. Approach behavior is an initial inspection of the subject and primarily triggered from distance by a color cue (wing color pattern). This behavior is also expressed in courtship and when approaching flowers in the forest. It is expressed by both sexes.

Fanning is hovering above a perched individual with no physical contact. The term fanning was described by Mallet (1986b) from his observations of *Helicoius erato* roosting aggregations. It is the behavioral unit with the highest frequency during roosting

interactions (Table 2-1), thus it is very important and probably serves primarily for recognition of a subject at close range. Both sexes express it. Fanning is not expressed exclusively during roosting interactions, but it is also expressed when butterflies are identifying flowers for feeding, when females search for a suitable oviposition site, and when males court females for mating (Klein & de Araújo, 2010). Fanning involves fluttering of the wings while holding a somewhat stationary position in front of the subject (5-15 cm from it) that is being inspected, and hence creates wind flow. This wind flow, together with the fact that is a close range interaction that occurs in different contexts, suggests that it could be a behavior that enhances volatile chemical compounds to reach chemosensillae in the antennae and other parts of the fanning individual.

Clutching involves brief contacts between an approaching individual's tarsal claws and the wings of a perched individual (Figure 2-1). Usually the contact involves the mesothoracic legs but the metathoracic pair is sometimes involved. The term “clutching” was coined by Mallet (1986b). Clutching behavior occurs also during courtship and mating (Klein & de Araújo, 2010). This behavior is the second most frequent behavior expressed during roosting (Table 2-1) and it is often combined with fanning. Scanning electron microscopy revealed several contact chemosensillae in the first 4 tarsal segments of *H. erato* males and females (Salcedo unpub. data). Contact between individuals seems to be critical for intraspecific recognition during roosting interactions with almost every individual expressing it during roost formation. The contact suggests detection of contact chemical cues.

During the “antenna-wing” behavior, an approaching individual touches a perched individual wing with the antennae (Figure 2-1). This behavior is very difficult to observe with the naked eye and even with slow motion video. Antenna-wing is not frequent (Table 2-1) and may occur incidentally as a result of an individual trying to touch another when clutching. In addition, *Heliconius* butterflies do not use antennal contact as in other insects, such as ants, where behavioral interactions require antennation (i.e. physical contact of the antennae with other individuals and subjects) for behavioral interactions and hence are equipped with antennae that have mechanoreceptors and contact chemoreceptors. This suggests that sensillae in the *Heliconius* antennae are used primarily to detect non-contact volatile chemical cues but the detection of contact chemical cues cannot be discarded. Additionally this behavior may be related to an auditory interaction, Hay-roe described a similar behavior when *H. cydno* produced sounds at roosting time (Hay-roe pers. com), and interestingly Swihart (1967) described that the greatest number of auditory receptors in *H. cydno* appeared at the optic lobes near the base of the antennae.

“Fending-off” behavior includes vigorous fluttering of the wings by one or several individuals upon fanning and/or clutching of an approaching individual that is seeking for a space to perch. The term was coined by Mallet (1986b). During fending-off, *Heliconius* males flutter their wings and expose androconial patches on their hindwings; females flutter their wings and expose their abdominal “stink clubs” (scent glands) (Mallet, 1986b) in a behavior similar to that used in diurnal rejection of males (Gilbert, 1976). Fending-off may also contribute to the dissemination of volatile chemical cues that may serve for intraspecific recognition or as spacing pheromones in the aggregations.

Figure 2-1. Behavioral interactions expressed during *Heliconius* roost formation. A: antenna-wing, B: clutching (leg-wing), C: fanning, D, E: fending-off. See table 2-1 for descriptions.

Table 2-1. Ethogram of *Heliconius* roosting interactions. See Figure 2-1 for photographs.

Behavioral unit	Description
males and females	
Approach	Direct flight towards an individual.
Fanning	Hovering over a perched individual with no physical contact.
Clutching	Brief contact between approaching individual's tarsal claws at the wings of a perched individual. Usually contact is on the forewing distal area.
Antenna-wing	An approaching individual touches a perched individual wing with the antennae.
Fending-off	Vigorous flapping of the wings by one or several perched individuals caused by fanning and/or clutching of a flying individual.

Table 2-2. Frequency (occurrences per minute of interaction) of behavioral traits of *Heliconius erato* and *H. sara* when forming roosting aggregations.

Observation period (min)	fanning	approach	fending off	clutching	antenna-wing
H. erato-captive 10 males 10 females					
10.57	3.60	0.53	0.00	0.76	0.57
10.67	4.03	1.05	0.09	0.00	0.00
36.25	3.03	1.56	0.11	0.28	0.14
45.68	3.94	1.12	0.15	0.74	0.09
65.80	0.88	0.43	0.00	0.02	0.02
H. sara-wild 5 males 3 females					
12.27	4.16	0.41	0.24	1.88	0.00
18.35	4.09	0.33	0.11	1.96	0.05
29.00	0.52	0.07	0.41	0.59	0.00
51.98	5.50	0.12	0.52	2.23	0.02
15.98	0.34	0.01	0.03	0.14	0.00

CHAPTER 3
ENVIRONMENTAL ELEMENTS AND ROOSTING BEHAVIOR IN *HELICONIUS* BUTTERFLIES

Introduction

An animal aggregation can be defined as any assemblage of individuals that results in a significantly higher density of individuals than in the surrounding area (Camazine et al., 2001). Aggregations may be synchronized with circadian rhythms (day-night cycles), or seasons, or they can be permanent (Waller & Gilbert, 1982). This behavior has been documented in several insect groups such as butterflies, moths, dragonflies, bees, and wasps for over a century, and in each case authors have proposed different hypotheses that usually involve important adaptations (Rehfeldt, 1993, Evans & Linsley, 1960, Benson & Emmel, 1973, Joseph, 1982, Greig & De Vries, 1986, Brower et al., 2008). *Heliconius* butterflies express nocturnal communal roosting (Wallace, 1870). In the typical situation, several individuals (males and females) begin to land usually on twigs, tendrils, and dry leaves under the shade of a tree just before sunset. After sunset, a group has usually been formed and most of its members remain together until sunrise (Crane, 1955, Crane, 1957, Jones, 1930, Mallet, 1986b, pers. obs). Few studies have addressed this particular behavior and much of the available information only outlines anecdotal observations and speculations (Young, 1978, Young & Thomason, 1975, Jones, 1930, Crane, 1955, Crane, 1957a, Young & Carolan, 1976, Gilbert, 1972, Turner, 1975). Although more recent studies have provided key information on some elements of the ecology of night roosting in *Heliconius* (Mallet, 1986b, Mallet, 1986a, Mallet & Gilbert, 1995a, Waller & Gilbert, 1982), there are no data on the environmental characteristics of these sites. Recent data show how large monarch (*Danaus p. plexippus*) aggregations in Mexico provide key microclimatic

advantages that confer metabolic and survival benefits by providing thermal buffering against cool temperatures and achieving higher humidity to reduce evaporation and desiccation (Brower et al., 2008). Although *Heliconius* nocturnal aggregations are much smaller and variable in number of individuals (from few individuals to up to 30 or more), the architecture of the roost sites (dry vines or branches under relatively dense vegetation mats) may provide microclimatic advantages as well as important cues for site finding. Additionally color cues are another important environmental element in the different contexts that *Heliconius* butterflies experience. Color vision is key in several interspecific and intra specific behavioral interactions such as sexual selection, where wing color pattern can be an important prezigotic isolation tool (Jiggins et al., 2001). Similarly color is important to locate pollen host plants from the distance in the forest.

This chapter presents data on humidity, temperature, light, and use of color cues at *Heliconius* roost sites and discusses their possible role on the expression of the behavior.

Methods

Study Organisms

Roosting behavior can occur at different heights depending on the species and the mimicry ring they belong to (Mallet & Gilbert, 1995a), making field methodologies practical for relatively low roosting species. Based on field observations, *Heliconius erato* and *H. sara* found in Central America were chosen for this study. *H. erato* roost sites are usually located at 0.5 m to 3 m from the ground, which make them ideal for indoor controlled experiments and installation of data logging equipment at natural roost sites. *H. sara* roosts higher up, in the 1.5 m to 4 m range, which is still manageable for observations and equipment installation in the field.

Field Sites

The study was carried out in two main natural areas in Central America. La Selva field station, of The Organization for Tropical Studies in Costa Rica, provided an excellent site where several *Heliconius sara* roosting sites were found. La Selva is located at the confluence of two major rivers in the Caribbean lowlands of northern Costa Rica. The area comprises 1,600 ha of tropical wet forests and disturbed lands. La Selva has also several kilometers of trails and forest edges that are ideal areas for *Heliconius* roosting sites. La Selva was visited during March and April 2009, at the beginning of the rainy season, which extends from May to early December in Costa Rica.

The second site was the Gamboa field station of the Smithsonian Tropical Research Institute in Panama. Gamboa is located 30 km north of Panama City, and is a small town on the east bank of the Panama Canal, north of the Chagres River. Gamboa and surrounding areas, including the Soberania National Park, provided an excellent area to find mostly *H. erato* and occasionally *H. sara* roost sites. Gamboa was visited in 2008 in July and August and in 2009 from May to September. The rainy season in Panama usually covers May until November.

Light Levels at Roost Sites

Light levels at one *Heliconius erato* roost site were measured. Measurements were taken at the actual perch and at a randomly chosen site 2-3 m next to it using a hand-held light meter (Enviro-Meter^{TM} Fischer Scientific). The recordings were made by holding the device at a 45^{o} angle with respect to a white sheet of paper where the ambient light was reflected. The light meter was calibrated every day just before taking the reading. Measurements were done on 41 days over a 2-mo period.

Relative Humidity, Temperature, and Wind Recordings at Roost Sites

Temperature and relative humidity (RH) was recorded inside and immediately outside *Heliconius erato* and *H. sara* natural roost sites. The temperature and RH loggers (iButton Hygrochrons model DS1923, Dallas Semiconductor Corporation) were inconspicuous small electronic discs (1.59 x 0.64 cm) that did not affect roosting behavior or physical conditions of the perch. The data loggers were set to record an instantaneous reading once every twenty min during 12 h (from sunset until sunrise). Using Velcro®, one data logger was attached to a branch at the roosting perch and a control was installed at a random point approximately 2 m outside the roost site. Installation and removal was done in the morning after the butterflies left the roost site.

The data loggers' accuracy was evaluated by comparing their readings under identical conditions. All data loggers were placed in pairs in the same plastic bag to record temperature and humidity every 20 min during 48 h of warm, room temperature conditions (144 records). The average of the test readings was compared for every pair of data loggers used in an inside-outside comparison at roost sites. When the average reading of one data logger under test conditions was less than the other, that difference was added to the measurements from the field of the first data logger.

Data was recorded at four natural *H. erato* roost sites in Gamboa, and five *H. sara* roost sites, four being at La Selva and one at Gamboa. At least 22 nights of recordings were done for each site and in total 829 nights were recorded (24,870 recordings).

Wind speed was recorded at one natural *H. erato* roost site in Gamboa using a hand held wind meter (Enviro-Meter^{TM} Fischer Scientific). Measurements were made at the time of roost formation on 43 days over a two-month period.

Importance of Color Cues at Roosting Aggregations

In this experiment, the wing color pattern (i.e. red forewing patch and yellow hind wing stripe) was covered dorsally and ventrally with a permanent-ink black marker (Sharpie®) so the entire individual looked black. Individuals were placed in a 2 x 3 x 3 m cage with appropriate artificial perches and feeding sources. Three groups (each composed of 4 males and 4 females of *Heliconius erato*) were used. After sunset, when all the butterflies were perched, it was observed whether an aggregation had formed or not. An aggregation was recorded as "successful" when two or more individuals were perched with the wings folded and with no more than 15 cm between each other. Three groups (four males and four females per group) were used as a control. Control individuals were covered with a black Sharpie® on the black areas of the wing dorsally and ventrally so the butterflies would keep their color pattern unchanged but had an equivalent area of Sharpie® permanent ink compared to the experimentally blackened butterflies; this would rule out the possibility that the ink itself is interfering with the behavior, possibly through odor aversion, pheromone masking, etc. After sunset it was observed whether the butterflies formed an aggregation or not. For every group, observations were made during at least five days. In total, 17 trials were made for each type of treatment.

Results and Discussion

Results from the color experiments suggest that, even in low light conditions, the absence of color in wing patterns interferes in the formation of the aggregations (Table 3-2). Butterflies with black wings were able to successfully form aggregations in 23.5% of the trials (4 trials out of 17). The control groups (butterflies with ink only in the black areas of the wings) successfully formed aggregations in 100% of the 17 control trials.

This suggests that the *Heliconius* eye detects wing color pattern in these light conditions, which can be as low as 5 ft-cd, and that color pattern is probably the first intraspecific recognition cue they use when arriving at roost sites. *Heliconius* butterflies have apposition compound eyes which are not typical in dim-light active insects, which instead have superposition compound eyes optimized for increased photon capture (Frederiksen & Warrant, 2008). Nevertheless, *Heliconius* eyes may have one or more adaptations found in some nocturnal and crepuscular insects (such as some species of bees, wasps, and butterflies) that have apposition compound eyes (Frederiksen & Warrant, 2008). These adaptations include large eye and facet size and coarsened spatial and temporal resolution (Frederiksen & Warrant, 2008). The *Heliconius* ability to see at dusk may also be used for navigation purposes when searching for roost sites in low light conditions. *Heliconius* navigational abilities could be evaluated in a large outdoor screened enclosure (50 x 4 x 10 m), where several potted flower plants and small trees could be moved at will so different arrangements could be set-up to test the use of landmarks of different sizes and color. Furthermore, crepuscular vision can be key in detecting and avoiding predator attacks.

Light levels at the *H. erato* roost site were considerably lower compared to an area outside the roost (Figure 2-1; K-S D = 0.7780 P = 0.001). The average light level for the roost site was 46.14 ft-cd, while light levels outside the roost averaged 153.53 ft-cd, suggesting that *Heliconius* butterflies may use negative phototaxis at dusk to locate the site. This is consistent with observations made in several other roost sites of *Heliconius erato*, *H. charithonia*, and *H. sara*. Negative phototaxis could be tested in controlled experiments using an outdoor-screened enclosure, where an area of the

enclosure provides darker light levels at dusk. If groups of butterflies consistently fly and perch in the shaded area, this would suggest the use of negative phototaxis. Besides the possibility of providing an environmental cue, low light levels at roost sites can have added advantages such as making the butterflies less conspicuous for birds and other predators at the time of roost formation when they interact in flight while getting ready to perch. The vegetation that usually covers the sites also offers protection from rain during roost formation and overnight which is crucial during the rainy season when it is not uncommon to have multi-hour rain sessions at sunset and overnight.

Relative humidity (RH) data suggest that butterflies at roost sites experience a drier environment while roosting at night (Figure 2-2). Recordings for RH and temperature generated by each pair of data loggers in each site were analyzed using ANOVA single factor (Table 3-1, Figure 3-2, 3-3). In seven roost sites (three *H. sara* and four *H. erato*), RH was significantly lower in the roost site, with differences up to 2.45% compared to the vicinity. Despite the fact that increased RH reduces desiccation, the RH in all cases was above 90%, which does not represent a physiological stress to the butterflies. Higher RH outside the roost sites could be explained by the site architecture. The live vegetation mats and surrounding plants contribute to a higher RH outside the roost site, while inside the roost site, dry and dead twigs and vines probably provide a drier environment. These differences in RH may also constitute an additional environmental cue for the butterflies when seeking the roost sites at dusk and the phenomenon suggests the use of humidity sensitive sensillae (hygroreceptors). Hygroreceptors have been found in several insect orders and they constitute essential environmental indicators that can be used in several ways, including host-plant

selection and hygropositive navigation towards beneficial micro environments (Altner & Loftus, 1985, Altner & Prillinger, 1980, Tichy & Loftus, 1996, Steinbrecht, 1998, Yokohari, 1999). Hygroreceptors are usually located in the antennae and other head organs (Altner & Loftus, 1985). Cage experiments testing for hygrosensitivity are very difficult because creating a humidity gradient that mimics the one found in natural sites is logistically complex. A meticulous examination of the *Heliconius* adult would probably reveal several types of sensillae that could be electrophysiologically tested for hygrosensitivity.

Temperature results were mixed, showing significant differences only in four sites (Figure 3-3 and Table 3-1). In two of these, temperatures were colder at the roost site and in two other sites, the trend was the opposite. Recorded temperatures were very mild, ranging from 22 ^{o}C to 26 ^{o}C, thus not generating physiological stress to the butterflies. Further in-cage observations made in fall and winter on native *H. charithonia* in northern FL, USA reveal that the butterflies do cluster tightly at low temperatures (5-15^{o}C) (pers. obs.). As for Andean tropical *Heliconius*, similar trends may be expected in their highest altitudinal range (2000 m), where night temperatures have comparable ranges to subtropical winter nights. This suggests that roost site architecture, level of clustering, and environmental temperature may be linked. A correlation between level of clustering and environmental temperature could be tested in air-conditioned indoor enclosures by varying the room temperature and recording the level of clustering. Aggregation level of clustering can be measured using spatial statistics (Appendix A).

Wind recordings at the time of roost formation showed virtually no wind (0 m/s) in 43 d of recordings over a two-month period, with only two recordings of 0.1 m/s and 0.3 m/s. In addition, more than 800 h of video recordings in four roost sites revealed only very occasional nocturnal movements, due to wind or rain, of the perched butterflies, hanging twigs, and vines at roost sites. These observations confirm that roost sites are well protected from wind and rain.

Aside from the environmental factors and use of color cues in the formation of *Heliconius* aggregations, chemical cues seem to be involved (Chapter 6, 7). Use of color patterns at dusk may be important for initial intraspecific recognition in the medium range (0.5-2 m), but at short range (0-0.5 m) different non-contact interactions and contact interactions are frequent among roostmates (Mallet, 1986b, Salcedo unpub. data). This, together with preliminary chemical analyses that reveal potential short-range volatile chemical cues and contact chemical cues (Salcedo unpub. data), suggest that chemical communication is also part of the equation that leads to the formation of nocturnal aggregations in *Heliconius* butterflies.

Table 3-1. ANOVA test to compare outside and inside readings of temperature and relative humidity at *Heliconius erato* and *H. sara* roost sites. No less than 50 nights (500 h) of recordings were analyzed per site.

				Relative humidity			Temperature		
Site**	GPS	Species	Roost size (# of ind.)	Location of highest reading	$F_{calculated}$	P	Location of highest reading	$F_{calculated}$	P
CPa	9°07'20.53" N 79°42'27.30" W	*H. erato*	3-5	outside	302.810*	0.001	outside	21.275*	0.001
183A	9°06'58.26" N 79°41'54.24" W	*H. erato*	6-14	outside	29.523*	0.001	inside	1.870	0.172
PR2KM	9°07'58.6" N 79°43'09.1" W	*H. erato*	3-5	outside	359.013*	0.172	inside	2.697	0.101
161	9°06'58.61" N 79°41'57.65" W	*H. erato*	3-5	outside	6.153*	0.013	outside	14.550*	0.001
CPb	9°07'31.98" N 79°42'23.26"W	*H. sara*	5-8	outside	82.695*	0.001	inside	9.779*	0.002
STR4500	10°26'01.6" N 84°02'07.8" W	*H. sara*	5-10	outside	117.482*	0.001	inside	2.471	0.116
SAZ1050	10°26'18.8" N 84°00'23.8" W	*H. sara*	3-8	outside	0.964	0.326	inside	1.875	0.171
SAZ830	10°26'13.5" N 84°00'19.7" W	*H. sara*	3-5	outside	41.933*	0.001	outside	4.548*	0.033
SAZ1000	10°26'18.8" N 84°00'19.7" W	*H. sara*	8-20	outside	1.100	0.294	equal	0.123	0.726

Table 3-2. Number and size of aggregations formed by a group of 4 males and 4 females of *Heliconius erato* butterflies with the color pattern covered by permanent black marker. Control individuals were treated by covering only the black areas of the wings.

	Experimental group		Control group	
Trial	Aggregations formed	Aggregation size	Aggregations formed	Aggregation size
1	0	-	1	4
2	0	-	1	3
3	0	-	2	2
4	0	-	1	4
5	1	2	1	5
6	1	2	2	2
7	0	-	1	2
8	0	-	1	3
9	0	-	1	3
10	0	-	2	2
11	0	-	1	3
12	0	-	1	3
13	0	-	1	3
14	2	2	2	2
15	1	2	1	4
16	1	2	1	5
17	0	-	1	5

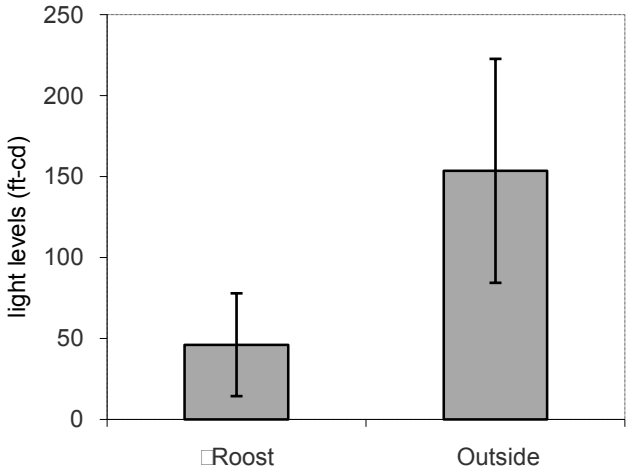

Figure 3-1. Light levels at a *Heliconius erato* roost site and its vicinity. Error bars indicate 95% CI for the means.

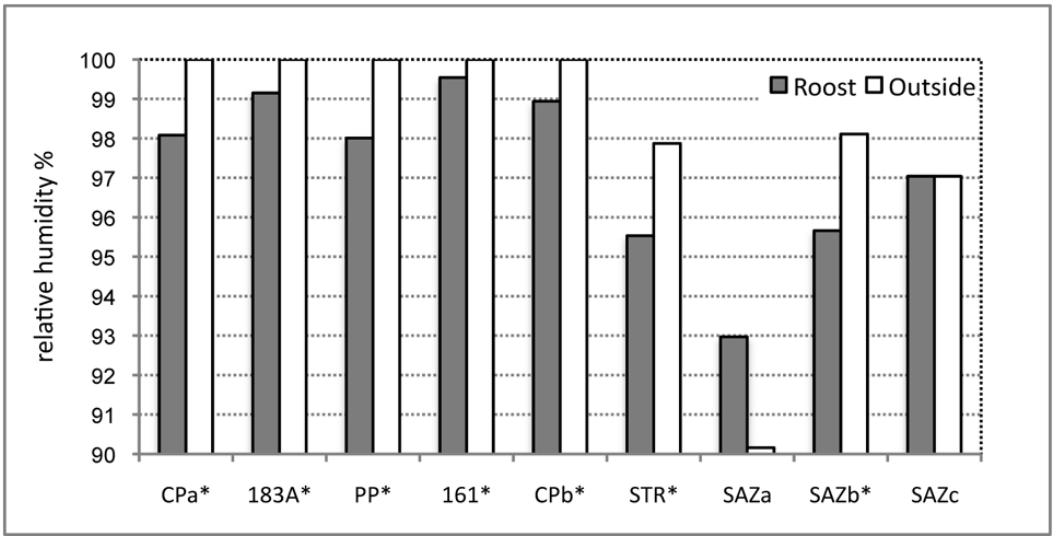

Figure 3-2. Average relative humidity recorded by sensors placed at roost sites of *Heliconius erato* and *H. sara*. Asterisks represent significant differences.

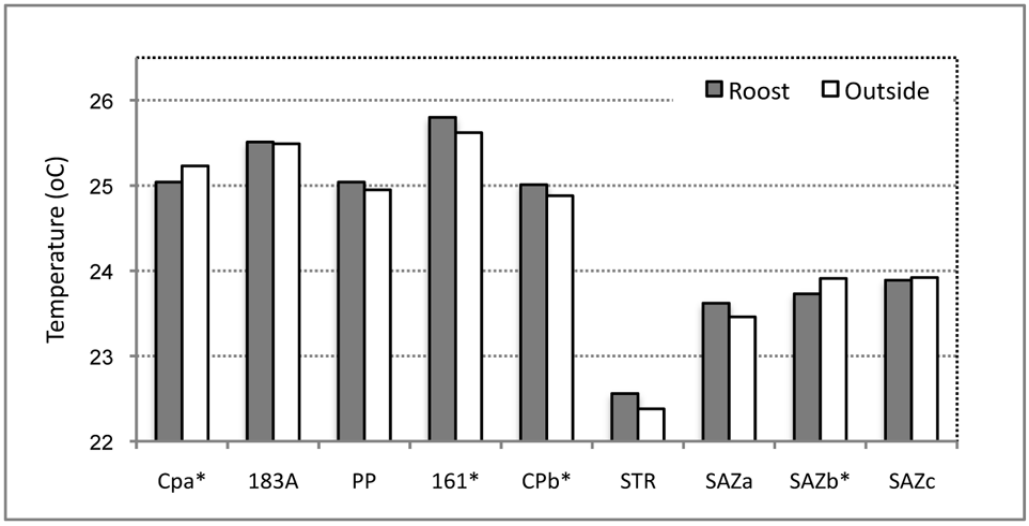

Figure 3-3. Average temperature recorded by sensors placed at roost sites of *Heliconius erato* and *H. sara*. *In two sites the sensor placed at the roost recorded significantly lower temperatures compared to the sensor placed outside the roost. In two other sites the trend was the opposite.

CHAPTER 4
HELICONIUS ROOSTING BEHAVIOR AND POLLEN FEEDING

Introduction

Many holometabolous insects have developed considerably different ecological and nutritional requirements in the larval and adult stages. For herbivorous insects, feeding on plant tissue is restricted to the larval stage, whereas the adult stage feeds primarily on plant-produced food supplements such as nectar and pollen. An interesting example with which to study these nutritional partitioning are mimetic butterflies from the genus *Heliconius*. Some species in this genus present the habit of diurnal pollen feeding as adults (Gilbert, 1972). *Heliconius* butterflies are known to rely significantly on their natural pollen host (Dunlap-Pianka et al., 1977, Brown et al., 1991). For example, *Heliconius charithonia* in a free-flying greenhouse colony with access to pollen lived for an average of 35 days, with a maximum of 105 days, which is a similar lifespan to that recorded in the field (Boggs, 1979). Oogenesis is also strongly influenced, with marked decreases in oviposition rates when pollen is absent in the diet (O'Brien et al., 2003). Similarly, lifetime fecundity (number of eggs throughout the lifespan) drops drastically without the pollen nutritional complement (Dunlap-Pianka et al., 1977). Further, essential amino acid transfer from pollen to butterfly eggs has been demonstrated in *H. charithonia* using stable isotope variation (O'Brien et al., 2003). Pollen-feeding genera include the monotypic *Laparus,* and *Heliconius* with 38 species, which is a significant figure in terms of the New World heliconiine diversity (Gilbert, 1991). *Heliconius* are unique in their systematic exploitation of pollen. During the day, adults spend long periods on a single flower collecting pollen and they occupy home ranges based largely on a network of pollen plants (Gilbert, 1991). Pollen grains stick to the proboscis and

are mixed with nectar to dissolve out amino acids, which are then ingested in solution (Gilbert, 1972, Krenn, 1998, O'Brien et al., 2003, Beltran et al., 2007). Pollen host plants include several species from the family Cucurbitaceae, including *Gurania* sp. and *Psiguria* sp., and a third one from the Rubiaceae, the genus *Psychotria* sp. These plants have inflorescences that bear bright orange and red colors, which are important long-range cues used by *Heliconius* adults to locate the plants when they are navigating the forest while foraging. By living close to the most important nutritional resource, adults maximize their fitness. However, inexperienced adults may be at risk when searching these conspicuous plants in the forest by increasing their exposure to predators. In addition, *Heliconius* pollen host plants are also pollinated by hummingbirds (Murawski & Gilbert, 1986, Stone, 1996, Cardoso de Castro & Cardoso Araujo, 2004), so minimizing search time is important in order to secure a meal before the daily supply is exhausted. Pollen host plant search time can be decreased in several ways. Butterflies may be born with no specific preference for a particular pollen host plant and hence they need to learn this preference. Recent evidence in bumblebees shows that inexperienced individuals learn floral odors from experienced foragers by associating flower-scented nectar, brought to the nest by the experienced foragers, with a particular chemical cue (Molet et al., 2009). *Heliconius* butterflies could have a similar mechanism to learn pollen odors at nocturnal aggregation roost sites. Recent field observations reveal that some individuals arrive to roost sites with pollen loads (Salcedo unpub. data) but it is unknown if these individuals are transferring any information on the foraging resources to their roostmates. Additionally these aggregations are stable and often are located relatively near to pollen host plants (Mallet, 1986b).

Given the ecological importance of pollen feeding and the fact that females arrive to roost sites bearing pollen loads, two questions arise from these observations: (1) Do young, inexperienced *Heliconius* butterflies learn pollen odors or taste from experienced foragers that arrive to nocturnal roost sites bearing loads of pollen? and (2) Do *Heliconius* females digest pollen overnight? In order to test these hypotheses, experiments with captive *H. erato* were performed (1) and pollen loads from wild *H. sara* roost members were analyzed (2).

Methods

Pollen Preference Experiments

To evaluate if the pollen preference in *Heliconius* is innate or learned, a group of *H. erato* butterflies was reared with *Passiflora biflora* as host plant and held in a 2 x 2 x 3 m outdoor cage. The butterflies were never exposed to their preferred pollen host-plants (i.e. *Psychotria* sp., *Gurania* sp., *Psiguria* sp.) and were fed only on sugar water solution. Greenhouses and cages were located at the Gamboa field station of the Smithsonian Tropical Research Institute, Panama. Each experiment was carried out in a 2 x 2 x 1.5 m outdoor cage. In each trial one individual was exposed to two feeding choices (a) 30% bee-collected pollen (Apiarios Malivern S. A. Panama), 10% sugar, 60% water; and (b) 30% *Psychotria* sp. pollen, 10% sugar, 60% water. The solutions were placed in identical red colored feeders (artificially made liquid supplier) attached to the roof of the cage by 2 mm wire holders and were 1.2 m from the ground. Based on preliminary tests, colored feeders were used because the butterflies needed the color cue to be able to recognize the feeders as a foraging source. Each trial was done in the morning and the butterflies were not fed before each trial. Each butterfly was released in

the cage and then the time spent in each foraging choice was recorded in 10-min trials. A total of 17 individuals were used.

Nocturnal Pollen digestion

Study organisms and field sites

The study was carried out in La Selva field station of the Organization for Tropical Studies in Sarapiquí, Costa Rica. La Selva is located at the confluence of two major rivers in the Caribbean lowlands of northern Costa Rica. The area comprises 1,600 ha of undisturbed and disturbed tropical wet forests. La Selva also has several kilometers of trails and forest edges that are ideal areas for *Heliconius* roosting sites. La Selva was visited during April 2009, at the end of the dry season. *Heliconius sara* populations are stable and roost sites are accessible. *H. sara* has a widespread neotropical distribution from south Mexico to south Brazil (Holzinger & Holzinger, 1994). It expresses nocturnal communal roosting at heights ranging from 1.5 m to 4 m (Mallet & Gilbert, 1995a).

Roost finding

Roost site architecture is a key visual aid when searching for roost sites in the field. *Heliconius* butterflies prefer shaded areas with plenty of thin dry vines and branches under relatively dense vegetation mats. These sites are often located near disturbed areas, forest edges, and trails. In order to identify potential roost sites, a preliminary search was done by exploring the area looking for sites with these characteristics. Roosting aggregations were found by hiking through La Selva trails and along forest edges from 1600 to 1730 h. When a butterfly was spotted, it was followed until it arrived at the roost site. After dark, searches were also done, taking into account the sites found in the preliminary search.

Collection and Analysis of Pollen Samples

Heliconius sara individuals bearing pollen loads in their proboscis were captured when they arrived at the roost sites at 1630-1740 h and after they perched at 2000-2200 h. Pollen loads were removed from the proboscis using an insect pin and then the butterflies were released immediately. Microscope slides of pollen loads were prepared in the field. Pollen grains were directly washed off from the insect pin in small drops of glycerin onto glass slides. The slides were covered with cover glasses and sealed with nail enamel (Krenn et al., 2009). The samples were observed under a compound light microscope with a digital camera attached (Olympus BX60 and Hitachi KP-D50). At least 20 different photographs were taken for each slide. The most abundant pollen grains (90% or more on each sample) belonged to *Psiguria sp*. Neck. ex Arn. Cogn. (Cucurbitaceae), so for the analyses only this species was taken into consideration. Pollen identification was done by comparing microscope photographs of pollen samples directly collected from *Gurania sp*., *Psiguria* sp. and *Psychotria sp*. from the surrounding areas, which are naturally preferred pollen host plants for *Heliconius*. Counts of 250 pollen tetrads were done per slide and the tetrads were classified as “intact” if the exine was intact and if their content was as homogeneous as in previously observed pollen from the anthers of the respective flower (Krenn et al., 2009) (Figure 4-2). Pollen grains were classified as “damaged” if they were deformed, had heterogeneous content, or if the pollen exines were broken and/or empty (Krenn et al. 2009) (Figure 4-2). Tetrad counts were done with Adobe Photoshop CS3 (version 10.0.1) by overlapping a digital grid over each photograph and then marking each tetrad with a color dot: blue for intact and red for damaged, this technique assured zero error in the counts. A maximum of two individuals were sampled per site per day to avoid disturbing the butterflies. Usually,

if there are repeated disturbances at roost sites the butterflies will abandon the site and relocate (per. obs.). Eighteen samples were collected at three roost sites over a 30-day period. In addition, 15 individuals that were observed bearing pollen loads while perched after dusk were marked with a permanent marker on the wing and observed the next day before leaving the roost for evidence of any pollen in their proboscis.

Results and Discussion

Pollen Preference Experiments

Time spent feeding on *Psychotria* sp. by *Heliconius erato* was significantly higher than time spent feeding on bee pollen (K-S D = 0.647 P = 0.001) (Table 1). This suggests that preference for natural pollen host plants is innate, and hence learning of pollen preference at roost sites is unlikely. In terms of foraging ecology, it has been suggested previously that young, inexperienced butterflies first meet older experienced individuals at foraging sites and then follow them to roost sites (Waller & Gilbert, 1982). This evidence further supports the fact that *Heliconius* butterflies do not acquire foraging information at roost sites.

It is unknown which chemical cues the butterflies are using to recognize their preferred pollen. Volatile and contact chemical cues may be involved. Based on field and in-cage observations of feeding behavior, butterflies first use color in the long range (2-10 m) to recognize their potential host plants, then fly towards the flower and hover over it. Pollen host plants may have short-range volatile chemical cues that could be emitted by the flower and also by the pollen grains themselves, which is not uncommon in insect-pollinated plants (Dobson & Bergström, 2000). It is likely then that chemical cues originated from the *Psychotria* sp. pollen grains, in addition to the color cue, may be enough to assure preference for *Psychotria* sp. pollen. Nevertheless, a combination

of flower and pollen volatiles is probably necessary in the forest, where chemical noise from numerous other sources is present. Further analyses are needed in order to identify the initial chemical cues involved in the *Heliconius* pollen feeding behavior.

Nocturnal Pollen Digestion

All 33 *Heliconius sara* individuals found at roosting sites bearing pollen loads were females. Three roosting sites were sampled on a daily basis and, on average, each site had 2-3 females and 4-5 males. All counts presented at least 50% of *Psiguria* sp. pollen tetrads with damage (Figure 4-1). However, when comparing percentage of damaged pollen tetrads between the two collection periods there are obvious differences. The first period (1630-1740 h) had a mean of 67.6% of damaged tetrads while the second period (2000-2200 h) had a statistically higher percentage of damage 85.1% (t-test $P<0.01$). In addition, the 15 marked individuals that were observed bearing pollen loads after dusk had no pollen on their proboscis before leaving the roost next day. Normally, after the pollen load has been digested, it is passively discarded (physically detaches from the proboscis by gravity) (Krenn et al., 2009). It is clear then that only females are digesting pollen overnight.

Heliconius butterflies start to actively forage during the morning, when *Psiguria* sp. inflorescences open and have supposedly maximum levels of pollen and nectar. *Psiguria sp*. flowers supposedly do not produce nectar or pollen in the afternoon. However, there are no accurate data on how the pollen levels of *Psiguria sp*. flowers change through the afternoon. *Heliconius* butterflies digest and discard pollen within several hours so it is unlikely that the analyzed pollen loads came from early morning collection events. Pollen amount was not recorded but the pollen loads observed and analyzed were comparable to the ones seen regularly in the morning. This, together

with the fact that about 50% of all the pollen analyzed had some level of damage by the time the butterflies start arriving to the roost sites, suggests that *Heliconius* females continue to find *Psiguria* sp. and efficiently forage until 1400-1500 h, and possibly just before starting to head to roosting sites. It is also important to add that *Psiguria* sp. flower availability can be affected by a number of factors, including rain levels (Murawski, 1987), natural disturbances, and anthropogenic disturbances. The different levels of pollen damage found in the samples collected at dusk probably reflect digestion efficiency and intra- and interspecific competition among *H. sara* females (Boggs, 1981, Cardoso, 2001).

Diurnal observations have shown that females tend to carry larger pollen loads than males (Gilbert, 1972, Boggs et al., 1981). This is linked to the female's physiological and ecological requirements, because pollen provides nutrients for egg production, increased longevity (Gilbert, 1972, Boggs et al., 1981, Dunlap-Pianka et al., 1977, Boggs & Gilbert, 1979, Cardoso & Gilbert, 2007), and for *de novo* synthesis of cyanide glycosides (Nahrstedt & Davis, 1983). The results here support these studies by providing evidence that *H. sara* females forage more intensively than males and that nocturnal digestion could be a strategy to maximize pollen feeding benefits. Additional observations in Pico Bonito, Honduras, revealed that *H. charithonia* females also arrive at roosting sites bearing pollen loads.

Despite the likelihood of nocturnal digestion as an advantageous adaptive strategy for *Heliconius* females, it is unclear whether this strategy is a local one that results from abundant *Psiguria* sp. availability in the study area. *H. erato*, another roosting species,

has never been seen consistently (in periods of at least 30 days, in two different years) bearing pollen loads at roost sites found in Panama.

The findings presented here show a potential key strategy that *Heliconius* females may use to maximize pollen feeding. Further field research on *H. sara* and other roosting and non-roosting species is needed to determine if (1) roosting environmental conditions offer specific advantages for optimized pollen digestion, (2) nocturnal pollen feeding is spread among other roosting and non-roosting species, and (3) to reveal if there are correlations between nocturnal pollen feeding and local availability of pollen sources.

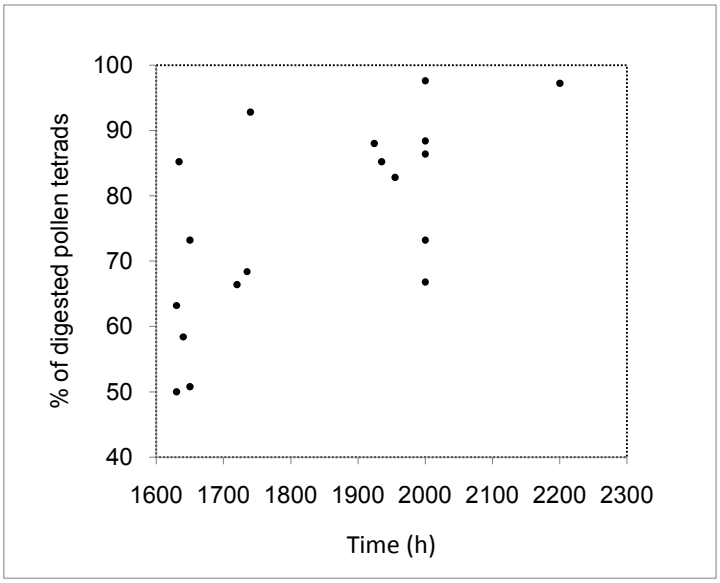

Figure 4-1. Percentage of damaged *Psiguria.* sp. pollen tetrads from samples collected from *Heliconius sara* individuals at roosting aggregations at La Selva field station, Costa Rica. Each dot represents a sample taken from a single individual.

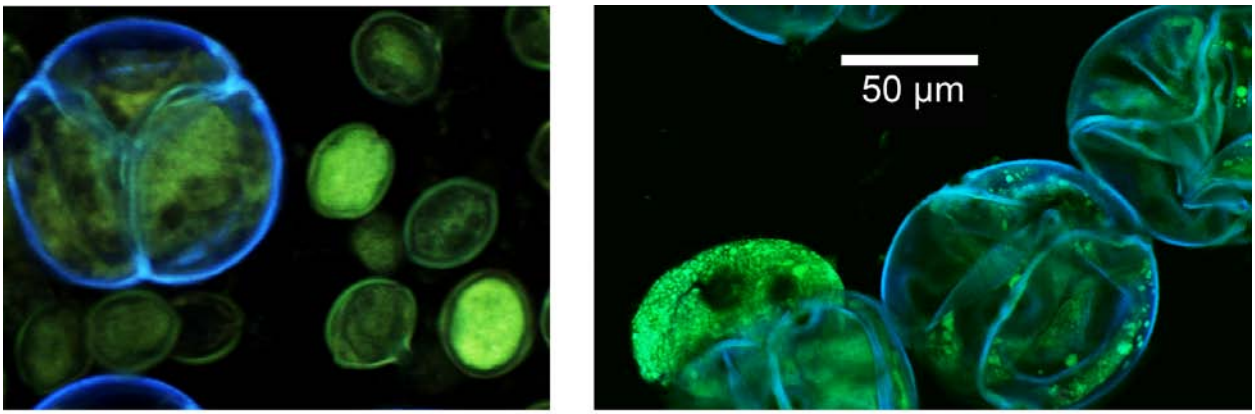

Figure 4-2. A: Undigested *Psiguria* sp. pollen tetrad removed from a *Psiguria* sp. flower. B: Digested pollen tetrads removed from the proboscis of a *Heliconius sara* female perched at a roosting aggregation at La Selva field station, Costa Rica.

Table 4-1. Time spent feeding in 10-minute trials by a group of 3 males and 3 females of *Heliconius erato* exposed to two choices of pollen in cage experiments.

	Time spent foraging (s)	
Sex	Bee pollen	*Psychotria* sp.
m	97	486
m	375	477
m	109	291
f	172	262
f	90	271
m	180	174
f	116	125
f	19	221
f	148	449
f	153	19
f	36	418
f	82	543
m	70	401
f	188	60
f	117	405
f	146	252
f	206	155

CHAPTER 5
IMPORTANCE OF *HELICONIUS* ROOSTING AGGREGATIONS AS AN ANTI-PREDATOR STRATEGY

Introduction

Kin selection favors the reproductive success of relatives, even at a cost to the individual's survival and/or reproduction (Hamilton, 1971, Gamberale & Tullberg, 1998). One way in which this is achieved involves unpalatability and aggregative behaviors. Many unpalatable species use conspicuous color patterns to give a clear signal (aposematism) to predators of their noxiousness (Cott, 1957, Edmunds, 1974, Guilford et al., 1987), and in many cases the message can be strengthened through aggregations (Gagliardo & Guilford, 1993). For example, aposematic monarch butterflies form large overwintering aggregations that are efficient in diluting predation (Brower et al., 1967). However, there are cases where the advantages of an aggregation are not clear or not necessarily the result of an evolutionary process that fixed the aggregative behavior trait to improve fitness (Odendaal et al., 1988, Gomes-Filho, 2000, Benson & Emmel, 1973).

Some species of aposematic *Heliconius* butterflies form nocturnal aggregations. Individuals aggregate at dusk in roost sites that are fairly stable, and depart from the site at sunrise. The most common predators of *Heliconius* are diurnal birds (Chai, 1986). Predator-mediated selection has modeled color patterns that characterize the unique Müllerian mimicry rings *Heliconius* present in the neotropics (Franks & Noble, 2003). Only one study has reported frequent disturbance events (mostly by birds) at *Heliconius* roosting sites at sunrise and occasionally at sunset (Mallet, 1986a). However, there is no evidence to date of overnight predation and disturbance.

In order to evaluate the importance of *Heliconius* roosting aggregations as a nocturnal anti-predation strategy, continuous sunset, sunrise, and overnight video recordings were made in several natural roosting sites of *Heliconius erato* and *H. sara* to document disturbance and predation events. In addition, potential kin-selection influence on the origin of the behavior was tested by breeding several broods (groups where all descendants came from a single female) of *H. erato*, which were separately bred in captivity and then combined to see whether closely related butterflies would roost together or not.

Methods

Nocturnal Video Recording of Roost Sites

Natural areas in La Selva, Costa Rica and Gamboa, Panama were explored to locate at least four roosting sites. Each site was videotaped using a weatherproof infrared camera system (Figure B-1) during at least 10 days. Videos were then analyzed for predation events using QuickTime Player 7 for Mac.

Kin-selection Experiments

Heliconius erato wild females were collected from 3 different populations (3-5 females per population). From six females, six separate broods were bred (broods A, B, C, D, E, F). Once the broods reached 10 individuals and started displaying roosting behavior, broods were split and combined into five different groups to test whether members of different broods roost together or not. The new groups were observed for 3-5 days. All individuals were marked with a Sharpie® on the underside of both forewings with the letter of the brood they belonged to and a number (e.g. A1, individual 1 from brood A).

Results and discussion

Nocturnal Video Recording of Roost Sites

In 870 h of dusk, nocturnal, and sunrise recordings, only one predation event was observed early in the evening when setting up the camera at an *Heliconius erato* roost, in which a butterfly got caught in a recently established spider web and was subsequently attacked and eaten by the spider. At one *H. erato* roost site, unidentified bats came an average of 2 times per night (73 visits in 38 days of recordings) and in one night a clear predation attempt was registered (Object 5-1). The bat hovered repeatedly by the roost until it quickly caught one butterfly with its mouth and left. Immediately after the bat left the camera field of view with the butterfly, a butterfly was observed entering the field of view. This individual was presumably the one attacked and was probably rejected and still able to fly back to the roost. No evidence of regurgitation (crushed body or wings) was found near the roost site the next morning. This site was very stable over the period of the recordings and held a high number of butterflies (10-16) compared to the average roost sizes (3-4 individuals) observed for *H.* erato (Mallet, 1986b, pers. obs.). Disturbance events by unidentified birds at these sites were scarce (only 2) and, other than a couple of insects, a few small mammals also passed by without apparently noticing the roost. However, these mammals did create some level of disturbance since the aggregation was very low (50 cm from the ground) (Table 5-1, Object 5-2, 5-3, 5-4). New recordings were made in the same site a year after after the observed bat predation attempt, but the site was destroyed and a small group of only 3-6 butterflies established a new roost site nearby, which relocated again after the new site was destroyed during a storm. The rest of the recordings had smaller roost sizes (3-4 for *H. erato* and 8-10 for *H. sara*) and showed very low disturbance

events (Table 5-1, 5-2, 5-3). These results suggest that overnight predation is not frequent in roost sites and that the butterflies are well concealed from nocturnal and crepuscular predators. It is unlikely that there are bats specialized in foraging at *Heliconius* at roost sites. Mist nets were set up near one of the roost sites and several bats were captured. The species captured, *Artibeus jamaicensis* and *Carollia perspicillata*, are frugivorous and occasionally prey on insects to supplement their diet (Estrada, pers. comm.). The frequent bat visits and the single predation attempt to the largest *H. erato* aggregation (10-14 individuals) (Figure 5-1, 5-2) can lead to different hypotheses to explain the importance of large *Heliconius* aggregations. Oversized aggregations may increase the risk of attack by predators, and even if the predators are tolerant to distastefulness or cannot learn, the aggregation still will dilute danger of predation on an individual basis. Alternatively, there could be an initial period of attacks after which predators would learn that the butterflies are distasteful. The data for large aggregations presented here only accounts for one site in a five-week period, so it is not conclusive and more sites need to be studied to test these hypotheses. On the other hand smaller aggregations or clusters (2-4 individuals), which are more common than large aggregations (Mallet, 1986b, Salcedo, 2006, pers. obs.), may have evolved for several reasons, including lack of nocturnal predators (i.e. not necessary to have a large aggregation to educate nocturnal predators) and to reduce the attractiveness (i.e. to reduce disturbance of other non predatory animals). The results here make this hypothesis is more plausible given the fact that in there was virtually no clear predators for *Heliconius* in three different roosting sites in two different seasons, an with observations in two different species (Table 5-1, 5-2, 5-3). Aggregation size and level of

attractiveness to predators could be further tested in cages where different sizes of artificial decoy-made aggregations are exposed to predators. In addition, aggregations seem to be extremely inefficient in alerting other individuals when there was an attack or a disturbance at night. It seems that the butterflies enter into a “deep-sleep” state. In all observations the butterflies remained motionless during disturbance events, except on very few occasions when one or two individuals briefly opened and closed their wings after a disturbance but remained perched in the same place (Object 5-1, 5-4). However, at dusk and sunrise butterflies seem to be very sensitive to movement or touch and will disband and spread in the roost periphery quickly (Object 5-2, 5-3). This behavior may be related to the higher activity levels of their most common predators, birds, which are more active at these times of the day.

Kin Selection Experiments

Establishing colonies was difficult because females constantly died and heavy storms killed many individuals and destroyed cages. Out of the 6 bred broods, only five had enough individuals that survived during the course of the experiments and out of all the groups formed only three of them gave meaningful data. In total, three groups of combined broods successfully formed aggregations. Each group was monitored for at least 3 days for a total of 11 days of observations where 26 aggregations were formed. Out of these, 12 were composed of members of the same brood, and seven aggregations were composed of 3-4 relatives and one non-relative (Table 5-4). The remaining seven had a 1:1 ratio of relative : non-relative. The fact that only 46% of the aggregations were composed of members of the same brood does not support the hypotheses of a kin-selected behavior. Nevertheless, the results are relevant because this was the first time this was tested in controlled experiments and gives support to

previous field evidence. Once emerged, teneral adults migrate and establish a home range and start or join a roost. Older adults move among roosts, there is overlapping of daily home ranges of individuals from different roosts, foreign individuals have the ability to join gregarious roosts, and there are high levels of heterozygosity within populations (Mallet, 1986a).

Table 5-1. Disturbance events at *Heliconius erato* aggregations in Gamboa, Panama August-September 2008. Data account for 466 h of infrared nocturnal footage obtained between dusk and dawn.

Description	Number of events
bats	73
agoutis	3
armadillos	1
rabbits	1
birds	2
arthropods	2
unidentified	6

Table 5-2. Disturbance events at *Heliconius erato* aggregations in Gamboa, Panama May-June 2009. Data account for 373 h of infrared nocturnal footage obtained between dusk and dawn.

Description	Number of events
bats	4
agoutis	0
armadillos	0
rabbits	0
birds	0
arthropods	7
unidentified	8

Table 5-3. Disturbance events at *Heliconius sara* aggregations at La Selva Biological Station, Costa Rica, April 2009. Data account for 31 h of infrared nocturnal footage obtained between dusk and dawn.

Description	Number of events
bats	2
agoutis	0
armadillos	0
rabbits	0
birds	0
arthropods	1
unidentified	2

Table 5-4. Aggregations formed when individuals from different broods were placed in the same cage. Each individual has a unique identification number: a capital letter that indicates the brood to which it belongs, and a numerical value, which is the individual unique identification number. Individuals between brackets [ ] formed an aggregation. Individuals in red belong to mixed brood-aggregations. Individuals that did not participate in aggregations are not reported.

Day	Families placed in the same cage	Individuals in cage	Aggregations formed
1	F+C	C1, C2, C3, C4, C6, F1, F2, F3, F4	[C2+C3+C6], [F4+C1], [F1+F3]
2			[C1+C2], [C2+C3+C4+F3]
3			[C1+C2], [C6+F1]
1	A+B	A1, A2, A3, A4, A5, B1, B2	[A1+A2], [A4+A3]
2	A+B		[A2+A3+B2], [A1+A5]
3	A+B		[A1+A2]
4	A+B		[A1+A2+B2], [A4+A5]
1	A+C	A1, A2, A3, A4, A5, A6, C3, C4, C5, C6, C11, C12, C13	[A1+A2+C6], [C5+C11], [C12+A3]
2	A+C		[A5+C6+C11], [A3+C12], [A1+A2]
3	A+C		[A1+C6+C11], [A4+C5], [A3+C4]
4	A+C		[A1+C2+C13], [A4+C5+C6], [C3+C4]

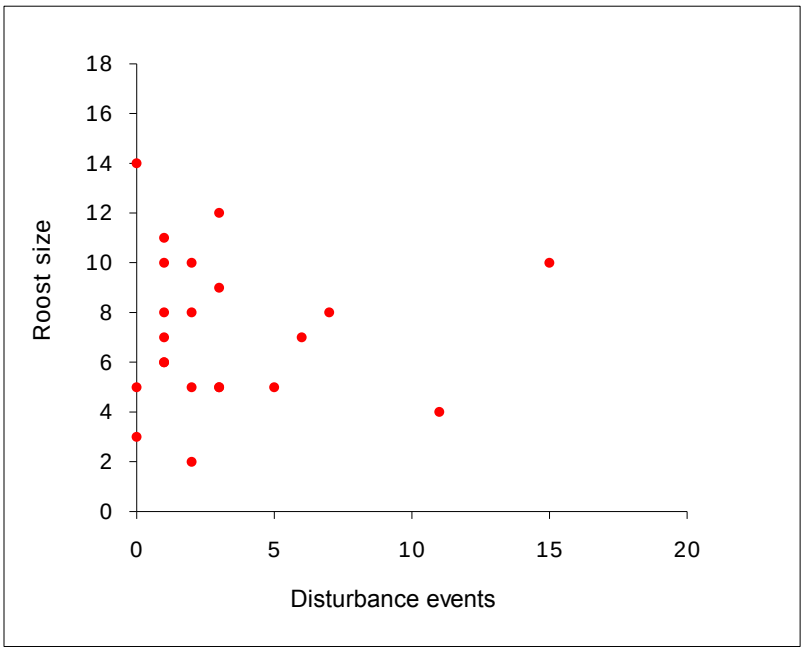

Figure 5-1. Roost size vs. Disturbance events in a *Heliconius erato* aggregation in Gamboa, Panama. Each data point represents number of individuals in the roost and the number of disturbance events registered in one night. The disturbance events were registered over a two-month period.

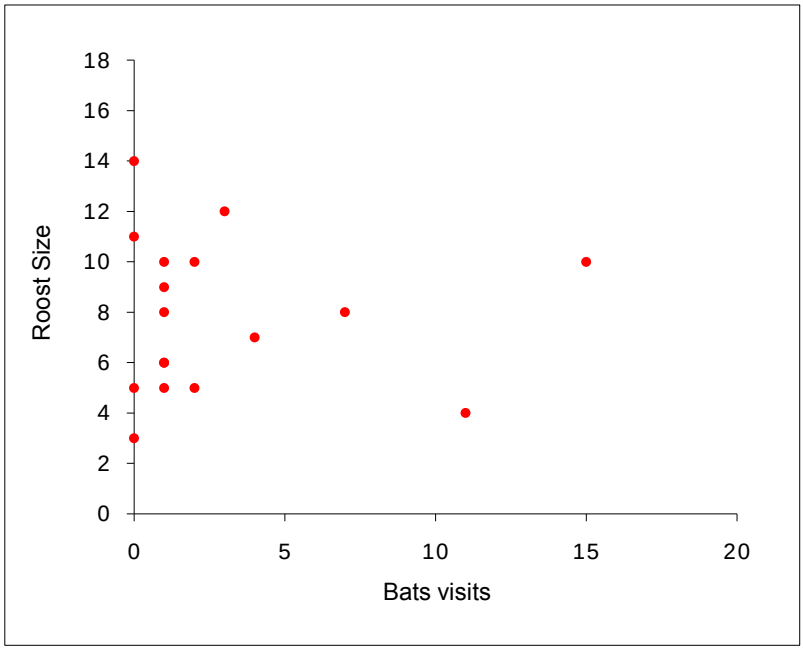

Figure 5-2. Roost size vs. Bat visits in a *Heliconius erato* aggregation in Gamboa Panama. Each of the 26 data point represents number of individuals in the roost and the number of bats visits registered in one night. The bat visit events were registered over a two-month period.

Object 5-1. Video of an unidentified bat predation attempt upon a *Heliconius erato* individual at a roosting site. (.MOV 18MB Quicktime required)

Object 5-2. Video of a disturbance event provoked by a Central American agouti (*Dasyprocta punctata*) at a *Heliconius erato* roosting site. (.MOV 10MB)

Object 5-3. Video of a disturbance event provoked by an unidentified bird (Aves) at a *Heliconius erato* roosting site. (.MOV 17MB)

Object 5-4. Video of a disturbance event provoked by a walking stick (Phasmidae) at a *Heliconius erato* roosting site. (.MOV 10MB)

CHAPTER 6
ANALYSIS OF *HELICONIUS ERATO* CUTICULAR EXTRACTS

Introduction

Insects in general are known for widespread,, use of chemical cues or semiochemicals. Semiochemicals have a wide range of functionality and play a key role, from simple behaviors such as host plant seeking (Bruce et al., 2005) to complex behaviors such as the ones presented by social insects, in which they facilitate mating, attract workers during nest relocation, identify the queen as a target for special worker care, and inhibit the production or development of new reproductive individuals (Winston, 1987, Hölldobler & Wilson, 1990, Landolt et al., 1998).

Although *Heliconius* butterflies are known to rely on vision to locate pollen host plants, navigate in the forest, form roosting aggregations, and mate, semiochemicals are likely to be involved in several of these components of their biology. *Heliconius* females have characteristic odors obvious to the human sense of smell and have been described subjectively more than one century ago; however, few attempts have been made to elucidate their biological function and chemical composition (Eltringham, 1925, Gilbert, 1976, Miyakado et al., 1989). Only recently have studies been published on semiochemicals in two *Heliconius* species, *H. melpomene* and *H. charithonia* (Schulz et al., 2008, Estrada et al., 2009). *Heliconius erato* presents an interesting case to study chemical ecology in *Heliconius*. *H. erato* is found in sympatry with *H. melpomene* across the neotropics in Müllerian mimicry rings (i.e. sets of aposematic species that mimic each others wing patterns to reduce individual predator burden), and presents unusual behaviors such as pupal mating and nocturnal gregarious roosting where semiochemicals may be important.

To find candidate chemical compounds potentially important in the communication systems of *Heliconius* roosting species, extracts from *H. erato* cuticle were collected from wild live individuals and analyzed.

Methods

Extraction of Cuticular Chemical Compounds from *Heliconius erato* Butterflies

Extracts were obtained from live individuals wild-caught in Gamboa, Panama and Pico Bonito, Honduras. In total, 18 males and 15 females were used. Each specimen was held by the wings with forceps, allowing methyl chloride (HPLC grade, Fisher Scientific) from a Pasteur pipette to drip slowly over the body of the butterfly. The body was held vertically, so that the methyl chloride bathed the whole surface, from the head to the end of the abdomen. The wings were not bathed. The methyl chloride dripped from the tip of the abdomen into a 5-mL vial. The sample was then passed through a glass wool filter to remove scales and other particles.

Analysis of *Heliconius erato* Cuticular Extracts

Each sample was concentrated by blowing nitrogen onto the open mouth of a vial with a 500 µL aliquot of extract until 400 µL were evaporated. Aliquots of 20-40 µL of the concentrated sample were used in gas chromatography-mass spectroscopic (GC-MS) analysis. MS analysis was performed by positive ion electron impact gas chromatography-mass spectrometry (EI GS-MS) on an HP 6890 gas chromatograph coupled to an HP 5973 MS detector. One µL of each sample was injected in the splitless mode (injector temperature – 240^{o}C, purge at 1 min) onto an Agilent HP-5MS dimethylpolysiloxane column (30 m×250 µm (i.d.)×0.25 µm film thickness, (Agilent Technologies, Palo Alto, CA, USA) and separated by temperatures programmed from 35°C (1.0 min hold) to 230°C at 10°C/min. Helium was used as a carrier gas at 1.2

ml/min. Compounds were tentatively identified by comparison of mass spectra (a) with mass spectra libraries (NIST and Department of Chemical Ecology, Göteborg University, Sweden) and (b) by comparing mass spectra and retention times of authentic standards when available.

Bioassays using *Heliconius erato* Cuticular Extracts

Cuticular extracts from males were also used to test behavioral response. Based on the GC-MS analysis, cuticular extracts were divided into two fractions based on molecular weight. The equipment used for GC fractionation was an Agillent Technologies 6890N GC with a J&H Scientific 30m DB-1 column (0.530 od, 0.5 micron coating) and cool on column injection. The analytical column was connected to a Y tube fitted with equal lengths of 0.1 µm (id) and 0.25 µm (id) deactivated fused silica tubing. The 0.1µm section of tubing went to the flame ionization detector and 0.25 µm section of tubing exited the GC and terminated in the heat block of a custom fabricated Brownlee-Silverstein collector. A total of 15 µL of methylene chloride containing the extract was injected for each run. Splitless injection at 280^{o}C was used with a temperature program rising from 35^{o}C to 270^{o}C, with heating rate of 10^{o}C/min. Carrier gas was helium at a pressure of 20.82 kPa. Initially all compounds eluting during the run were collected in a single collection. Subsequently, two fractions were collected. Fraction one contained all peaks eluting from injection until 23.35 min in the run (this corresponded to a Kovats index of 1170) and fraction two contained all peaks eluting after that. The fractions were collected in a 100 mm 1.1-1.2 inside diameter melting point capillary tube (Corning, 9530-1), the chemicals passed GC through a heated transfer line into the capillary. The chemicals were captured in the capillary where it passed through an aluminum block cooled by dry ice bathed in acetone. The fractions

were eluted from the capillary with 50 µL of methylene chloride in two washes for a total of 100 µL of solvent. These fractions were then used for bioassay. Collection efficiency was routinely 65%+. The extracts were analyzed afterwards with GC-MS to detect loss of compounds.

Butterfly decoys were made by using dead butterflies that were cleaned by dipping the specimens in methyl chloride for five min. Due to the number of contacts and fanning interactions previously observed during roost formation, bioassays all the trials were done during pre-roost formation between 1750-1630 h in a screened enclosure located in the Smithsonian Tropical Research Institute, Santa Cruz Greenhouse area, Gamboa, Panama. In each trial, no less than 6-8 individuals (3-4 males, 3-4 females) were exposed to two choices: a decoy treated with the chosen extract (3 µL) and an unscented control decoy. Counts of fanning (hovering over the decoy) and contacts towards the experimental choices were registered in 10-min trials. Data sets were tested for normality using D'Agostino Pearson omnibus tests and a paired Student's t-test was used to compare differences in between data sets. All the data was analyzed using Graphpad Prism 4.0b for Mac, GraphPad Software, San Diego California USA, www.graphpad.com .

Results and Discussion

Gas Chromatography-Mass Spectrometry Analysis

Mass spectrometry analysis revealed several compounds (Table 6-1) including molecules that are known to be volatile and found in other *Heliconius* species, insects, and plants, and heavier molecules with limited volatility that may be involved in contact communication. Average quantities per individual were calculated for ocimene, oleamide, and squalene (Figure 6-1, 6-2). Ocimene is common in nature, being a

component of many floral scents (Knudsen et al., 1993), and is one of the most common volatiles in plants whose release is induced by herbivore attacks (Paré & Tumlinson, 1997). Ocimene has been identified in sex glands and claspers of *Heliconius melpomene* males as an antiaphrodisiac that is transferred to females to prevent further matings (Schulz et al., 2008). Ocimene may also have the same antiaphrodisiac role in *H. erato,* but the fact that it used as an attractant by flowers suggests that it may also be advantegeous as an intra and/ or interspecific attractant in other contexts, such as the formation of roosting aggregations or to follow other individuals to foraging patches. Its presence in the cuticle may be derived from contamination when washing the cuticle to obtain extracts, because females often expose their sex glands during the washing process and males evert their abdominal claspers. The average concentrations found in male and female cuticle were different from the concentrations previously reported for *H. melpomene*. The concentration of ocimene in *H. melpomene* males is very variable and is considerably higher in mature males (>5 d old) ranging from 0.3 µg up to 63 µg per individual and decreases after mating, while females concentration is zero before mating and increases after mating, but to much lower levels compared to males (0.02 µg) (Schulz et al., 2008). The average concentration found in *H. erato* cuticle is very low (0.0-1.0 ng) and females have almost double the levels compared to males (0.028ng vs. 0.043ng). This could be again due to different levels of contamination when females exposed their sex glands when collecting the extracts, to the fact that the condition (mated or virgin) and age of wild individuals was unknown, or it may be an indication of a different role of this monoterpene in *H. erato* from that of *H. melpomene* biology.

Several fatty acids were found in males and females, including nonanoic acid, tetradecanoic acid, and hexadecanoic acid (Table 6-1). Fatty acids are volatile and have been found in many insect groups with different roles, including sex pheromones, defense pheromones, and attractants. All these fatty acids have been found in other Lepidoptera, including *H. melpomene* female sex glands (Schulz et al., 2008), and several species of the subfamily Danainae (male scent glands) (Schulz et al., 2008). The role of fatty acids in *Heliconius* is unknown but since they have been found in male and female sex glands in other lepidopterans it is likely that they form part of the pheromone bouquet used during courtship.

The only fatty acid amide identified was oleamide. Although oleamide has not been reported for any lepidopteran, it has been found on the cuticle and Dufour gland of *Polistes* wasps (Dani et al., 1995). Fatty acid amides are very unusual components for cuticular lipids, and since they are reported as contaminants from plastic materials (Ende & Spiteller, 1982), their occasional presence in cuticular extracts has been considered unnatural (Dani, 2009). Oleamide can be volatile (282 g/mol), but its biological role in Lepidopera has never been documented or tested.

Octadecyl acetate, a fatty acid-derived ester, was also identified. This ester is volatile and has been reported in the bouquets of several species of *Cypripedium* sp. (Orchidaceae) (Barkman et al., 1997), and as component of pheromone bouquets in Tortricidae, Noctuidae, and Danainae (Lepidoptera) (Yang et al., 2009, Schulz et al., 1993, Wenqui et al., 1999, Inomata et al., 2005). In these groups it is usually associated with androconial organs or sex glands of males and females and it has not been reported for *Heliconius.*

The terpene squalene was also identified. Squalene has been found in *H. melpomene* sex glands of males and mated females, which suggests that is a compound that is part of the antiaphrodisiac bouquet. The average concentrations in males and females are very low and very similar, 0.054 ng and 0.056 ng respectively.

A set of eight unsaturated (branched) hydrocarbons was found ranging from C23 to C34 (Table 6-1). The specific location and nature of the branching was not determined but the mass spectra are reported (Figures 6-3, 6-4, 6-5, 6-6, 6-7, 6-8, 6-9, 6-10). Cuticular hydrocarbons range from 300-450 g/mol and hence have limited volatility and are commonly used as contact semiochemicals. They have been studied in many insect groups including species of social insects, where they serve for kin recognition (Espelie et al., 1994) and to inform task decisions (Greene & Gordon, 2003). In *Drosophila* (Diptera) they are used for mate recognition (Howard et al., 2003), and in Lepidoptera they are pheromone components and sex attractants (Geometridae, Noctuidae, Arctiidae, and Lymantriidae) (Millar, 2003).

Bioassays using Cuticular Extracts

Bioassays were primarily designed to test the importance of cuticular extracts on communication at short range. Number of fanning interactions towards decoys treated with the first fraction was significantly higher ($t = -2.38$ $p = 0.004$). Differences for contact interactions when testing the first fraction were not as marked ($t = -1.87$, $p = 0.095$). The second fraction also generated a higher number of fanning interactions towards the treated decoy but at a lower level ($t = -2.38$, $p = 0.041$) and no differences in the number of contact interactions ($t = -0.246$, $p = 0.811$). These results suggest that a major component in short range attraction is present in the first fraction. From the identified compounds in this fraction (Table 6-1) it can be suggested that the active component

that generates attraction is E-(β)-ocimene. The decoy treated with the second fraction generated also a higher number of fanning interactions when compared to the control but at a much lower level. This suggests that there are components in this fraction that, together with ocimene, conform an attractive blend. The second fraction is largely made up by fatty acids, esters, and cuticular hydrocarbons (Table 6-1).

Bioassays designed to test the role ocimene alone and blends of ocimene and components from the second fraction, would be the next logical step to detect what components of the cuticular extracts are important in *Heliconius erato* chemical communication.

Table 6-1. Compounds found in *Heliconius erato* cuticle of wild individuals. *Compounds identified against known standards.

RT	MW	Compound	males	females
13.50	136	(E)-β-Ocimene*	+	++
19.77	158	Nonanoic acid	+	+
25.89	206	(1,1-dimethylethyl)-Phenol, 2,4-bis	+	+
31.5	228	Tetradecanoic acid	+	
35.82	256	n-Hexadecanoic acid	+	+
43.20	282	(Z)-9-Octadecenamide*	+	+
43.20	312	Octadecyl acetate	+	+
43.96	252	1-Octadecene	+	+
44.84	278	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	+	+
46.85	410	Squalene*	+	+
		Hydrocarbons		
43.08	324	Tricosane (C23) branched*	+	+
44.80	352	Pentacosane (C25) branched*	+	+
46.09	380	Heptacosane (C27) branched*	+	+
48.14	436	Hentriacontane a (C30) branched*	+	+
48.45	436	Hentriacontane b (C30) branched*	+	+
50.04	464	Tritriacontane (C33) branched*	+	+
50.35	464-478	Tetra/Tri-triacontane (C33-C34) branched*	+	+
52.64	478	Tetratriacontane (C34) branched*	+	+

Table 6-2. Ocimene, oleamide, and squalene average amounts per individual found in the cuticle of *Heliconius erato* individuals.

Compound	males (ng)	females (ng)
(E)-β-Ocimene	0.028	0.043
(Z)-9-Octadecenamide	0.050	0.015
Squalene	0.056	0.054

Table 6-3. Bioassays to test *Heliconius erato* cuticular extract fraction containing compounds eluting from 0-23.55 min (split at 1170 KI) in GC-FID analysis. In each trial a group of 4 males and 4 females was exposed to two decoys prewashed with methyl chloride. Decoys were attached to an artificial perch that the butterflies learned to use for roosting and were 20 cm from each other. One of the decoys was treated with 3 µL of the fraction. Number of fannings and contacts were counted in 10-min trials at sunset. Only one trial was done per day to avoid disrupting roost formation and the same group was used for all the trials.

	Control		Fraction	
Trial	Contacts	Fanning	Contacts	Fanning
1	3	3	7	16
2	1	2	1	4
3	5	9	1	9
4	0	5	2	10
5	1	5	3	13
6	2	4	3	5
7	0	1	1	5
8	2	5	9	18
9	0	0	5	9
10	0	1	0	4

Table 6-4. Bioassays to test *Heliconius erato* cuticular extract fraction containing compounds containing compounds eluting from 0-23.55 min (split at 1170 KI) in GC-FID analysis. In each trial a group of 4 males and females was exposed to two decoys prewashed with methyl chloride. Decoys were attached to an artificial perch that the butterflies learned to use for roosting and were 20 cm from each other. One of the decoys was treated with 3 µL of the fraction. Number of fannings and contacts were counted in 10-min trials at sunset. Only one trial was done per day to avoid disrupting roost formation and the same group was used for all the trials.

	Control		Fraction	
Trial	Contacts	Fanning	Contacts	Fanning
1	8	14	6	20
2	0	3	1	5
3	1	2	1	3
4	0	7	0	4
5	0	4	1	5
6	0	8	0	6
7	0	3	0	8
8	0	6	1	10
9	0	8	2	13
10	3	6	1	10

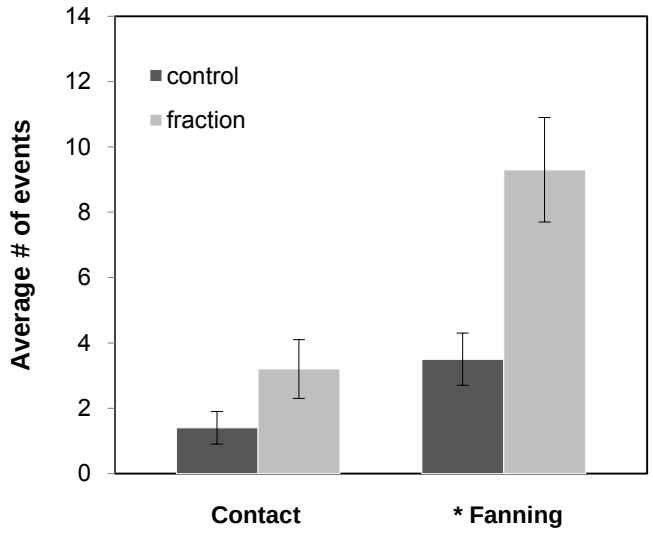

Figure 6-1. Average number of fanning and contact behavioral interactions in bioassays to test *Heliconius erato* cuticular extract fraction containing compounds eluting from 0-23.55 min in GC-FID. In each trial a group of 4 males and 4 females was exposed to two decoys prewashed with methyl chloride. Decoys were attached to an artificial perch that the butterflies learned to use for roosting and were 20 cm from each other. One of the decoys was treated with 3 µL of the fraction. Number of fannings and contacts were counted in 10-min trials at sunset. Only one trial was done per day to avoid disrupting roost formation and the same group was used for all the trials. Bars represent standard error. * Significant at α=0.005

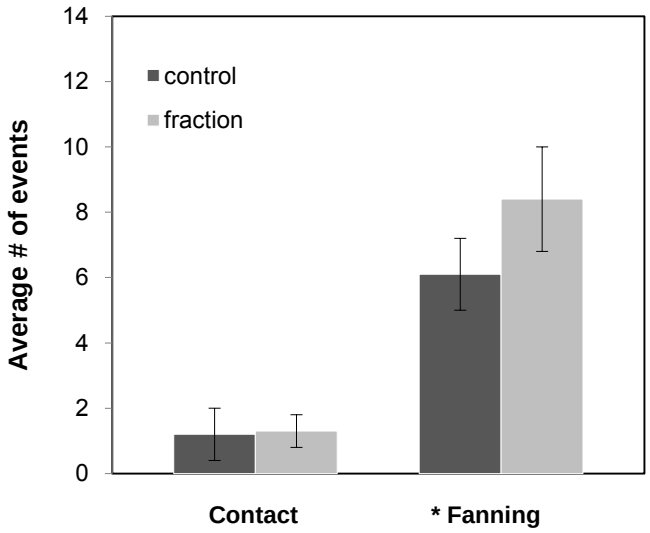

Figure 6-2. Average number of fanning and contact behavioral interactions in bioassays to test *Heliconius erato* cuticular extract fraction containing compounds eluting from 23.55-50 min in GC-FID. In each trial a group of 4 males and 4 females was exposed to two decoys prewashed with methyl chloride. Decoys were attached to an artificial perch that the butterflies learned to use for roosting and were 20 cm from each other. One of the decoys was treated with 3 µL of the fraction. Number of fannings and contacts were counted in 10-min trials at sunset. Only one trial was done per day to avoid disrupting roost formation and the same group was used for all the trials. Bars represent standard error. * Significant at α=0.05

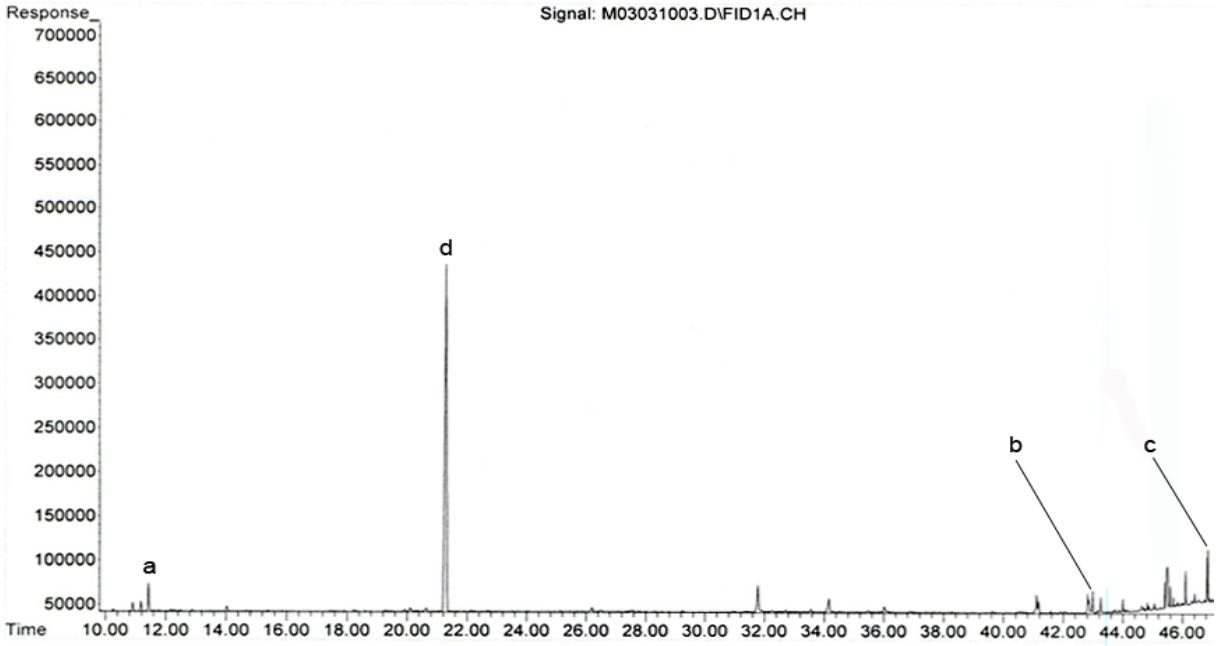

Figure 6-3. Gas chromatogram of a cuticular extract of wild *Heliconius erato* females from Gamboa, Panama. a: ocimene, b: oleamide, c: squalene, d: internal standard (nonyl acetate).

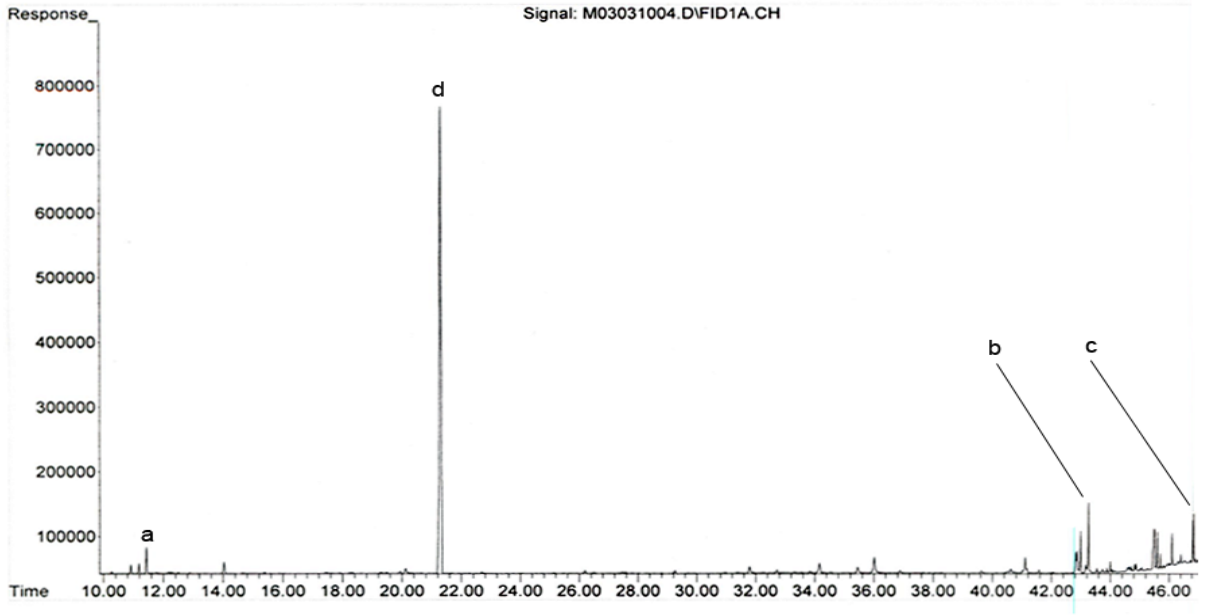

Figure 6-4. Gas chromatogram of a cuticular extract of wild *Heliconius erato* males from Gamboa, Panama. a: ocimene, b: oleamide, c: squalene, d: internal standard (nonyl acetate).

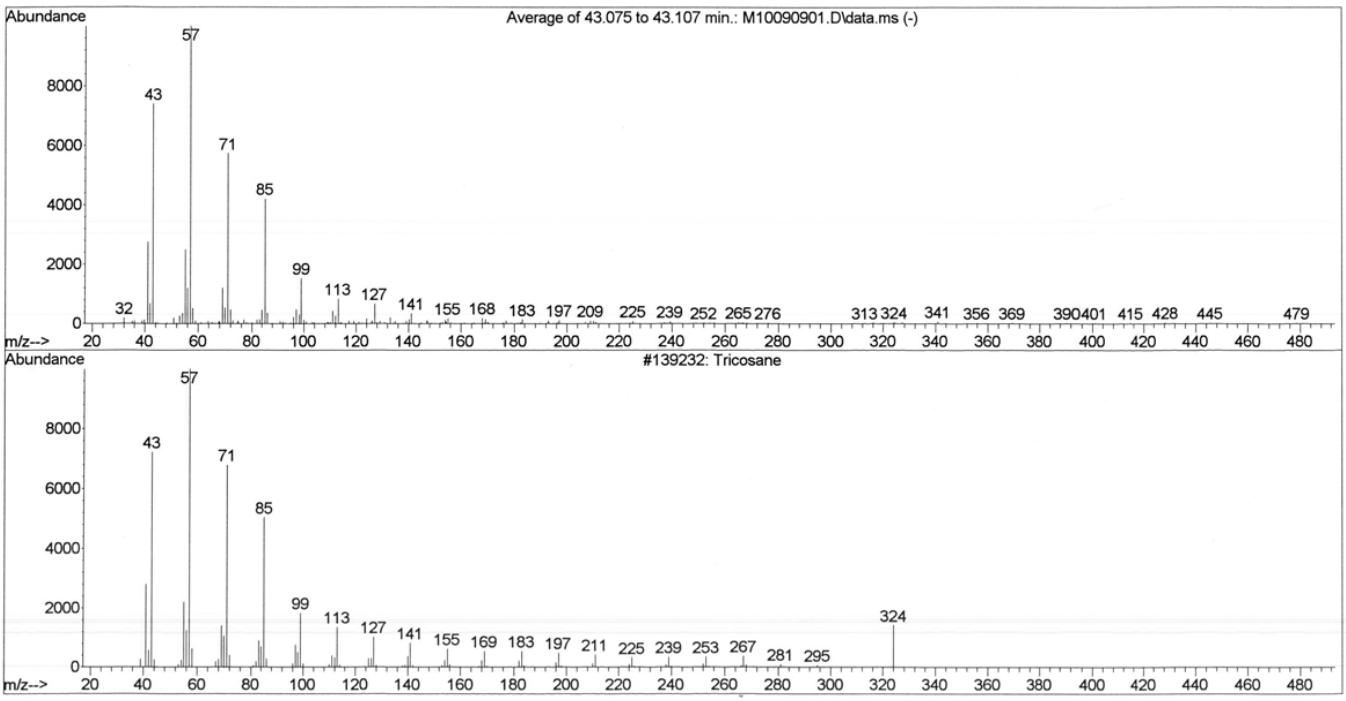

Figure 6-5. Mass spectra of branched Tricosane C23 identified from cuticular extracts of *Heliconius erato* males and females (above) and of a Tricosane standard (below).

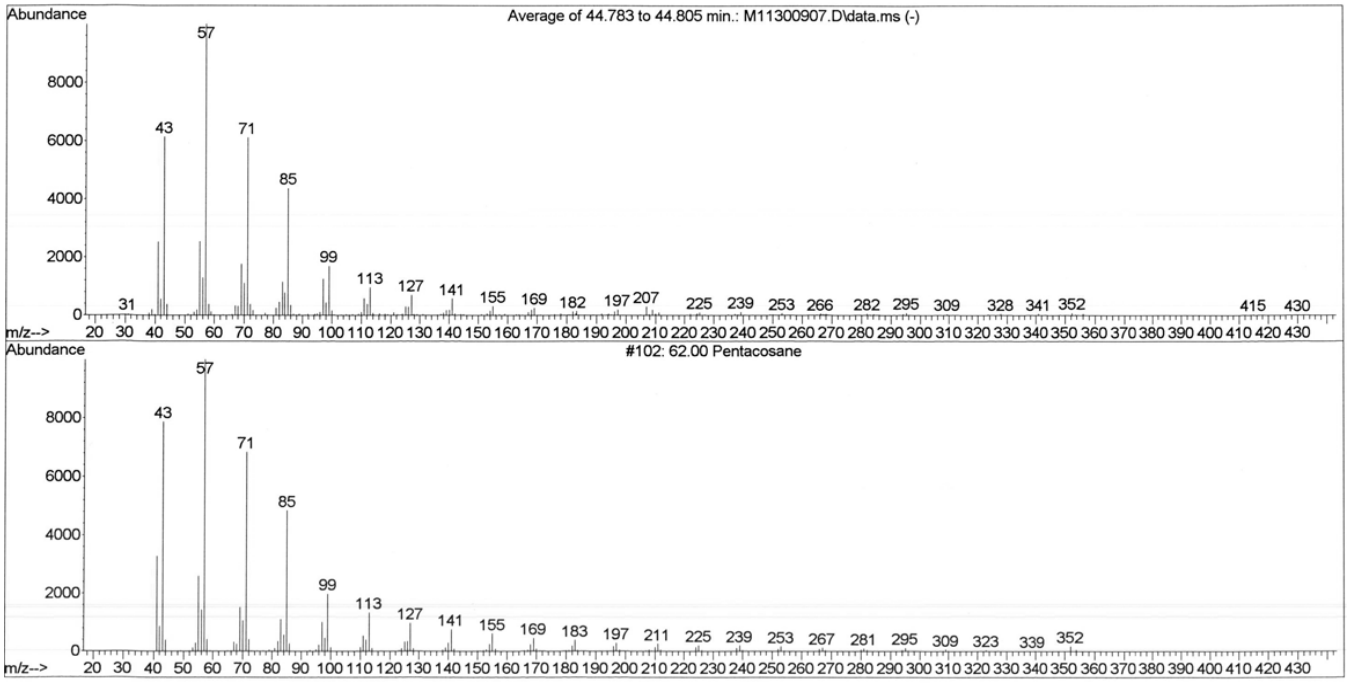

Figure 6-6. Mass spectra of branched Pentacosane C25 identified from cuticular extracts of *Heliconius erato* males and females (above) and of a Pentacosane standard (below).

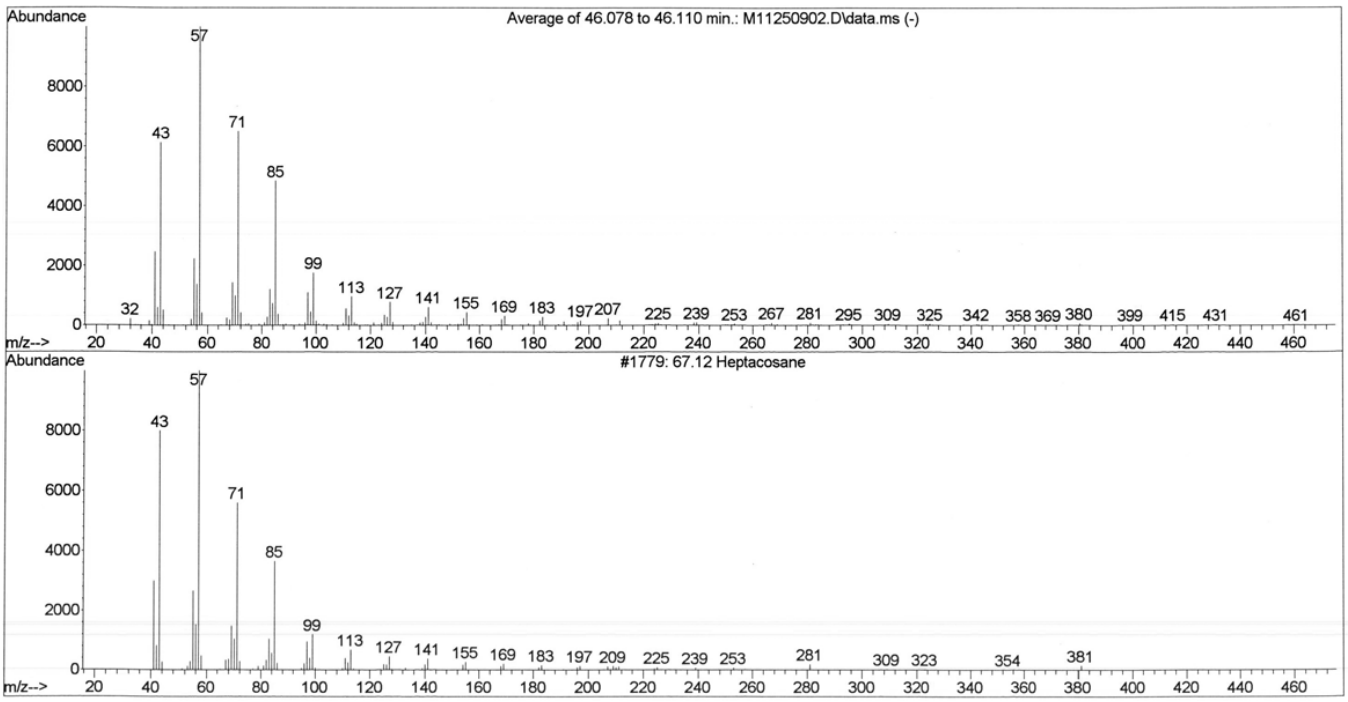

Figure 6-7. Mass spectra of branched Heptacosane C27 identified from cuticular extracts of *Heliconius erato* males and females (above) and of a Heptacosane standard (below).

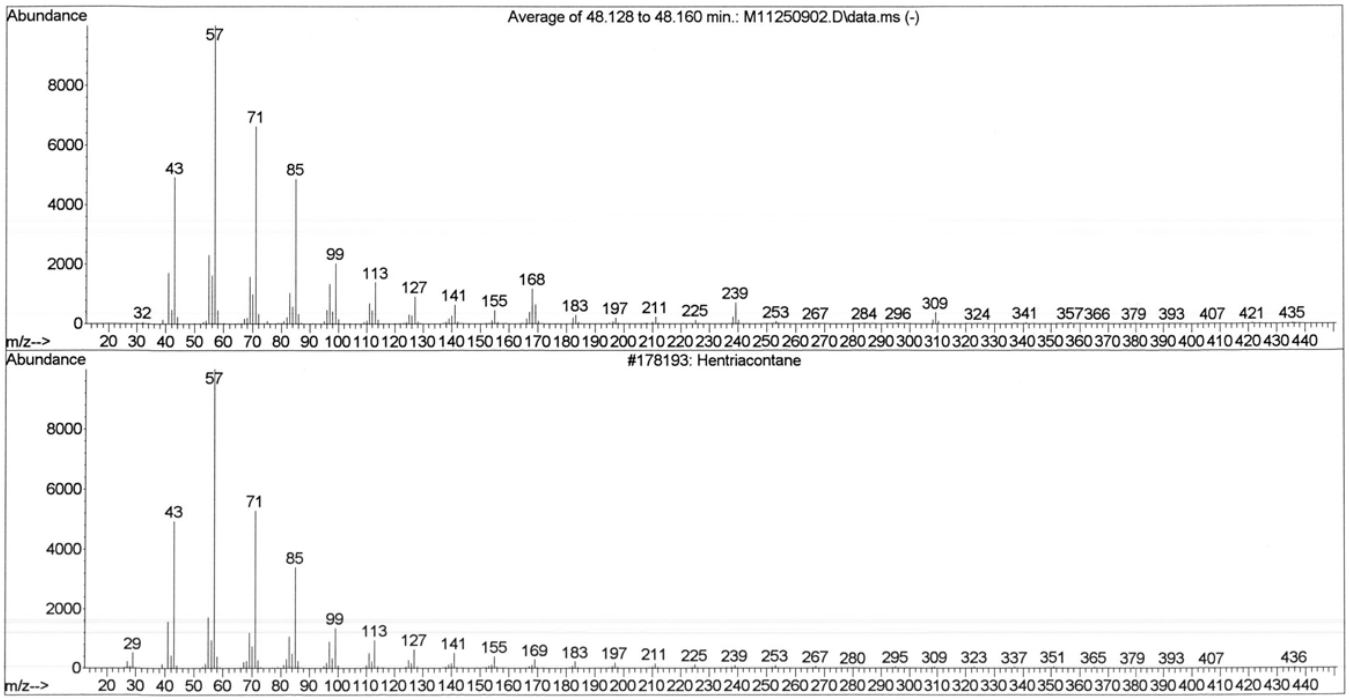

Figure 6-8. Mass spectra of a branched Hentriacontane C31 identified from cuticular extracts of *Heliconius erato* males and females (above) and of a Hentriacontane standard (below).

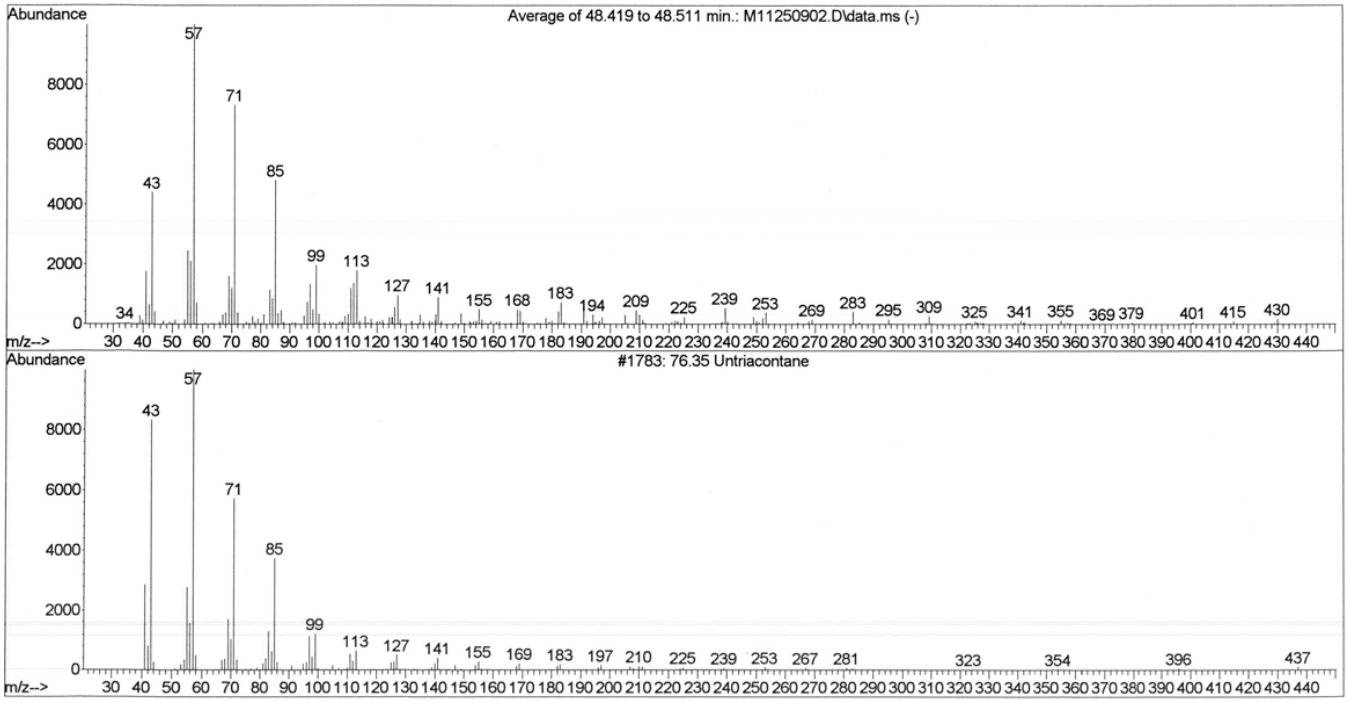

Figure 6-9. Mass spectra of a second branched Hentriacontane C31 identified from cuticular extracts of *Heliconius erato* males and females (above) and of a Hentriacontane standard (below).

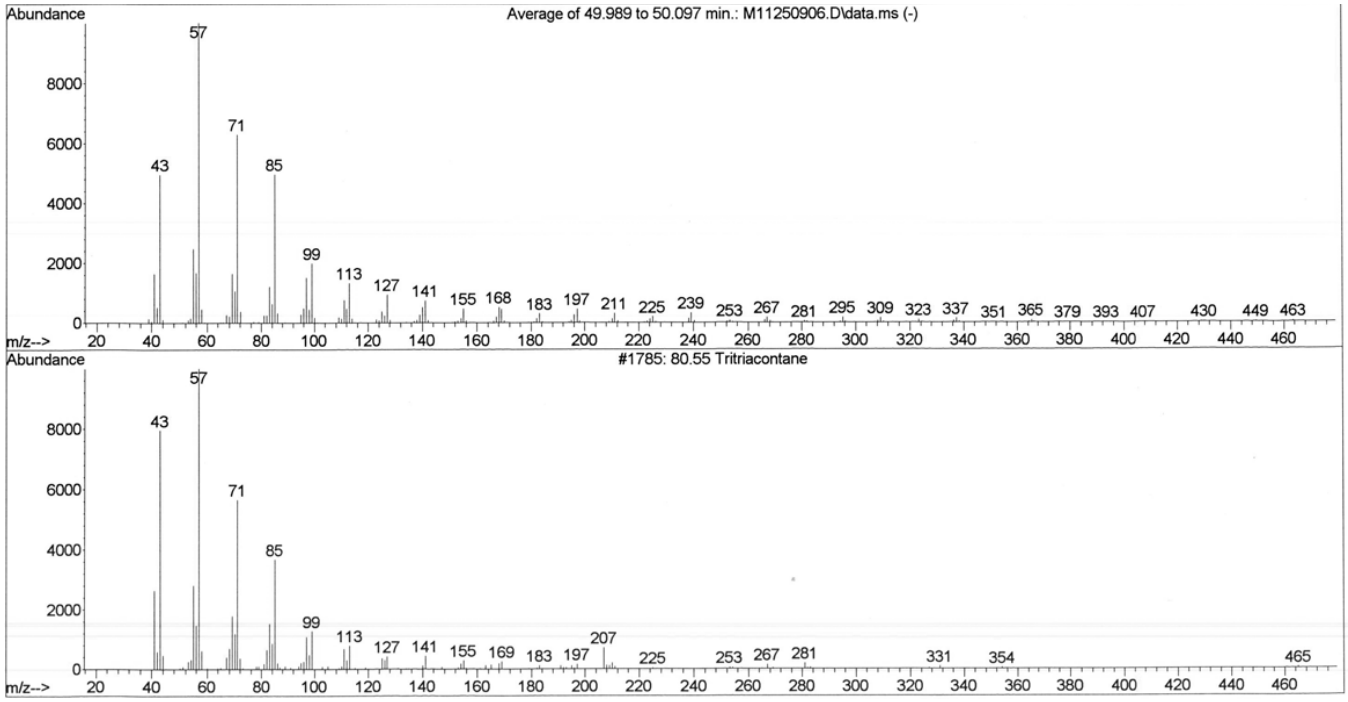

Figure 6-10. Mass spectra of a second branched Tritriacontane C33 identified from cuticular extracts of *Heliconius erato* males and females (above) and of a Tritriacontane standard (below).

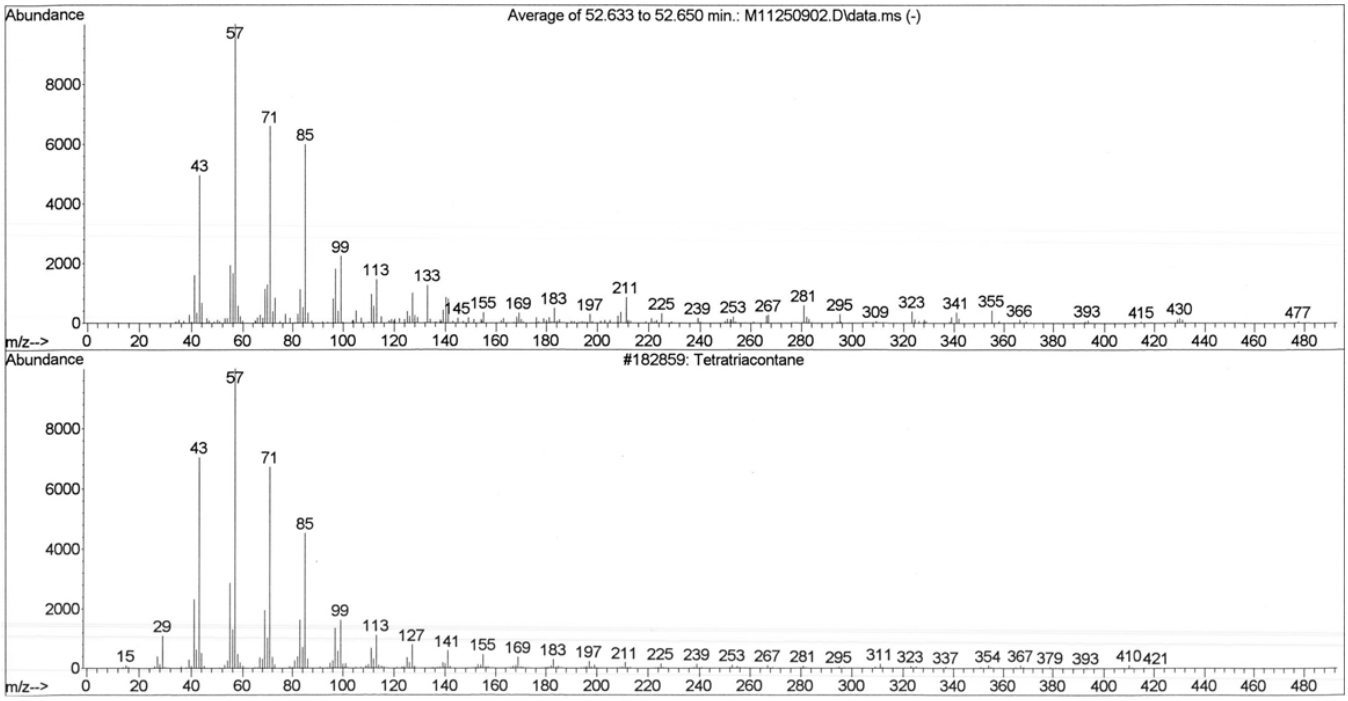

Figure 6-11. Mass spectra of a second branched Tetratriacontane C34 identified from cuticular extracts of *Heliconius erato* males and females (above) and of a Tetratriacontane standard (below).

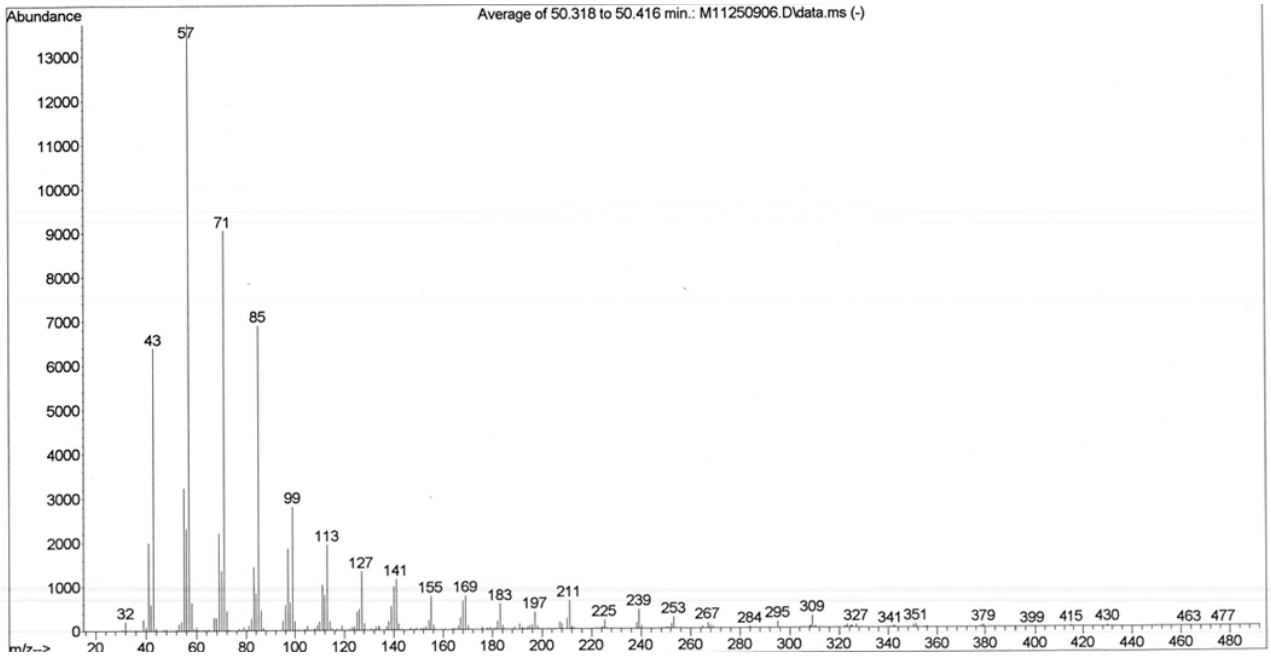

Figure 6-12. Mass spectra of a second branched Tetra or Tritriacontane C33-34 identified from cuticular extracts of *Heliconius erato* males and females. Compare to Figure 6-7 and Figure 6-8 standards.

CHAPTER 7
EVALUATION OF (E)-ß-OCIMENE AS AN ATTRACTRANT IN *HELICONIUS ERATO*

Introduction

The first studies that have accurately identified *Heliconius* semiochemicals and their role in behavior have only been recently published (Schulz et al., 2008, Estrada et al., 2009). Schulz et al. (2008) identified the monoterpene (E)-β-ocimene (EBO) as an antiaphrodisiac in *Heliconius melpomene* that males transfer to females during copulation to prevent further matings. EBO is a relatively widespread monoterpene in nature, being a component of many floral scents (Knudsen et al., 1993), and is one of the most common volatiles whose release is induced by herbivory (Paré & Tumlinson, 1997).

Preliminary evidence shows that *H. erato*, a co-mimic of *H. melpomene*, is attracted to EBO (Salcedo pers. obs.). In order to evaluate the relevance of EBO in the biology of *H. erato*, chemical analyses of *H. erato* abdominal glands were performed to determine if they produce EBO. In addition, behavioral bioassays were used to evaluate the role of EBO as an attractant in *H. erato*.

Methods

(*E*)-β-ocimene Preparation

(E)-β-ocimene (EBO) was supplied by Dr. Stefan Schulz (Institut für Organische Chemie, Technische Universität Braunschweig, Hagenring 30, 38106 Braunschweig, Germany) and was prepared according to the method of Matsushita and Negishi (Matsushita & Negishi, 1982), resulting in a 7:3 mixture of (*Z*)- and (*E*)-ocimene.

Analytical Procedures

Chemical analyses were carried out by Stefan Schulz on individual butterflies from a colony of *Heliconius erato* held in a greenhouse at Freiburg (Germany). The culture, originating from butterflies collected in Corcovado, Osa Peninsula, Costa Rica, was reared for about five generations with *Passiflora caerulea* as host plant and *Lantana camara* as source of pollen. The butterflies also had access to sucrose solution supplemented with pollen. Additional samples were obtained from a colony of *H. erato* held at the University of Texas at Austin, originating from individuals brought from Costa Rica. They were reared in greenhouses at about 32°C and high humidity (>80%). Butterflies had access to *Passiflora oerstedi* (larval host plant), sugar, and honey water, and sources of pollen from *Psiguria* sp., *Psychotria poeppigiana* and *L. camara* flowers. Butterflies were analyzed individually. The extracts were prepared either immediately after emergence from pupae or 5 d later. Claspers of males and the last abdominal segment of females were dissected from bodies of freshly killed butterflies and placed individually in vials with approximately 100 µL of pentane. The lower tips of the abdomens were also cut and stored in vials with pentane. Analyses of the latter served to identify compounds found in tissues surrounding scent glands. Samples were kept at −70°C until analyzed.

Gas chromatography and mass spectrometry (GC-MS) of pentane extracts were performed with a Hewlett-Packard model 5973 mass selective detector operated in the EI mode connected to a Hewlett-Packard model 6890 gas chromatograph with a BPX5-fused silica capillary column (SGE, 30 m°—0.25 mm, 0.25-µm film thickness). Injection was performed in splitless mode (250°C injector temperature) with helium as the carrier gas (constant flow of 1 ml/min, injector purge at 1 min). The temperature program

started at 50°C, was held for 1 min, and then rose to 320°C with a heating rate of 5°C/min. Ocimene was identified by comparison of the mass spectra and retention times with those of authentic reference samples in the different compound classes as well as by analysis of mass spectral fragmentation patterns.

Behavioral Bioassays

To evaluate the behavioral response of *Heliconius erato* butterflies to EBO, laboratory-reared individuals were used. Individuals were held in outdoor insectaries at Smithsonian Tropical Research Institute, Santa Cruz facility, Gamboa, Panama. Larvae were fed with *Passiflora biflora* and adults with artificial nectar (20% sugar water), and pollen host plants *Psiguria* sp. and *Psychotria* sp.

Individuals used in the experiments came from a stock cage of 40 butterflies, where individuals were no younger than 3 days old and females were non-virgin (mated). For each trial, individuals were taken randomly from the stock cage and let acclimate for 5-10 min in the experimental cage. All the trials were carried out in outdoor-screened cages of 2 x 2 x 1 m. The cages did not have any plants or feeders inside. Experiments were carried out from 1000 to 1200 h in mornings with no rain. Four trials were done per morning. In each trial, groups of three males and three females, five males only, or five females only were exposed simultaneously to two choices: filter paper only and filter paper treated with 2 µL (50 ng/µL) EBO. The filter papers were white, small pieces (1 x 2.5 cm) suspended from the roof of the cage by a 60 cm thread and 40 cm from each other. Counts of approaches (direct flight towards a paper filter) and fanning (sustained hovering over a paper filter) were registered in 10-min trials with 10-min rest periods. The same design was used with butterfly decoys (made from dead butterflies) replacing the filter paper. Decoys were washed with methylene chloride to

remove volatiles and let dry for at least one hour before experiments. For the experiments using filter papers 20 trials were done with female-only groups, 11 with male-only groups, and 20 with mixed sex groups. For the experiments using decoys, 20 trials were done with female-only groups, 20 with male-only groups, and 14 with mixed sex groups. D'Agostino Pearson omnibus test was used to determine normality in the data sets. When data had normal distribution, a student's paired t-test was used to compare data sets within the same experimental set-up, and a Wilcoxon test for non-normal datasets. For comparisons across experimental set-ups a Student's t-test was used with normal data and a Mann-Whitney test for non-normal data. All the data was analyzed using Graphpad Prism 4.0b for Mac, GraphPad Software, San Diego California USA, www.graphpad.com .

Results and Discussion

Results show that all *Heliconius erato* groups expressed a statistically higher number of "approach" interactions towards choices treated with EBO over the unscented controls (i.e. without the monoterpene). Mixed sex groups exposed to filter paper choices had an average of 6.35 approach events per trial vs. 3.45 for the control ($p=0.0015$); mixed sex groups exposed to decoys choices had an average of 7.42 approach events per trial vs. 3.14 for the control ($p=0.0171$); males exposed to filter paper choices had an average of 6 approach events per trial vs. 1.45 for the control ($p=0.001$); males exposed to decoy choices had an average of 5.95 approach events per trial vs. 2.35 for the control ($p=0.066$ not significant); females exposed to filter paper choices had an average of 7.25 approach events per trial vs. 2.15 for the control ($p<0001$); and females exposed to decoy choices had an average of 5.2 approach events per trial vs. 2.5 for the control ($p=0.0013$) (Tables 7-1, 7-2, 7-3, 7-4, 7-5, 7-6,

Figures 7-1, 7-2, 7-3, 7-4, 7-5, 7-6). For the fanning interactions, only one experimental set up, males exposed to scented vs. unscented filter papers, showed statistically higher number of events towards the EBO-treated filter paper (p=0.0078) (Table 7-3, Figure 7-3). The only experimental set-up where both number of approach and fanning interactions towards the EBO-treated choice were significantly higher, was males exposed to filter paper choices. When comparing decoy vs. filter paper "approach" data sets to evaluate if color pattern has an additive value in attraction the statistical analyses show no difference (mixed sex p=0.505, males p=0.981, females p=0.1103).

These results suggest that EBO alone is an attractant in the range were the approach behavior was registered (up to 1 m) but it does not discard the possibility of eliciting attraction at longer ranges. The presence of color pattern or wing shape does not seem to generate an additional stimulus. This does not discard a partial role of color pattern in attraction and recognition. Color pattern is necessary in roosting interactions (Chapter 2) and as an initial cue at longer ranges (pers. obs.). The results from the fanning behavior show that these interactions are not affected by EBO under the context set in these bioassays. Fanning is a behavior that is expressed in several contexts and usually is combined with repeated tarsal contacts towards the subject that is being fanned upon (pers. obs.). Fanning occurs when males court females, when recognizing individuals at a roost site, when females seek a host plant to lay eggs, when approaching a flower to feed, and when selecting a perch in a roost site (pers. obs.). This suggests that butterflies use other cues (contact, color, chemicals having lower volatility, etc.) to interact with subjects at very close distances. The fact that mixed sex groups had almost no fannings towards any choice (Table 7-1, 2), may indicate that

when both sexes are present they get distracted due to sexual interactions, which may include EBO detection, and hence disregard the experimental choices in the cage.

However, when comparing fanning data sets of males and females exposed to EBO-treated filter papers, males express a significantly higher number of fannings than females (2.54 vs. 0.25 average events per trial, p=0.001), which suggests that *H. erato* males detect more readily EBO. This concord with previous evidence that shows that females have almost double the levels of ocimene in the cuticle compared to males (Table 6-2) and may be an indication of a similar phenomenon to that occurring in *H. melpomene*, where males transfer EBO to females in copula (Schulz et. al, 2008). Behavioral bioassays and chemical analyses such as the ones performed by Schulz et. al to test the role of EBO as an antiaphoridisac in *H. melpomene* will test this hypothesis. Under a different context EBO could also be used as a recognition signal at *H. erato* roosting aggregations. Semiochemicals can often carry different messages by working together with other compounds and/or upon context (sexual selection, foraging, defense/alarm from predators, etc.). Roost assembly includes medium and short-range contact interactions and non-contact interactions (Chapter 2) so EBO may be used in short-range non-contact interactions together with visual cues to elicit contact interactions, which are probably the last step in intraspecific recognition when forming roosting aggregations at dusk (pers. obs). Cage experiments with roosting groups are needed to reveal the role of EBO in roosting behavior. In addition, EBO could also be used as an interspecific recognition signal. It is common to find one or two *H. melpomene* individuals perched just next to *H. erato* roosting aggregations. Roosting sites are virtually predator-free places and offer particular humidity conditions that may

be beneficial for the butterflies (Chapter 2, 4). It may not be surprising, then, if EBO has a second role in *H. melpomene*, where it may be used to recognize and follow *H. erato* to take advantage of co-mimic roosting sites and may be even used to follow other individuals towards foraging areas in the morning when leaving the roost site (Waller & Gilbert, 1982). Cage experiments, where *H. melpomene* butterflies are exposed to EBO-treated decoys at dusk, when roosting usually occurs, could reveal a very unique case where semiochemicals lead to roost site comensalism in Müllerian mimics. An additional possible role for EBO in *H. erato* and *H. melpomene* could be floral attraction. Color is an important cue for *Heliconius* to locate foraging patches in the forest, and in fact, their pollen host plants are bright red or orange (*Psychotria* sp.,*Psiguria* sp., and *Gurania* sp.). However, *Heliconius* also supplement their diet with nectar and pollen from flowers that have less attractive colors (white, purple, etc.) (pers. obs.). Chemical analysis of *Heliconius* host plants and bioassays using flower decoys of different colors treated with EBO could evaluate the role of EBO in flowers attractiveness to *Heliconius.*

Aside from EBO, there is a number of compounds found in the *Heliconius* cuticle (Salcedo unpub. data) and abdominal glands (Schulz et al., 2007, Estrada et al., 2009, Schulz unpub. data) whose biological importance is unknown. This, and other recent studies on butterfly chemical ecology (Estrada et al., 2007, Schulz et al., 2004, Schulz et al., 1993), show promising and interesting results that call for more research on this area to further understand and discover ecological mechanisms where semiochemicals are key to generate and sustain diversity in this and other tropical groups.

Table 7-1. Approaches (flight towards experimental choice) and fanning (sustained hovering over experimental choice) behavior counts by a group of *Heliconius erato* males (3) and females (3) exposed simultaneously to (1) Filter paper treated with 2 µL (50 ng/µL) (*E*)-β-Ocimene (EBO) and (2) clean filter paper. Each trial lasts 10 min.

	Filter paper + EBO		Clean filter paper	
Trial	Approach	Fanning	Approach	Fanning
1	3	0	5	0
2	2	0	0	0
3	3	0	1	0
4	9	1	2	0
5	3	0	2	0
6	1	0	3	0
7	5	0	2	0
8	10	0	6	0
9	13	0	6	0
10	18	0	9	0
11	12	0	9	1
12	3	1	7	0
13	3	0	0	0
14	3	0	1	0
15	5	0	1	0
16	10	0	2	1
17	5	0	6	0
18	7	0	2	0
19	10	0	4	0
20	2	0	1	0

Table 7-2. Approaches (flight towards experimental choice) and fanning (sustained hovering over experimental choice) behavior counts by a group of *Heliconius erato* males (3) and females (3) exposed simultaneously to (1) Decoy treated with 2 µL (50 ng/µL) (*E*)-β-Ocimene (EBO) and (2) clean decoy. Each trial lasts 10 min.

	Decoy + EBO		Clean decoy	
Trial	Approach	Fanning	Approach	Fanning
1	20	0	2	3
2	17	0	0	0
3	6	0	3	0
4	5	0	1	0
5	7	0	3	0
6	3	0	0	0
7	6	0	2	3
8	7	0	1	0
9	7	0	0	2
10	3	0	12	0
11	1	1	2	1
12	7	0	6	0
13	3	0	3	0
14	12	0	9	1

Table 7-3. Approaches (flight towards experimental choice) and fanning (sustained hovering over experimental choice) behavior counts by a group of *5 Heliconius erato* males exposed simultaneously to (1) Filter paper treated with 2 µL (50 ng/µL) (*E*)-β-Ocimene (EBO) and (2) clean filter paper. Each trial lasts 10 min.

	Filter paper + EBO		Clean filter paper	
Trial	Approach	Fanning	Approach	Fanning
1	6	5	0	0
2	4	1	0	0
3	1	0	0	0
4	7	2	3	0
5	12	2	7	0
6	8	5	1	0
7	11	0	0	0
8	6	4	2	0
9	3	6	0	0
10	5	1	2	1
11	3	2	1	0

Table 7-4. Approaches (flight towards experimental choice) and fanning (sustained hovering over experimental choice) behavior counts by a group of 5 *Heliconius erato* males exposed simultaneously to (1) Decoy treated with 2 µL (50 ng/µL) (*E*)-β-Ocimene (EBO) and (2) clean decoy. Each trial lasts 10 min.

	Decoy + EBO		Clean decoy	
Trial	Approach	Fanning	Approach	Fanning
1	3	0	0	0
2	7	0	0	0
3	4	0	0	0
4	6	5	0	0
5	16	3	1	0
6	13	1	5	0
7	17	1	2	0
8	21	0	2	0
9	13	3	0	0
10	11	0	2	0
11	0	0	6	0
12	0	0	4	1
13	0	0	2	0
14	0	0	3	0
15	2	0	10	2
16	3	0	4	0
17	3	4	3	0
18	0	0	0	0
19	0	0	3	0
20	0	0	0	0

Table 7-5. Approaches (flight towards experimental choice) and fanning (sustained hovering over experimental choice) behavior counts by a group of five *Heliconius erato* females exposed simultaneously to (1) Filter paper treated with 2 µL (50 ng/µL) (*E*)-β-Ocimene (EBO) and (2) clean filter paper. Each trial lasts 10 min.

	Filter paper + EBO		Clean filter paper	
Trial	Approach	Fanning	Approach	Fanning
1	15	1	6	0
2	4	0	2	0
3	7	0	1	0
4	6	1	1	0
5	8	0	0	0
6	9	0	0	0
7	4	0	1	0
8	9	0	0	0
9	4	0	1	0
10	5	0	1	0
11	4	0	2	0
12	3	0	0	0
13	7	0	4	0
14	8	0	6	0
15	10	2	3	0
16	10	0	6	0
17	7	1	2	0
18	10	0	4	0
19	13	0	2	0
20	2	0	1	0

Table 7-6. Approaches (flight towards experimental choice) and fanning (sustained hovering over experimental choice) behavior counts by a group of 5 *Heliconius erato* females exposed simultaneously to (1) Decoy treated with 2 µL (50 ng/µL) (*E*)-β-Ocimene (EBO) and (2) clean decoy. Each trial lasts 10 min.

	Decoy + EBO		Clean decoy	
Trial	Approach	Fanning	Approach	Fanning
1	6	1	0	0
2	2	0	0	0
3	0	0	0	0
4	3	0	0	0
5	10	0	1	0
6	2	0	1	0
7	1	0	2	0
8	2	1	0	0
9	0	0	0	0
10	1	0	0	0
11	10	0	11	0
12	6	0	3	0
13	10	1	7	0
14	1	0	3	0
15	8	0	5	0
16	12	0	3	0
17	11	0	8	0
18	7	0	5	0
19	9	0	2	0
20	3	0	0	0

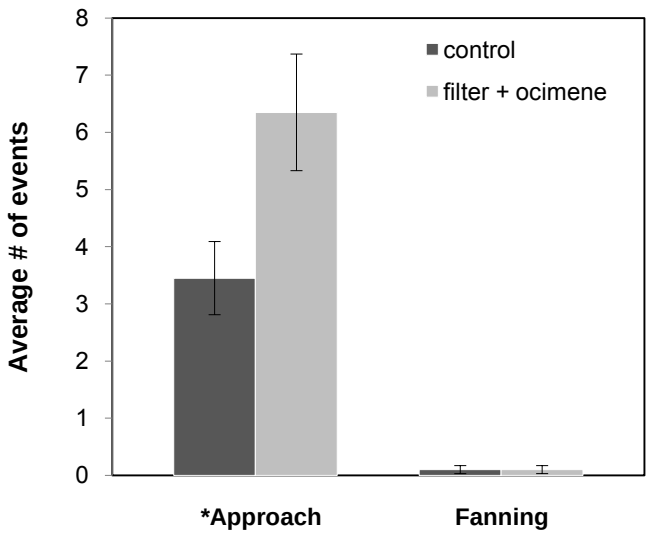

Figure 7-1. Average number of “approach” and “fanning” behavioral interactions in bioassays. A group of 3 males and 3 females of *Heliconius erato* exposed simultaneously to (1) Filter paper treated with 2 µL (50 ng/µL) (*E*)-β-Ocimene and (2) clean filter paper. Bioassays were done during the morning. Individuals were different in each trial and selected randomly from a stock colony of 40 individuals. Each trial lasted 10 min. * Significant differences p=0.002

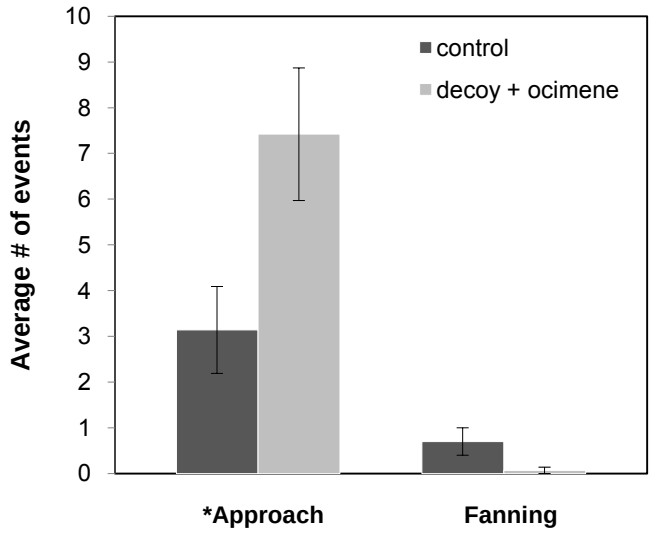

Figure 7-2. Average number of “approach” and “fanning” behavioral interactions in bioassays. A group of 3 males and 3 females of *Heliconius erato* exposed simultaneously to (1) Decoy treated with 2 µL (50 ng/µL) (*E*)-β-Ocimene and (2) clean decoy. Bioassays were done during the morning. Individuals were different in each trial and selected randomly from a stock colony of 40 individuals. Each trial lasted 10 min. * Significant differences p=0.034

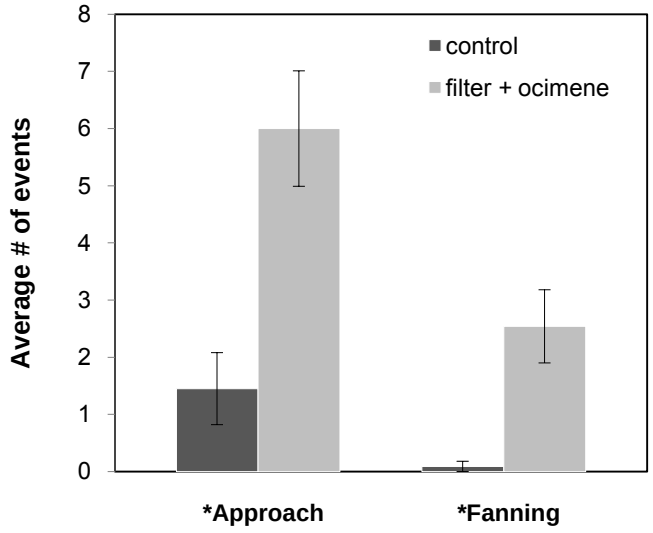

Figure 7-3. Average number of “approach” and “fanning” behavioral interactions in bioassays. A group of 5 males of *Heliconius erato* exposed simultaneously to (1) Filter paper treated with 2 µL (50 ng/µL) (*E*)-β-Ocimene and (2) clean filter paper. Bioassays were done during the morning. Individuals were different in each trial and selected randomly from a stock colony of 40 individuals. Each trial lasted 10 min. * fanning p = 0.004; approach p=0.0001

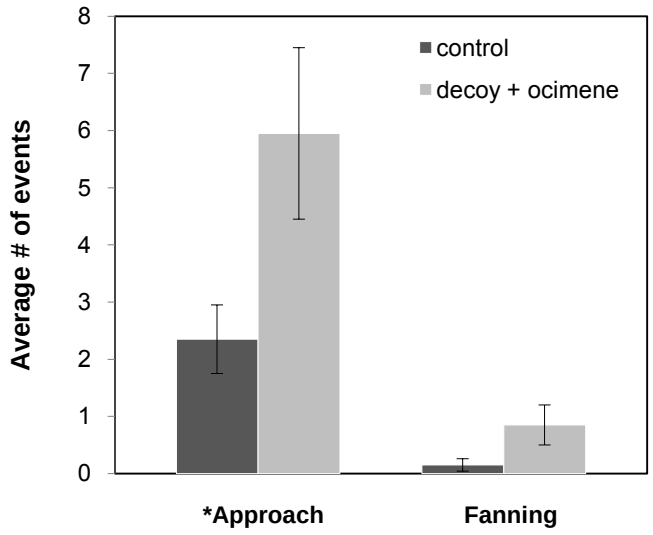

Figure 7-4. Average number of “approach” and “fanning” behavioral interactions in bioassays. A group of 5 males of *Heliconius erato* exposed simultaneously to (1) Decoy treated with 2 µL (50 ng/µL) (*E*)-β-Ocimene and (2) clean decoy. Bioassays were done during the morning. Individuals were different in each trial and selected randomly from a stock colony of 40 individuals. Each trial lasted 10 min. * Significant differences p=0.049

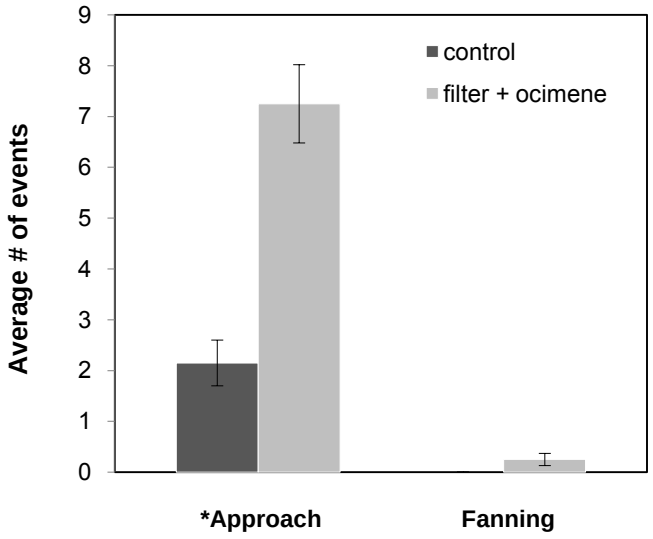

Figure 7-5. Average number of “approach” and “fanning” behavioral interactions in bioassays. A group of 5 females of *Heliconius erato* exposed simultaneously to (1) Filter paper treated with 2 µL (50 ng/µL) (*E*)-β-Ocimene and (2) clean filter paper. Bioassays were done during the morning. Individuals were different in each trial and selected randomly from a stock colony of 40 individuals. Each trial lasted 10 min. * Significant differences p=0.0001

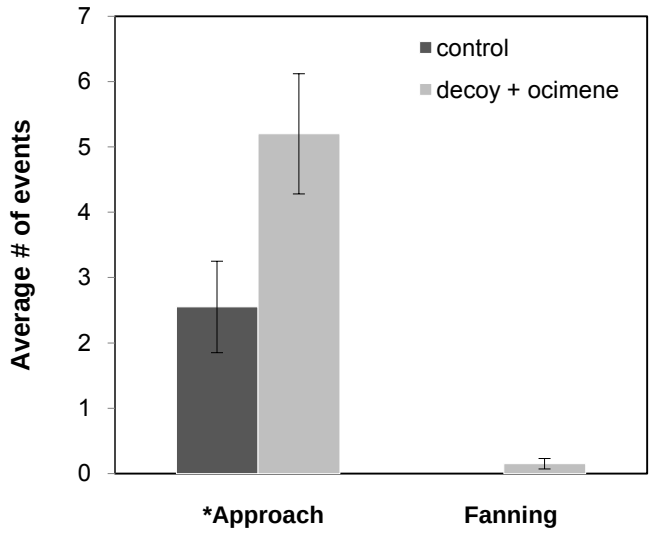

Figure 7-6. Average number of “approach” and “fanning” behavioral interactions in bioassays. A group of 5 females of *Heliconius erato* exposed simultaneously to (1) Decoy treated with 2 µL (50 ng/µL) (*E*)-β-Ocimene and (2) clean decoy. Bioassays were done during the morning. Individuals were different in each trial and selected randomly from a stock colony of 40 individuals. Each trial lasted 10 min. * Significant differences p=0.001

CHAPTER 8
CONCLUSION

More than one century after the first report of *Heliconius* roosting behavior (Wallace, 1870), the first in-depth attempt to reveal some of the mechanisms involved in the expression of the behavior and its ecological advantages has been made.

In order to successfully form roosting aggregations, *Heliconius* butterflies have a set of defined behavioral traits (antenna-wing, clutching, fanning, and fending-off) that involve long, medium, and short-range contact and non-contact interactions that ensure intraspecific recognition. These interactions are mediated by visual and chemical cues. *Heliconius* butterflies are able to recognize conspecific wing patterns as a key step in the aggregation process. A variety of chemical components were identified from the cuticle of *Heliconius erato* males and females. These included several volatile (ocimene, nonanoic acid, tetradecanoic acid, hexadecanoic acid, squalene) and reduced volatility compounds (unsaturated C20-C34 hydrocarbons), including compounds already reported for other *Heliconius* species as well as other lepidopteran and insect groups (Danainae, Tortricidae, Noctuidae, Geometridae, Arctiidae, Lymantridae, Diptera, and Hymenoptera). These components were tested in roosting bioassays, using low and high molecular weight fractions of the cuticular extracts. Bioassays showed that the fraction containing (*E*)-β-ocimene (a common insect attractant) elicited a higher number of fanning interactions vs. unscented controls. The second fraction elicited attraction but at a lower level, which implies the presence of other components that may be part of an attractive chemical blend used during roosting interactions.

(*E*)-β-ocimene, previously identified for *H. melpomene* (Schulz et al., 2008), was found to generate attraction during bioassays. Ocimene elicited higher number of approach interactions in groups of males, females, and mixed sex. And when comparing males and females ocimene elicited a higher number of fanning interactions in males.

Heliconius roost sites present a variety of characteristics that make them environmentally disctintive. They offer buffered lower relative humidity, protection from rain and wind, and reduced light conditions at dusk. Protection from wind and rain are evidently beneficial for a place where the butterflies are vulnerable and motionless during approximately 10 h every night. The advantages of lower relative humidity overnight are harder to identify but lower humidity may be related to the physical components of the perch, which usually is made of dry twigs and vines. Reduced light levels at dusk and dawn may reduce visibility to predators. Disturbance events at roost sites overnight are relatively frequent, but these do not deter the butterflies from coming back to the same sites everyday. Predation events are very infrequent and the evidence strongly suggests that there are no nocturnal predators specialized on *Heliconius* butterflies.

Females of *H. sara* arrive at roost sites with pollen loads and digest them overnight. This suggests that females from other *Heliconius* species may also maximize pollen feeding with nocturnal digestion. A link between female nocturnal digestion efficiency and roost site environmental conditions is can not be discarded. *Heliconius* butterflies naturally prefer pollen from *Psychotria* sp. over commercial bee pollen in choice tests. This suggests that the preference for *Psychotria* sp. and other *Heliconius*

pollen host plants, such as *Psiguria* sp. and *Gurania* sp., is innate and is not likely to be learned at roosting sites.

Previous evidence of frequent movements of butterflies among roost sites within a specific home range and the ability of butterflies from different populations to join roosts out their home range suggested that kin selection is an unlikely reason for the evolution of roosting aggregations in *Heliconius* (Mallet, 1986b). The results here complement this previous evidence by showing that butterflies from different broods cannot roost together.

This work supplements previous anecdotal observations and the scarce record of publications on *Heliconius* roosting aggregations. Data on the environmental conditions of roost sites, behavior, ecological interactions, and on potential and actual semiochemicals involved in the expression of roosting behavior, show that far from being apparently simple, *Heliconius* roosting is a complex behavior that little by little is opening more windows to a better understanding of the success of this and other tropical groups.

APPENDIX A
SPATIAL DYNAMICS OF NIGHT ROOSTING IN *HELICONIUS*

Introduction

The following appendix was the result of new analyses made with the data reported by Salcedo (2006). The new analyses revealed new data that is relevant to this work.

Heliconius butterflies are well known their brightly colored wings and their ability to sequester and synthesize toxic compounds from their host plants (Engler-Chaouat & Gilbert, 2007). These features are used in Müllerian mimicry rings to spread the burden of educating predators (Mallet & Gilbert, 1995a, Chai, 1986, Chai, 1990). *Heliconius* also use color for sexual selection, and are able to locate their pollen and nectar host-plants using color cues (Jiggins et al., 2001, Brown Jr., 1981). But when night falls, all the visual cues used during diurnal behaviors are probably inefficient, and so *Heliconius* butterflies must rely on different strategies to minimize potential nocturnal predation. During the night, *Heliconius* butterflies roost gregariously, perched from dead, hanging vines, leaves or twigs. Roosting sites are usually under vegetation mats, sometimes near streams, and in areas with almost no wind. Most of the work on *Heliconius* roosting behavior has been based on anecdotal observations of the behavior without statistical support (Young, 1978, Young & Thomason, 1975, Jones, 1930, Crane, 1955, Crane, 1957a, Young & Carolan, 1976, Gilbert, 1972, Turner, 1975), although more recent studies have provided key information on the ecology of night roosting in *Heliconius* (Mallet, 1986b, Mallet, 1986a, Mallet & Gilbert, 1995a, Waller & Gilbert, 1982). The adaptive significance of this distinctive behavior is still unknown.

Heliconius has been shown to demonstrate night roosting behavior when in captivity (Crane, 1957b), and laboratory experiments provide an excellent arena to further understand the development of this behavior. Using spatial statistics for point processes, a captive colony of *Heliconius erato* was studied to test whether roosting behavior is consistently expressed in captivity, and most importantly to evaluate how sex, age, and size (wingspan) can affect roosting patterns in captive colonies of *H. erato*.

Methods

Butterfly Rearing

Heliconius erato pupae were donated by the El Bosque Nuevo butterfly farm, Costa Rica. The experimental colonies were started with 10-15 individuals. Adults were kept in 2 x 2 x 2 m indoor insectaries at 27ºC, 80% ºH inside a temperature-controlled Lord & Burnham glass house (5 x 8 m) at the University of Florida, Department of Entomology and Nematology. Appropriate larval host plants (*Passiflora biflora, P. punctata,* and *P. auriculata*) were provided and mating and oviposition were observed. Once eggs were laid, they were collected and transferred to environmental chambers for hatching and larval growth until pupation and eclosion; chambers were maintained under constant laboratory conditions (27ºC, 75% ºH and a 14L: 10D photoperiod).

Butterfly Marking and Wing Measurement

After eclosion, each individual was sexed and marked on the underside of both forewings using a black permanent ink Sharpie® marker. Forewing length was measured using a dial caliper. The wing was measured from the wing base to the apex.

Roost Structure and Roosting Patterns

Our experiments were carried out in indoor insectaries. Observations were made after sunset during the months of July-November 2005. White light was found to awaken individuals after dawn; therefore, a low-intensity red-beam flashlight, which apparently did not disturb the butterflies, was used to identify each of them at night.

In order to study roosting patterns, the spatial patterns of the perched butterflies under two scenarios were studied: (1) with the insectary walls as the only perching surface, and (2), with a dry vine suspended from the insectary roof. We decided to use only one vine because after testing with several vines the butterflies only used one vine to roost gregariously.

To accurately track the position of every perched individual when walls were used as a perch, we used a grid system. The grid was created using natural fiber threads attached to the outside of the insectary screen. Each cell of the grid was 5 x 5 cm and was identified by a letter-number code. For the second experiment, the vine was marked, creating sections that enabled us to track the positions of the butterflies.

Statistical Analyses

Two different statistical analyses were performed, one prior to introduction of the vine to the insectary and another once the vine was introduced. For the first analysis, the hypothesis was that the butterflies demonstrated clustering as they settled on a surface at the end of the day. Clustering, of course, is dependent upon scale. The original data were the locations of the individual butterflies recorded to the level of the grid cell on the wall where they settled. This spatial scale was too fine for our analyses since only one butterfly could settle in an occupied grid cell. In addition, the total number of butterflies within the insectary ranged from nine to 24, whereas the total area in the

insectary on which they could have settled was 24 m^2 (4 walls, roof, and ground, each with an area of 4 m^2). Hence, we chose to test for clustering at the scale of the sides of the insectary, of which there are 6 (4 walls, a floor and a ceiling). We used a Monte Carlo Test procedure with the Index of Cluster Size (ICS) (David & Moore, 1954) as the statistic. Under the null hypothesis that the butterflies are independently selecting one of the walls, floor or ceiling for settling at the end of each day, the number of butterflies per side of the insectary is distributed as a Poisson random variable where the mean number of butterflies/side should equal the variance. Hence ICS = $(S^2/m) - 1$, where S^2 is the sample variance and m is the sample mean number of butterflies per side of the insectary, should equal zero under the null hypothesis. ICS is greater than zero when clustering of animals on one or a few sides of the insectary is observed and is negative when animals tend to be distributed very evenly, i.e., about equal numbers of butterflies on each side of the insectary.

In this experiment, the number of butterflies settling on each wall, floor, and ceiling of the insectary were observed for 35 days. In addition, butterflies were added at different times and survived for varying amounts of time ranging from a single day to up to 32 days. Testing the hypothesis of clustering cannot be done with parametric tests under this scenario (differing numbers of animals per day, and differing numbers of days that each animal was in the insectary), so we chose to do a non-parametric Monte Carlo test (Manly, 1997). The testing procedure followed the experimental design in that varying numbers of butterflies were used on each day and the number of repeated observations was fixed at 35 days. The Monte Carlo test was performed by running 1000 simulations of the entire experiment, with the number of animals selected for that

day within that simulation, with animals randomly selecting a wall, ceiling or floor for settling at the end of each day. These choices generate 6 counts (the number of animals /side of the insectary) and these counts are Poisson-distributed under a scenario of random selections. For a single simulation, we randomly selected the number of individuals to be used from the list of values used in the experiment for each day of 35 days. For each day, these individuals were randomly assigned to one of the six sides and the ICS was calculated. At the end of each simulated experiment, the mean ICS was calculated and stored. In addition to the simulated mean values from the Monte Carlo testing, we calculated the observed mean ICS for the actual experiment. The p-value of the test of the hypothesis that the butterflies do cluster (on a wall, roof or floor) is the proportion of Monte Carlo values exceeding the observed test statistic value. These tests were performed using the statistical software R (freeware available from the R Project for Statistical Computing at http://www.r-project.org/).

Once the vine was placed within the insectary, a different type of experiment was run. We were interested in testing (1) whether there was a preference for the vine rather than the walls of the insectary and (2) whether the distribution of individuals along the vine was dependent on gender, age, time since entering the insectary, or wingspan. In this experiment, the number of butterflies in the insectary on any given day ranged from two to 37 and the choices of the butterflies at the end of each day were recorded for 93 days. To test preference for the vine (hypothesis 1), we classified the location of a butterfly each day as either on or off the vine. Two analyses were performed. First, we calculated the proportion of days in the experiment that the number of butterflies on the vine exceeded the number of butterflies not on the vine. We tested the hypothesis that

butterflies preferred the vine to the walls using a large sample z-test of a binomial proportion. We then tested whether the choice of vine or sides of the insectary was related to the butterfly's age, gender or wingspan. For this we ran a logistic regression with on or off the vine as the response and with gender, age, wingspan, and all interactions of these as the explanatory variables. Since the data were repeated measures on the same individual for varying lengths of time, we added a random block effect for individual.

We did test whether the butterflies clustered on sub-regions of the vine. For this test, similar to the one performed for testing clustering on the walls, we divided the vine into 6 areas and tested whether there was differential numbers of butterflies in the areas relative to that expected under the null hypothesis of random selection of locations on the vine.

Finally, we were interested in whether the butterflies that did show a preference for the vine showed preferences for areas along the vine based on several different factors. We divided the vine into three equal-length sections (lower, middle, and upper, where upper is near the point of attachment of the vine to the insectary) and tested the hypothesis that the location along the vine depended on gender, age or wingspan. We used a generalized logistic model with section as the response and gender, age, wingspan, and all interactions as the explanatory variables. We included individual as a random block effect to account for repeated observations on the same individual.

We also tested whether the choices made by individuals that settled on the vine changed with time, that is, the longer they were in the insectary. This is distinct from age in the sense that we were interested in testing whether the daily choices evolved with

experience. A generalized logistic model with section as the response and day since entering and wingspan as explanatory variables was run. We included individual as a random block effect to account for repeated observations on the same individual. These analyses were performed using SAS v 9.1 (SAS Institute, Cary NC).

Results and Discussion

Clustering Patterns and Perch Choice

Our results show that *Heliconius erato* do cluster consistently in captivity. The butterflies exhibited clustering when the cage walls were the only surface to perch (ICS = 6.433, $p < 0.001$).

The butterflies showed a slight preference for the vine (55%, $Z = 1.1425$, $p = 0.1271$). However, this was not statistically significant so we cannot conclude that there was a preference for the vine over the walls. In addition, there was no change in the choice of wall/vine as a function of time in the cage ($p > 0.05$); hence once the individuals selected a perch (wall or vine), they continued to use the same perch consistently. We also did not find any statistically significant effects of sex, age or wingspan on the choice of wall or vine by the butterflies ($p > 0.05$ for all main effects and interactions).

When a vine was placed in the cage, the butterflies that perched on it showed clustering along the vine (ICS = 1.524, $p < 0.001$); i.e., they did not randomly settle on the vine. This pattern of clustering at smaller scales has been observed in the field in *H. erato* races from Colombia, Panama, and Peru (pers. obs. and Mallet, 1986b). The relevance of small-group clustering in *H. erato* is unknown; however, a subgroup

formation may be a survival strategy by exposing only a fraction of the roost (i.e., 3-4 individuals) to potential predation or disturbance events.

Roost Structure

The results of the generalized logistic regression of choice of vine location as a function of wingspan, age and gender indicates a complex response to choice along the vine (Table I). The interaction term age*wingspan is statistically significant at $\alpha = 0.05$ which implies that the choice of location on the vine depends on a combination of age of the butterfly and its size as measured in wingspan. (Recall that age is the actual age of the butterfly and not the time spent in the cage). The sex*wingspan interaction is not statistically significant at 0.05, but the relatively small p-value (0.058) indicates that there is some evidence of an effect of the combination of sex and wingspan on the choice of vine location.

There is a higher predicted probability of larger and older butterflies to be on the lower and middle sections of the vine than on the upper section. Concomitantly, the probability of being on the upper section decreases with age and wingspan. This implies that larger butterflies are found on the middle and lower sections of the vine while smaller and/or younger butterflies are found in the upper section of the vine. Different arthropods, including spiders, walking sticks, and scorpions, have been reported to crawl in the twigs or vines at roosting aggregations (pers. obs, Janzen pers. comm.). Hence, larger and more experienced individuals would benefit from perching in the mid and lower sections of a natural vine or twig since they would more efficiently avoid potential predators or disturbances. No other interaction was found to be significant regarding individual distribution within the roost.

The final analysis to determine if there was any alteration to behavior with increasing time that the butterfly was in the insectary did not show any statistical significance ($p = 0.3915$).

Conclusion

The results of this work quantitatively evaluated for the first time *Heliconius* spatial roosting patterns and confirmed that captive colonies can be useful to study this behavior. Although the analysis revealed trends that may be used as survival strategies, (sub-groups and preference of larger and older individuals to perch on mid and lower sections of the roosting vine), further experiments, including wild aggregations, need to be done to confirm the adaptive importance of such patterns. Additional experiments should gather evidence regarding the possible role of predation at natural roost sites and evaluate if the larger and older individuals located in the mid-low heights of vines within the roost have better fitness over the younger and smaller individuals located in the upper section of the roost.

APPENDIX B
A STANDALONE WEATHERPROOF LOW COST CAMERA SYSTEM TO RECORD DIURNAL OR NOCTURNAL ANIMAL BEHAVIOR CONTINUOUSLY FOR EXTENDED PERIODS

Introduction

Field research on nocturnal behavior can be a difficult task due to the inherent conditions of the night. This has hindered and biased research towards the study of diurnal behaviors. In many cases, continuous field recordings are necessary to evaluate behavioral traits, predation events, or physical changes of the subject or the environment, etc., and hence stand-alone camera systems are needed. There are video systems that meet these needs on the market, but they are often out of the reach of researchers due to high costs. Alternatively, low-cost systems can be developed, but challenges met in the process include weatherproofing and development of systems, long time-period recording capabilities, long-lasting battery systems, video quality, and ease of installation.

The camera system presented here, although developed for videotaping of nocturnal aggregations of *Heliconius* butterflies in tropical rainforests, can be used to study many other subjects, even in daylight conditions. The system is relatively inexpensive, easy to operate, safe, and reliable. A basic understanding of electricity principles is needed to assemble the system. It is a weatherproof system that can record monochromatic video continuously during 8 h in complete darkness, or color video in daylight conditions. The system can be easily installed or attached to trees or any other supporting device. Footage recorded has a resolution of 720 x 480 lines and renders standard video in MPEG-2, a common and widely accepted video format compatible with many of the available behavioral and video editing software. Most

importantly, the parts and assembly concepts presented here can be used to assemble similar systems if the specific components described here are unavailable to the researcher.

System Description

A. Sony® Hand-held camera DCR-SR220: this is the most important component of the system. The hand-held camera has a 60 GB hard drive that can record up to 14 h in High Quality mode and provides fast video transfer to a computer. Autofocus feature enables the camera to focus the subject automatically. A remote control (provided with the camera) is essential to operate the camera and focus the subject. The camera can record in zero light conditions (0 lux) thanks to infrared capability (Super NightShot®). NightShot® mode is operated by a simple switch that needs to be turned on for night recording. Switching to Nightshot® mode physically displaces the camcorder's internal glass filter called "IR Cut Filter (ICF)", which means that much more NIR light (Near Infrared Wavelength) reaches the CCD (the Charge-Coupled Device is the sensor that captures the image). Sony's Nightshot® camcorders have excellent sensitivity level for the NIR, which is the same wavelength used in night vision goggles. If this model is not available, any handheld camcorder from Sony® with Super NightShot® or NightShot® can be used. I know of no other commercial camcorder manufacturer that provides 0 lux videotaping capabilities with NIR filters.

B. IR illuminator (IR23 Supercircuits, Inc.): essential for night recording. The infrared illuminator provides a long-range infrared beam (up to 75 feet) that is necessary to properly illuminate the subject. A built-in photocell automatically senses darkness and switches the unit on and off as needed. If this model is not

available, a variety of online suppliers specialized in surveillance and security tasks can provide similar illuminators. It is very important to take into account the power requirements of the IR illuminator in order to select the appropriate battery.

C. 12V 4.5AH/20HR Sealed lead-acid battery and charger: powers the IR illuminator. These types of batteries (lead-acid) are reliable, durable, and inexpensive. A cable connection must be assembled to connect the two devices. A charger with 1 A capacity is necessary to charge the battery.

D. Transparent weatherproof utility dry box #GSI0052 (GSI Outdoor, Inc): encloses all the components of the system and provides weatherproofing. No modifications are necessary because the camera can capture video through the transparent polycarbonate with no significant distortion. If a transparent box is not available, similar utility weatherproof boxes can be purchased from a variety of manufacturers, but a modification will be necessary. Using a rotary tool, cut two square sections of the box, one to make a window to capture video and to allow the IR beam to illuminate the subject, and the second to allow observation of the camera LCD screen to focus and adjust field of view. Cover the windows with 5 mm plexiglass and glue with marine-grade silicone.

E. Sony® NP-FH100 Info-Lithium battery: this is the highest capacity battery provided by the manufacturer. It is absolutely necessary to use the original Sony® battery if long, continuous periods (up to 8 h) of recording are required, because third-party manufacturer batteries do not provide the same performance.

F. Sony® Hand held camera DCR-SR220 LCD Display

Electrical Connections for LSA Battery, IR Illuminator, and Charger:

To make this connection, purchase the correct dimension DC power jacks and plugs in order to be able to charge the battery and to connect the battery to the IR illuminator. For the most common scenario, where the IR illuminator comes with a DC power jack, purchase a DC power plug of the same dimensions to the one of the charger. Replace the IR DC power jack with a DC power plug. A matching DC power jack need to be attached to the battery. Use the appropriate electrical wire, and properly solder and seal all the connections. Make sure all positive and negative connections are correct using a voltmeter. This assembly is very simple and only requires basic knowledge of electrical principles; however, if you are not familiar or do not feel comfortable with soldering and measuring low-voltage electrical currents, please ask for help.

Preparation of the System Before Use

Familiarize yourself with the capabilities and operation of the camera by reading the owner's manual and by trial videotaping. Charge the camera and the LSA battery. The LSA battery used in this system will be fully charged in 5 h with the recommended charger. Place all the components of the system inside the case as shown in Figure 1. Make sure you flip the camera LCD screen. When placing the camera and the IR illuminator, make sure they are in direct contact with the lateral wall of the box and avoid scratching the internal and external part of the box where the image reaches the camera lens. To avoid movement of the components, use high-density foam. The next step is to test the installation of the system. The system can be attached to any artificial

holder or to a tree using bungees or straps with Velcro® attachments. Now the system is ready to be installed and used.

Operation of the System

Connect the IR illuminator to the battery. Turn on the camera and turn on the NightShot® mode. Close the box, making sure there are no objects in the edges of the box, and seal. Install the camera, making sure your object is in the field of view. Adjust zoom and field of view using the remote control and press “rec”. After the recording session is finished, do not open the box in the field, because debris and water can get inside. Once you are in your workstation or laboratory, dry the outside of the box if wet and open it. Disconnect the IR illuminator from the camera. Remove the camera to download video and put the LSA battery and the camera battery to charge. If in a high-humidity environment, use a dry room to store and charge all the components; this will increase the reliability and life of all the electronics.

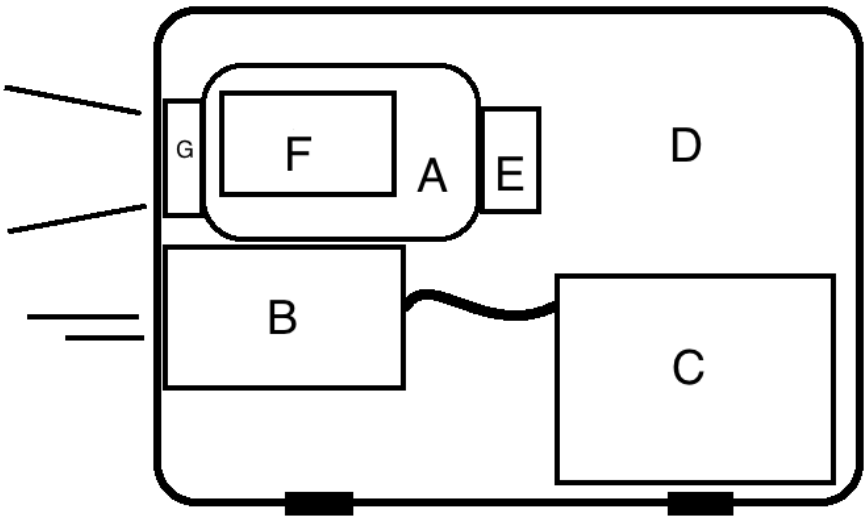

Figure B-1. Camera system. A. Sony® DCR SR220, B. IR 23 infrared illuminator, C. 12V 4.5AH/20HR SLA battery, D. Weatherproof case, E. Camcorder battery, F. Camcorder LCD screen, G. Camera lens.

LIST OF REFERENCES

Altner, H. & Loftus, R. 1985. Ultrastructure and function of insect thermo- and hygroreceptors. *Annu. Rev. of Entomol.* **30**: 273-295.

Altner, H. & Prillinger, L. 1980. Ultrastructure of invertebrate chemo-, thermo-, and hygroreceptors and its functional significance. *Int. Rev. Cytol.* **67**: 69-139.

Barkman, T. J., Beaman, J. H. & Gage, D. A. 1997. Floral fragrance variation in *Cypripedium*: Implications for evolutionary and ecological studies. *Phytochemistry* **44**: 875-882.

Beltran, M., Jiggins, C. D., Brower, A. V. Z., Berminghan, E. & Mallet, J. 2007. Do pollen feeding, pupal-mating and larval gregariousness have a single origin in *Heliconius* butterflies? Inferences from multilocus DNA sequence data. *Biological Journal of the Linnean Society* **92**: 221-239.

Benson, W. W. & Emmel, T. C. 1973. Demography of gregariously roosting populations of the nymphaline butterfly *Marpesia berania* in Costa Rica. *Ecology* **54**: 326-335.

Boggs, C. 1979. Resource allocation and reproductive strategies in several Heliconiine butterfly species. PhD dissertation. University of Texas, Austin.

Boggs, C. L. & Gilbert, L. E. 1979. Male contribution to egg production in butterflies: evidence for transfer of nutrients at mating. *Science* **206**: 83-84.

Boggs, C. L., Smiley, J. T. & Gilbert, L. E. 1981. Patterns of pollen exploitation by *Heliconius* butterflies. *Oecologia* **48**: 284-289.

Brower, L., Williams, H. W., Fink, L. S., Zubieta, R. R. & Ramirez, M. I. 2008. Monarch butterfly clusters provide microclimatic advantages during the overwintering season in Mexico. *J. Lepid. Soc.* **62**: 177-188.

Brower, L. P., Brower, J. V. Z. & Corvino, J. M. 1967. Plant Poisons in a Terrestrial Food Chain. *Proc. Natl. Acad. Sci.* **57**: 893-898.

Brown Jr., K. S. 1981. The Biology of *Heliconius* and Related Genera. *Annu. Rev. Entomol.* **26**: 427-457.

Brown, K. S., Trigo, J. R., Francini, R. B., Barros de Morais, A. B. & Motta, P. C. 1991 Aposematic insects on toxic host plants: coevolution, colonization, and chemical emancipation. In: *Plant-animal interactions: Evolutionary ecology in tropical and temperate regions*, (Price, P. W., Lewinsohn, T. M., Fernandes, G. W. & Benson, W. W., eds.). pp. 375–402. John Wiley & Sons, New York.

Bruce, T. J. A., Wadhams, L. J. & Woodcock, C. M. 2005. Insect host location: a volatile situation. T*rends Plant Sci.* **10**: 269-274.

Camazine, S., Deneubourg, J.-L., Franks, N. R., Sneyd, J., Theraulaz, G. & Bonabeau, E. 2001. *Self-organization in Biological Systems*. Princeton University Press, New Jersey.

Cardoso de Castro, C. & Cardoso Araujo, A. 2004. Distyly and sequential pollinators of *Psychotria nuda* (Rubiaceae) in the Atlantic rain forest, Brazil. *Plant Systematics and Evolution* **244**: 131-139.

Cardoso, M. Z. 2001. Patterns of pollen collection and flower visitation by *Heliconius* butterflies in southeastern México. *J. Trop. Ecol.* **17**: 763-768.

Cardoso, M. Z. & Gilbert, L. E. 2007. A male gift to its partner? Cyanogenic glycosides in the spermatophore of longwing butterflies (*Heliconius*). *Naturwissenschaften* **94**: 39-42.

Chai, P. 1986. Field observations and feeding experiments on the responses of rufous-tailed jacamars (*Galbula ruficauda*) to free-flying butterflies in a tropical rainforest. *Biol. J. Linnean Soc.* **29**: 161-189.

Chai, P. 1990. Relationships between visual characteristics of rainforest butterflies and responses of a specialized insectivorous bird. In: *Adaptive Coloration in Invertebrates: Proceedings of a Symposium Sponsored by the American Society of Zoologists.* pp. 31-60.

Cook, L. M., Thomason, E. W., & Young, A. M. 1976. Population structure, dynamics, and dispersal of the tropical butterfly *Heliconius charithonius*. *J. Anim. Ecol.* **45**: 851-863.

Cott, H. B. 1957. *Adaptive coloration in animals*. London: Meuthen.

Crane, J. 1955. Imaginal behavior of a Trinidad butterfly, *Heliconius erato hydara* Hewitson, with special reference to the social use of colour. *Zoologica* **40**: 167-196.

Crane, J. 1957. Imaginal behavior in butterflies of the family Heliconiidae; changing social patterns and irrelevant actions. *Acta Zool.* **40**: 167-196.

Dani, F. 2009. Cuticular lipids as semiochemicals in paper wasps and othe social insects. *Ann. Zool. Fennici* **43**: 500-514.

Dani, F. R., David Morgan, E. & Turillazzi, S. 1995. Chemical analysis of sternal gland secretion of paper wasp *Polistes dominulus* (Christ) and its social parasite *Polistes sulcifer* (Zimmermann) (Hymenoptera: Vespidae). *J. Chem. Ecol.* **21**: 1709-1718.

David, F. N. & Moore, P. G. 1954. Notes on contagious distributions in plant populations. *Ann. Bot.* **18**: 47-53.

DeVries, P.J., Schull, J. & Greig, N. 1987. Synchronous nocturnal activity and gregarious roosting in the neotropical skipper butterfly *Celaenorrhinus fritzgaertneri* (Lepidoptera: Hesperiidae). *Zool. J. Linn. Soc*. **89**: p. 89-103.

Davenport, J. W. & Conner, W. E. 2003. Dietary alkaloids and the development of androconial organs in *Estigmene acrea*. *J Insect Sci.* **3**: 3.

Dobson, H. E. M. & Bergström, G. 2000. The ecology and evolution of pollen odors. *Plant Syst. Evol.* **222**: 63-87.

Dunlap-Pianka, H., Boggs, C. L. & Gilbert, L. E. 1977. Ovarian dynamics in heliconiine butterflies: programmed senescence versus eternal youth. *Science* **197**: 487-490.

Edmunds, M. 1974. *Defence in animals : a survey of anti-predator defences*. Longman, England.

Eltringham, H. 1925. On the abdominal glands in *Heliconius* (Lepidoptera). *Trans. Entomol. Soc. London* **73**: 269-275.

Emsley, M. G. 1965. Speciation in *Heliconius* (Lep., Nymphalidae): morphology and geographic distribution. *Zoologica (NY)* **50**: 191-254.

Ende, M. & Spiteller, G. 1982. Contaminats in mass spectrometry. *Mass Spectrom. Rev.* **1**: 29-62.

Engler-Chaouat, H. S. & Gilbert, L. E. 2007. De novo synthesis vs. sequestration: Negatively correlated metabolic traits and the evolution of host plant specialization in cyanogenic butterflies. *J. Chem. Ecol.* **33**: 25-42.

Espelie, K. E., Gamboa, G. J., Grudzien, T. A. & Bura, E. A. 1994. Cuticular hydrocarbons of the paper wasp, *Polistes fuscatus*: a search for recognition pheromones. *J. Chem. Ecol.* **20**: 1677-1687.

Estrada, C., Schulz, S., Yildizhan, S. & Gilbert, L. E. 2007. Pupal mating, intraspecific signaling and evolution of mimicry among closely related species. In: *5th International Conference on the Biology of butterflies.* pp. 62. Tor Vergata University, Italy.

Estrada, C., Yildizhan, S., Schulz, S. & Gilbert, L. E. 2009. Sex-specific chemical cues from immatures facilitate the evolution of mate guarding in *Heliconius* butterflies. *Proc. R. Soc. Lond. B*. **277**(1680):407-13.

Evans, H. E. & Linsley, E. G. J. 1960. Notes on a sleeping aggregation of solitary bees and wasps. *Bull. Southern Cal. Acad. Sci.* **59**: 30-37.

Franks, D. W. & Noble, J. 2003. The origins of mimicry rings. In: *Proceedings of the eighth international conference on Artificial life.* pp. 186-191. MIT Press.

Frederiksen, R. & Warrant, E. J. 2008. Visual sensitivity in the crepuscular owl butterfly *Caligo memnon* and the diurnal blue morpho *Morpho peleides*: a clue to explain the evolution of nocturnal apposition eyes? *J. Exp. Biol.* **211**: 844-851.

Gagliardo, A. & Guilford, T. 1993. Why do Warning-Coloured Prey Live Gregariously? *Proc. R. Soc. Lond. B*. **251**: 69-74.

Gamberale, G. & Tullberg, B. S. 1998. Aposematism and Gregariousness: the combined effect of group size and coloration on signal repellence. *Proc. R. Soc. Lond. B*. **265**: 889-894.

Gilbert, L. E. 1972. Pollen feeding and reproductive biology of *Heliconius* butterflies. *Proc. Natl. Acad. Sci.* **69**: 1403-1407.

Gilbert, L. E. 1976. Postmating Female Odor in Heliconius Butterflies: A Male-Contributed Antiaphrodisiac? *Science* **193**: 419-420.

Gilbert, L. E. 1991. Biodiversity of a Central American *Heliconius* community: Pattern, process, and problems. In: *Plant-animal Interactions: Evolutionary ccology in tropical and temperate Regions*, (Price, P. W., Lewinsohn, T. M., Fernandes, G. W. & Benson, W. W., eds.). pp. 403-427. John Wiley & Sons, New York.

Gomes-Filho, A. 2000. Aggregation Behavior in the Neotropical Owlfly *Cordulecerus alopecinus* (Neuroptera: Ascalaphidae). *J. N.Y. Entomol. Soc.* **108**: 304-313.

Greene, M. J. & Gordon, D. M. 2003. Social insects: Cuticular hydrocarbons inform task decisions. *Nature* **423**: 32-32.

Greig, N. & De Vries, P. J. 1986. Observations on the diurnal gregarious roosting of *Ocalaria* sp. (Noctuidae) in Costa Rica. *J. Lepid. Soc.* **40**: 124-126.

Guilford, T., Nicol, C., Rothschild, M. & Moore, B. P. 1987. The biological roles of pyrazines: evidence for a warning odor function. *Biol J Linnean Soc.* **31**: 113-128.

Hall, D. W. & Butler J. F. *2000. Eumaeus atala* Röber (Insecta: Lepidoptera: Lycaenidae). Online publication EENY-169. University of Florida. http://entnemdept.ifas.ufl.edu/creatures/bfly/atala_hairstreak.htm

Hamilton, W. D. 1971. Geometry for the selfish herd. *J Theor. Biol.* **31**.

Hay-Roe, M. M. 2004. Comparative processing of cyanogenic glycosides and a novel cyanide inhibitory enzyme in *Heliconius* butterflies (lepidoptera: Nymphalidae: heliconiinae) *Entomology and Nematology.* PhD dissertation. University of Florida, Gainesville.

Hay-Roe, M. M. & Nation, J. 2007. Spectrum of cyanide toxicity and allocation in Heliconius erato and Passiflora host plants. *J. Chem. Ecol.* **33**: 319-329.

Hölldobler, B. & Wilson, E. O. 1990. *The Ants*. Harvard University Press, Cambridge, MA.

Holzinger, H. & Holzinger, R. 1994. *Heliconius and realted genera*. Sciences NAT, Venette, France.

Howard, R. W., Jackson, L. L., Banse, H. & Blows, M. W. 2003. Cuticular Hydrocarbons of *Drosophila birchii* and *D. serrata*: Identification and Role in Mate Choice in *D. serrata*. *J. Chem. Ecol.* **29**: 961-976.

Inomata, S., Watanabe, A., Nomura, M. & Ando, T. 2005. Mating communication systems of four plusiinae species distributed in japan: identification of the sex pheromones and field evaluation. *J. Chem. Ecol.* **31**: 1429-1442.

Jiggins, C. D., Naisbit, R. E., Coe, R. L. & Mallet, J. 2001. Reproductive isolation caused by colour pattern mimicry. *Nature* **411**: 302-305.

Jones, F. M. 1930. The sleeping heliconias of Florida: A Biological mystery in a fascinating setting. *J. Nat. Hist.* **30**: 635-644.

Joseph, K. J. 1982. The roosting behaviour of *Chalybion bengalense* (Dahlbom) (Sphecidae: Hymenoptera). *Entomon. India.* **7**: 403-405.

Klein, A. & de Araújo, A. 2010. Courtship behavior of *Heliconius erato phyllis (*Lepidoptera, Nymphalidae) towards virgin and mated females: conflict between attraction and repulsion signals? *J. Ethol*. March 15, 2010. Video article. doi:10.1007/s10164-010-0209-1

Knudsen, J. T., Tollsten, L. & Bergstrˆm, L. G. 1993. Floral scents-a checklist of volatile compounds isolated by head-space techniques. *Phytochemistry* **33**: 253-280.

Krenn, H., Eberhard, M., Eberhard, S., Hikl, A.-L., Huber, W. & Gilbert, L. 2009. Mechanical damage to pollen aids nutrient acquisition in *Heliconius* butterflies (Nymphalidae). *Arthropod-Plant Inte.* **3**: 203-208.

Krenn, H. W. P., C. M. 1998. Mouthparts of Heliconius butterflies (Lepidoptera: Nymphalidae): a search for anatomical adaptations to pollen feeding behavior. *International J. Insect Morphol. Embryol.* **27**: 301-309.

Landolt, P. J., Jeanne, R. L. & Reed, H. C. 1998. Chemical communication n social wasps. In: *Pheromone Communication in Social Insects*, (Vander Meer, R. K., Breed, M. D., Winston, M. L. & Espelie, K. E., eds.)*.* pp. 216–235. Westview Press, Boulder, CO.

Mallet, J. 1986a. Dispersal and gene flow in a butterfly with home range behavior: *Heliconius erato* (Lepidoptera: Nymphalidae). *Oecologia* **68**: 210-217.

Mallet, J. 1986b. Gregarious roosting and home range in *Heliconius* Butterflies. *National Geoographic Research* **2**: 198-215.

Mallet, J. & Gilbert, L. E. 1995a. Why are there so many mimicry rings? Correlations between habitat, behaviour and mimicry in Heliconius butterflies. *Biol. J. Linn. Soc.* **55**: 159-180.

Mallet, J. & Gilbert, L. E. 1995b. Why are there so many mimicry rings? Correlations between habitat, behaviour and mimicry in *Heliconius* butterflies. *Biol. J. Linn. Soc.* **55**: 159-180.

Mallet, J. & Jackson, D. A. 1980. The ecology and social behaviour of the Neotropical butterfly *Heliconius xanthocles* Bates in Colombia. *Zool. J. Linn. Soc.* **70**: 1-13.

Manly, B. F. J. 1997. *Randomization, bootstrap and Monte Carlo methods in biology*. Chapman & Hall, New York.

Matsushita, H. & Negishi, E. 1982. Palladium-catalyzed reactions of allylic electrophiles with organometallic reagents. A regioselective 1,4-elimination and a regio- and stereoselective reduction of allylic derivatives. *J. Org. Chem.* **47**: 4161-4165.

Millar, J. G. 2003. Polyene hydrocarbons and epoxides: A Second Major Class of Lepidopteran Sex Attractant Pheromones. *Annu Rev. Entomol.* **45**: 575-604.

Miyakado, M., Meinwald, J. & Gilbert, L. E. 1989. (R)-(Z,E)-9,11-octadecadien-13-olide: An intriguing lactone from *Heliconius pachinus* (Lepidoptera). *Experientia* **45**: 1006-1008.

Molet, M., Chittka, L. & Raine, N. 2009. How floral odors are learned inside the bumblebee (*Bombus terrestris*) nest. *Naturwissenschaften* **96**: 213-219.

Müller, F. 1879. *Ituna and Thyridia*: a remarkable case of mimicry in butterflies. *Proc. Entomol. Soc. London.* **1879**: 20-29.

Murawski, D. A. 1987. Floral Resource Variation, Pollinator Response, and Potential Pollen Flow in *Psiguria Warscewiczii*. *Ecology* **68**: 1273-1282.

Murawski, D. A. & Gilbert, L. E. 1986. Pollen flow in *Psiguria warscewiczii*: a comparison of *Heliconius* butterflies and hummingbirds. *Oecologia* **68**: 161-167.

Nahrstedt, A. & Davis, R. H. 1983. Occurrence, variation, and biosynthesis of the cyanogenic glycosides linamarin and lotaustralin in species of the Heliconiini (Insecta: Lepidoptera). *Comp. Biochem. Phys.* **75B**: 65-73.

O'Brien, D. M., Boggs, C. L. & Fogel, M. L. 2003. Pollen feeding in the butterfly Heliconius charitonia: isotopic evidence for essential amino acid transfer from pollen to eggs. *Proc. R. Soc. Lond. B*. **270**: 2631-2636.

Odendaal, F. J., Turchin, P. & Stermitz, F. R. 1988. An Incidental-Effect Hypothesis Explaining Aggregation of Males in a Population of Euphydryas anicia. *Amer. Nat.t* **132**: 735-749.

Paré, P. W. & Tumlinson, J. H. 1997. De Novo Biosynthesis of Volatiles Induced by Insect Herbivory in Cotton Plants. *Plant Phys.* **114**: 1161-1167.

Penz, C. M. 1999. Higher level phylogeny for the passion-vine butterflies (Nymphalidae: Heliconiinae) based on early stage and adult morphology. *Biol. J. Linnean Soc.* **127**: 277-344.

Pinheiro, C. E. G.; Medri, I. M. & Salcedo, A. K. M. 2008. Why do the ithomiines (Lepidoptera, Nymphalidae) aggregate? Notes on a butterfly pocket in central Brazil. *Rev. Bras. entomol.* **52**: 610-614.

Rehfeldt, G. E. 1993. Heterospecific tandem formation in *Sympetrum depressiusculum* (Selys) (Anisoptera: Libellulidae). *Odonatologica* **22**: 77-82.

Salcedo, C. (2006) Spatial Dynamics of Night Roosting in *Heliconius erato petiverana* (Lepidoptera: Nymphalidae). MSc Thesis. University of Florida, Gainesville.

Schulz, S., Beccaloni, G., Brown, K. S., Boppre, M., Freitas, A. V. L., Ockenfels, P. & Trigo, J. R. 2004. Semiochemicals derived from pyrrolizidine alkaloids in male ithomiine butterflies (Lepidoptera: Nymphalidae: Ithomiinae). *Biochem.Sys. and Ecol.* **32**: 699-713.

Schulz, S., Boppre, M. & Vane-Wright, R. I. 1993. Specific Mixtures of Secretions from Male Scent Organs of African Milkweed Butterflies (Danainae). *Philos. Trans. R. Soc Lond. B. Biol. Sci.* **342**: 161-181.

Schulz, S., Estrada, C., Yildizhan, S., Boppré, M. & Gilbert, L. E. 2008. An Antiaphrodisiac in *Heliconius melpomene* Butterflies. *J. Chem. Ecol.* **34**: 82-93.

Schulz, S., Yildizhan, S., Stritzke, K., Estrada, C. & Gilbert, L. E. 2007. Macrolides from the scent glands of the tropical butterflies *Heliconius cydno* and *Heliconius pachinus*. *Org. Biomol. Chem.* **5**: 3434-3441.

Steinbrecht, R. A. (1998) Bimodal thermo- and hygrosensituve sensilla. In: *Microscopal Anatomy of Invertebrates*, (Harrison, F. W. & Locke, M., eds.). pp. 405-422. Wiley-Liss, New York.

Stone, J. L. 1996. Components of pollination effectiveness in *Psychotria suerrensis*, a tropical distylous shrub. *Oecologia* **107**: 504-512.

Swihart, S.L. Hearing in butterflies (Nymphalidae: *Heliconius, Ageronia*). *J. Insect Physiol.* **13:** 467–476.

Tichy, H. & Loftus, R. 1996. Hygroreceptors in insects and a spider: Humidity transduction models. *Naturwissenschaften* **83**: 255-263.

Turner, J. R. 1981. G. Adaptation and evolution in *Heliconius*: a defense of neo-Darwinism. *Annu. Rev. Ecol. Syst.* **12**: 99-121.

Turner, J. R. G. 1975. Communal roosting in relation to warning colour in two Heliconiine butterflies (Nymphalidae). *J. Lepid. Soc.* **28**: 221-227.

Wallace, A. R. 1870. *Contributions to the Theory of Natural Selection*. McMillan, London.

Waller, D. A. & Gilbert, L. E. 1982. Roost recruitment and resource utilization: Observations in *Heliconius charithonia* L. roost in Mexico (Nymphalidae). *J. Lepid. Soc.* **36**: 178-184.

Wenqui, W., Cottrell, C. B., Hansson, B. S. & Löfstedt, C. 1999. Comparative study of pheromone production and response in Swedish and Zimbabwean populations of turnip moth, *Agrotis segetum*. *J. Chem. Ecol.* **25**: 177-196.

Williams, C. M. & Mander, L. M. 2001. Chromatography with silver nitrate. *Tetrahedron* **57**: 425-447.

Winston, M. L. 1987. *The Biology of the Honey Bee*. Harvard University Press, Cambridge, MA.

Yang, C. Y., Han, K. S. & Boo, K. S. 2009. Sex pheromones and reproductive isolation of three species in genus *Adoxophyes*. *J. Chem. Ecol.* **35**: 342-348.

Yokohari, F. 1999. Hygro- and thermoreceptors. In: *Atlas of Arthropod Sensory Receptors: Dynamic Morphology in Relation to Function*, Eguchi, E. & Tominaga, Y., eds. pp. 191-210. Springer, Berlin.

Young, A. M. 1978. A communal roost of the butterfly *Heliconius charithonius* L. in Costa Rican premontane tropical wet forest (Lepidoptera: Nymphalidae). *Entomol. News* **89**: 235-243.

Young, A. M. & Carolan, M. E. 1976. Daily instability of communal roosting in the neotropical butterfly *Heliconius charitonius* (Lepidoptera: Nymphalidae). *J. Kansas Entomol. Soc.* **49**: 346-359.

Young, A. M. & Thomason, J. H. 1975. Notes on communal roosting of *Heliconius charitonius* (Nymphalidae) in Costa Rica. *J. Lepid. Soc.* **29**: 243-255.

BIOGRAPHICAL SKETCH

Christian Salcedo was born in Bogotá, a city in the Andean mountains of South America located at 2600 m (8900 ft) and the capital of Colombia. He was exposed to nature as a child by periodically visiting tropical dry forests in the Caribbean coast of Colombia during summer vacations. His interest in biology was later revived in his last years of high school (1997-1998) during his biology lectures. In search of a benign and intellectually spirited climate, Salcedo went to the Universidad de los Andes in Bogotá. Coursework in invertebrate life, evolution, and ecology, together with fieldtrips to local natural parks, gave him confidence and enthusiasm towards a future in biological sciences. Two years before graduation, Salcedo volunteered at the Population Genetics Institute at Universidad de los Andes. There he performed his first formal research project under the supervision of Dr. Mauricio Linares, which later became his undergraduate thesis. After graduation (2003), he was accepted in the Master's program at the Entomology and Nematology Department at the University of Florida, under the supervision of Dr. Thomas C. Emmel. During his master's thesis he studied spatial dynamics of *Heliconius* butterfly roosting behavior. In fall 2006 he received his Master's degree. He continued his work on *Heliconius* roosting behavior as a Ph.D. student in the same academic unit, integrating classic ecology, behavior, and chemical ecology approaches in his investigations.