Signaling and Communication in Plants

Jorge M. Vivanco
Tiffany Weir *Editors*

Chemical Biology of the Tropics

An Interdisciplinary Approach

Springer

Signaling and Communication in Plants

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Preface

I first set foot in the Tambopata National Reserve (TNR) in Peru in June 2006. I had assembled a team of colleagues and students from Colorado State University to accompany me on an exploratory expedition to establish collaborative ties with Peruvian researchers and to familiarize ourselves with the countless opportunities to study chemical ecology in a tropical field setting. For years, I had been studying chemical interactions between organisms in a laboratory. I had published numerous papers on the interactions that occur between plants and other soil-dwelling microorganisms. Then one day a colleague criticized me for defining nature through my office window, and as stung as I was by this observation, I realized that he was right. I had yet to venture outside the safety and comfort of the laboratory to see how my expertise could be applied in the field, and I made it a goal of my sabbatical to broaden my scientific horizons. Serendipitously, an old friend from high school was working at the Field Museum in Chicago and decided to pay me a visit at the same time that I was planning my sabbatical to Peru. He had spent several years working as a free-lance guide in the Amazon, and particularly in the TNR, and shared stories and slides that inspired me to focus my sabbatical research in this incredibly biodiverse area. During the planning stages of my sabbatical, I learned of the work of Drs Eric Cosio and Waltraud Kofer from the Pontificia Universidad Catolica del Peru in Lima, who are proponents of tropical chemical ecology in Peru and who had created a successful research enterprise between their university and a private lodge in Tambopata. Collaboration was formed and my venture into tropical chemical ecology began to materialize.

That muggy June morning as I pulled on my rubber boots and drenched myself with insect repellant in preparation for my first hike in the TNR, I was not sure what I was looking for or what I expected to find. Our guide introduced us to the strangler figs, trees that germinate in the canopy of a host tree and grow downward, eventually engulfing and smothering their host with a massive tangle of fused roots. We saw the castaña, or Brazil nut trees, giants of the jungle who provide a livelihood to so many indigenous families, and the walking palms that are said to use their stilt-like root system to crawl across the jungle floor seeking open spaces where they can harvest the sunlight. Birds with brilliantly colored plumage peeked out of nest holes and flocked to pick at the clay on riverbanks, socializing and neutralizing toxins accumulated from their diet of plants, which produce these chemicals to defend themselves against the barrage of herbivores that they encounter daily. Monkeys screeched and howled overhead while insects crawled down below, but the things that truly captured my attention were the ants. Meandering trails of army and leaf cutter ants criss-crossed our paths. Lone bullet ants, as big as my thumb, crawled up trees. Ground-dwelling ants had constructed piled up mounds of dirt that were nothing less than mini-metropolises crawling with activity. However, what interested me the most were the specialized adaptations that numerous plant species had developed to accommodate these ants. On that first hike I watched ants feeding from nectaries on *Inga*, encountered heart-shaped domatia of the *Tococa* plants, and felt the bite of the *Pseudomyrmex*, the aggressive patrollers of the *Triplaris* trees. How specific were these ant–plant interactions and what was the basis of the recognition of an ant for a particular plant species? Despite the attention that has been given to studying ant chemistry and ecology, there are still many unanswered questions. I now knew what would be the focus of my sabbatical research.

My wife, a microbiology graduate student at that time, also accompanied me on this trip, and as one might expect, her attention was drawn in a completely different direction. She would impatiently flick the ants off her clothing while focusing her camera lens on the numerous fungi that we encountered on our trek. Her academic pursuits concentrated on the chemical communication between microbes and how plants can contribute or interfere in those conversations, and she was drawn to the idea of studying the incredible microbial diversity of this forest and decoding how some of the chemical signals used by microbes translate into functional microbial communities. She has since began pursuing these studies and her enthusiasm for conducting research in the rainforest matches my own, making her an integral member of my research team, as well as the co-editor of this book.

Although many people are drawn to the rainforest, the research that is done there has largely remained ecological. It is not often that those who are trained in chemistry or molecular biology and typically work in controlled laboratory environments with highly expensive and technical equipment wander out into the jungle and try to untangle the tight web of organismal interactions. We had a steep learning curve to climb if we wanted to make this opportunity successful. Some of the issues we faced were technical. For instance, it is difficult to relocate forest plots marked with fluorescent green tree ties (pink or red work much better), and you should always use rainproof sample labels. A good sense of direction (or a good GPS) is critical; stepping a few yards off the trail could leave a lab-dwelling researcher hopelessly disoriented. Of course, the most important thing we learned was an appreciation for the complexity of this ecosystem, and how important it is to reach beyond our comfort zone (and reach out to those who work outside of that zone) if we want to advance our understanding of it. It was from this lesson that the Pan-American Studies Institute (PASI) – Chemical Biology in the Tropics was born.

The PASIs are symposia funded by the National Science Foundation (NSF) designed to foster collaborations between researchers and institutes in North and

South America. Although the primary goal of these symposia is to bring together international researchers in a given discipline, I wanted to stretch that concept to bring together researchers from very different disciplines. Many of the workshop invitees had never visited or worked in the tropics, but I believed they had special knowledge and skills to contribute to a better understanding of tropical ecosystems. Our final list of participants included landscape and restoration ecologists and seasoned tropical biologists, but it also incorporated microbiologists, analytical chemists, cancer researchers, and plant physiologists. While I had hoped that the different disciplines would complement one another and that individuals would form collaborations that would result in new ways to look at old questions, the actual culmination of the PASI far exceeded my expectations.

Perhaps the success of the PASI was due to the outstanding group of participants assembled, or maybe it was the lack of electricity in individual rooms, which drove everyone to congregate in the lodge after 5 pm to discuss the day's topics over a beer and plan night hikes and collecting trips. Whatever the reason, it was a transformative experience for many of our colleagues, particularly those who had pledged themselves to a life in the laboratory; and many new collaborations and friendships were forged. This book is a product of those interactions, and our goal is to share the beauty and intricacy of tropical forests with a broader audience while introducing some of the interdisciplinary ideas that contributed to the success of the PASI. We have assembled a group of scientists to write chapters that ignited their interest in the tropical forests, and the final product is a book that includes general information on chemical ecology and biodiversity, a few first-hand accounts of the triumphs and trials of working in a tropical forest, and some informative essays detailing interesting ecological phenomena. It is our hope with this book to convey our excitement about this field of research to a broader audience and to provide incentive to a new generation of scientists to travel to the tropics and to use their ingenuity to understand species interactions. A deep understanding of this system is needed in order to preserve it. Ecologist and conservationist have done research in the tropics for decades and have helped to develop a global understanding of the functioning of this system. We now need another breed of scientists who can successfully work in interdisciplinary teams to explore how molecules and genes influence community structure and ecosystem functioning and ensure the preservation of these resources for generations to come.

Finally, I would like to thank everyone who made this book a possibility. I want to dedicate this book to all of the participants of the 2008 PASI – Chemical Biology in the Tropics; organizing this conference and participating in it has been by far the most collegial and satisfying academic experience in my career. It is because of your excitement and enthusiasm for the subjects presented here that this book was compiled. I would especially like to thank Mark W. Paschke, Margaret Lowman, Aaron Dossey, Caroline S. Chaboo, Dan Manter, Tiffany Weir, Waltraud Kofer, Art Edison, Stephan Halloy, and Eric Cosio for contributing to chapters in this book. I want to acknowledge Emily Wortman-Wunder who took care of much of the stateside planning and logistics for the PASI and also contributed to the writing of this book. Thank you to Max Gunther, the owner of Rainforest Expeditions, whose business vision included a place where research and tourism combine to advance the appreciation, understanding, and ultimately the conservation of one of our planets' greatest resources; and to the staff of the Explorer's Inn, especially the management and our numerous knowledgeable guides, for making our experiences there thoroughly educational and enjoyable. I also want to acknowledge my friend, Guillermo Knell, for igniting my passion for the Amazon rainforests. Finally, thank you to the Guggenheim and Fulbright Foundations for funding my research during my sabbatical, to the NSF for their support of the PASI and other research/educational experiences in TNR, to the Pontificia Universidad Catolica del Peru, and especially Dr. Eric Cosio for hosting my stay in Peru and providing logistic and technical assistance in initiating my tropical studies.

Fort Collins, CO, USA Jorge M. Vivanco
April 2011

Contents

Biodiversity

Emily Wortman-Wunder, Jorge Vivanco, and Mark W. Paschke

Abstract This chapter provides a framework for the rest of the book, by defining biodiversity, describing techniques to measure biodiversity, and listing the Earth's biodiversity hotspots as identified by Norman Myer. The chapter then gives a brief discussion of theories of the causes of biodiversity, starting with the earliest theories, published in the 1960s, and ending with the most recent theories explaining various regional areas of biodiversity, several of which are controversial. Finally, the chapter concludes by exploring possible future directions of biodiversity research, and notes the importance of incorporating a more thorough knowledge of the chemistry underlying ecological interactions in areas of high biodiversity.

1 Introduction

In early August 1993, a young woman limped into a remote settlement in southwestern Ecuador (Sullivan 1993). She had hiked 5 miles through dense jungle on a broken ankle, and she was the bearer of bad news: she had survived a plane crash that killed her fiancé, the ornithologist Theodore Parker, the botanist Alwyn Gentry, and the ecologist Eduardo Aspiazu, along with two other Ecuadorians. The conservation

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world reeled in shock: Parker and Gentry were naturalists of the first order. Between the two of them they possessed over two-thirds of the unpublished knowledge of Latin American biodiversity – knowledge that had now been destroyed in a single crash. Furthermore, they died conducting critical aerial surveys of biodiversity for Conservation International's Rapid Assessment Program (RAP). This program, founded by Parker and Nobel prize-winning Dr. Murray Gell-Mann, was just a few years old and had been designed to help assess the flora and fauna of biodiversity "hotspots" to identify crucial areas for conservation. Their death was a blow to the program and an even greater blow to the world of international conservation. Furthermore, the loss of so much of the world's biodiversity knowledge in a single event made clear its vulnerability.

That the flora and fauna changes with location on Earth is one of the pillars of the ancient art of travel writing; most observers over the millennia have also noted that certain areas teem with more abundance than others. However, it took fear of this abundance being stamped out to prompt humans to coin a term for it, biodiversity, and to make a serious attempt to catalog and study it. The study of biodiversity centers on three basic questions: how do you measure biodiversity, what places on earth are most biodiverse, and why do some places have more biodiversity than others?

2 Measuring Biodiversity

How to measure biodiversity? The earliest counts of biological diversity were simply that: counts of the number of plant and animals (usually birds and mammals) divided by area. Botanist Alwyn Gentry's landmark 1988 study of tree species in the upper Amazon is a typical example: he cordoned off a hectare of Peruvian rainforest and proceeded to count all the tree species found there (Gentry 1988). Early assessments of biodiversity based on this type of species count identified tropical rainforests as the areas of highest biodiversity levels, and based those assertions on numbers of plants and vertebrate animals. Entomologists later cried foul (Stork 2007); so did biologists of marine environments, in which birds, mammals, and vascular plants tend to be underrepresented (Roberts et al. 2002; Myers et al. 2000). Microbial biologists came late to the table (Nee 2004), and to some extent are still trickling in: the first methodical study of microbial diversity across latitudinal gradients was completed only in 2006 (Fierer and Jackson 2006). Its surprise conclusion that bacterial diversity is actually far higher in temperate zones has recently been called into question. New methods for finding and counting bacteria and fungi in a sample demonstrate one of the basic problems of biodiversity counts: after a certain point, it is not possible to manually count that many species, or even, in some cases, to recognize them. Not even the most assiduous beetle collector is going to be able to recognize all 350,000 estimated beetle species, for example. The world's most compendious ornithologist, Theodore Parker, was thought to be able to recognize some 4,000 bird species, a mere fraction of the 10,000 estimated species worldwide; Gentry had collected some 70,000 tropical plants (less than a third of the putative 275,000 thought to exist). Clearly, simple counting will not suffice: statistical estimation methods are essential.

In addition to measuring number of taxa within a particular area, biodiversity researchers need to know the distribution of taxa. If a spot contains 100 species, but 95 of those species are confined to a single class, it is not as robustly diverse as an area where the species are distributed more evenly across the classes. Likewise, if 95 of those species are represented by single individuals, and the remaining five species dominate the field in numbers and biomass, the diversity quality is distinctly different. Ecologists now have several tools in their statistical toolbox to address these questions, including the Simpson index (measures how dominated a particular area is by a particular subset of species), the Shannon–Wiener index (measures how evenly diversity is spread over the taxa present in an area), and a simple measure of species richness (Whittaker 1972). Another significant measurement is how many species of an area are endemic (i.e., occur nowhere else). Regions that have very high biodiversity counts tend to also have high numbers of endemic species. Lately, there have also been attempts to measure how various organisms interact, and it is related to this subject that chemical ecology is most pertinent. The myriads of different chemical interactions between all organisms in a given area have never been properly characterized or categorized, nor has any study been made that compares how changes in some species' chemical interactions affect the other species in a region.

3 Where Are They? A Short List of the World's Biodiversity Hotspots

What are the most biodiverse places on earth, and what do they look like? More critically, how threatened are they, and what can the world conservation communities do to help? These are the questions that British environmentalist Norman Myers set out to illuminate in 1988 with his catalog of the world's ten biodiversity "hotspots". The inflamed language was not an accident: his hotspots were not simply abundant in plant and animal life, but they were in danger of losing vast swaths of that life to human development. A few years later Myers published a revised list of hotspots, this time taking nonvascular plants, invertebrates, and marine habitats into account (Myers et al. 2000).

Myers' original "hotspots" were concentrated mostly on the South American continent and in the Malay Archipelago. This region, a vast scattering of islands between Southeast Asia and Australia, has a rich and complicated geological history. Likewise, South America has a long history of isolation and a fragmented geography. Additional hotspots include the island of Madagascar and the steep isolated valleys of the eastern Himalayas. All these regions are characterized by a unique evolutionary/geological history, tropical conditions, and a wide range of ecological zones.

3.1 Madagascar

This country lies off the eastern coast of Africa and has long been isolated from the rest of the world; it is sometimes referred to as the “eighth continent”. As a consequence, 80% of its flora and fauna are endemic and occur nowhere else (Myers 1988), including eight endemic families of flowering plants, eight endemic carnivores and lemurs, an endemic family of primitive primates.[1] All eight carnivores are thought to have descended from a single ancestor, which swam the Mozambique Channel between 18 and 24 million years ago (Yoder et al. 2003). The island is bisected by a mountain range and thus contains a variety of both hot and wet and cool and dry climatic zones, from the xeric spiny thickets to the humid lowland forests.

3.2 Eastern Himalayas

This area consists of Nepal, Bhutan, and several states in northern India, plus part of Myanmar and the Yunnan Province of China; it is characterized by deep tropical valleys separated by steep and rugged mountain passes that effectively isolate the resident flora and fauna, leading to endemism rates of up to 60% in some areas (Myers 1988). The various countries of this region vary in political and economic stability.

3.3 Malay Archipelago

Four of the hotspots are located within the Malay Archipelago. The wondrous flora and fauna of this region led Alfred Russel Wallace to develop his theory of evolution, independent of Charles Darwin; he was also the first to articulate the distinct differences in species found in the western part of the Malay Archipelago compared with those from the east. The Wallace Line, as it is known, passes between islands fewer than 25 km apart in some cases and yet is a clear continental boundary: the species assemblage to the west is Asian and that to the east is Australian. We now know this line is due to continental drift and that 50 million years ago they were separated by an ocean more than 1,500 km wide; the Philippines were a separate cluster of islands, drifting west. Within this archipelago, there are islands that so closely resemble the flora and fauna of the ancient super continent Gondwana that they are sometimes called real-life Jurassic Parks. The nature of human colonization of these islands varies widely. In some places, such as New

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[1] Biodiversityhotspots.org. Conservation International Foundation 2007. Produced by J. Carr and P. Langhammer. Accessed October 2008.

Caledonia, humans have coexisted with the ancient biodiversity for more than 50,000 years (Diamond 1999); other places were visited for the first time within the past few thousand years (Diamond 1999). Later colonizations tended to have a more devastating effect on the local biodiversity, as humans with highly developed hunting technology, and accompanied by carnivorous dogs, pigs, and rats, descended upon areas that had been predator-free for eons (Daws and Fujita 1999).

3.3.1 Peninsular Malaysia

Over 8,500 tree species once occurred in the rainforests of the Malaysian peninsula, nearly 27% of them endemic (Myers 1988); this area is also famed for its mangrove swamp forests (which played a critical role in protecting coastal communities from the 2004 tsunami), its limestone crag region, dotted with limestone spires and caves and home to several endemic bats and orchids, and the quartz spires outside of Kuala Lumpur, which are a UN World Heritage Site. All are threatened by mining and deforestation, as this region is one of the most densely populated and fastest growing areas on earth (see footnote 1).

3.3.2 Northern Borneo

The tropical forests of the island of Borneo contain over 10,000 plant species, of which 34% are endemic (Myers 1988). The area is characterized, again, by high geographic diversity (with the 13,435-ft Kinabalu mountain at its core, Borneo is the world’s highest island), with ecoregions ranging from lowland forests, peat forests, and heath forests to freshwater swamp forests. The Bornean rainforests are the only remaining refuge of the Bornean orangutan and are also critical habitat for the Asian elephant, the Sumatran rhinoceros, and the Bornean clouded leopard; unfortunately, these rainforests are currently being decimated by the Malaysian plywood industry, as well as a massive relocation program sponsored by the Indonesian government designed to give landless peasants productive land and to ease urban crowding (Daws and Fujita 1999). The plan has mostly failed and has led to the logging and degradation of thousands of acres of forest.

3.3.3 Philippines

This country consists of over 7,000 islands of varying geological origin and once comprised a rich zone of lowland tropical forest characterized by a 44% endemism rate (Myers 1988). Many of these endemic species are critically endangered, such as the Cebu flowerpecker, found only on the island of Cebu and thought to be extinct until 1992 (Stattersfield and Capper 2000). As in other island countries in this region, the Philippines’ prosperity has brought both an acute awareness of its precious biological heritage and an accelerating destructive development cycle, as forests are logged, industry pollutes the water and air, and formerly pristine islands are paved and carved into golf courses and condominiums (see footnote 1).

3.3.4 New Caledonia

Like Madagascar, this island broke off from the super continent of Gondwana several million years earlier than other parts of the globe and thus contains a mind-boggling number of endemic species (it contains the relatively low total plant species number of around 1,575, but 89% of those species are endemic) (Myers 1988). Its most notable flora and fauna include the small flightless bird the kagu and a few emblematic species of pine; until the arrival of rats and dogs, the region was home to only one species of mammals, the flying fox (actually a vegetarian bat). Like many small countries, New Caledonia faces a delicate balancing act as it tries to develop a robust economy while preserving its unique biological heritage. Its mineral resources have led to a relative degree of prosperity for the country (Pascal et al. 2008); unfortunately, they have also led to incredible levels of habitat destruction. The country has very few fully protected natural preserves and is in grave danger of losing some of its unique bioregions (see footnote 1).

3.4 South America

Four of Myers' hotspots are located on the continent of South America. While Alfred Wallace is most closely associated with the Malay Archipelago, he cut his explorer's teeth on the rich biota of the Amazon, where he spent 4 years collecting and surveying for a private expedition. It was this early experience that helped him develop into the visionary scientist he became, and his lack of specific work on this region is due not to its less-interesting geological history but to an exploring disaster: on his return trip to England the ship he was on caught fire and sank, taking all of his carefully preserved specimens and most of his field notes with it. Likewise, Charles Darwin's travels along the western coast of South America are well known to have led to his theories of evolution, although he did not even come close to the richest ecological zones of this continent. Similarly, while the biodiversity of this continent easily rivals that of the diverse and island-strewn south Pacific, the root causes of this diversity are much less understood. Some have argued that its history of fragmentation during glacial periods should be credited (Brown and Gibson 1983); others that its long isolation from the rest of the world is responsible. Finally, new and exciting ideas have emerged from an unexpected source: archeologists. Recent discoveries in the Amazon basin seem to point to a human role in increasing biodiversity of this region – a striking reversal of thought regarding human interaction with local biota. These ideas will be explored more fully in the next section; for now we will simply describe the most biodiverse regions of South America.

3.4.1 Atlantic Coast of Brazil

This is a diverse zone containing tropical lowland forest, mountains, and arid regions; this area once exceeded the Amazon basin in diversity and numbers of species (Myers 1988). Approximately half its 10,000 plant species are endemic, with a similar endemism rate for animals. Approximately 5% of the pre-European settlement forest was thought to survive in 1988.

3.4.2 Western Ecuador

This is, again, one of the hottest of the hotspots, with over 10,000 known species of plants, a high rate of endemism, and rampant deforestation (Myers 1988). The region is particularly known for its endemic epiphytes. This is the region Theodore Parker and Alwyn Gentry were surveying when their plane went down in 1993.

3.4.3 Colombian Chocó

This section of northeast Colombian coast is sandwiched between mountains and the Panamanian Isthmus and has the rainiest lowlands in the world, with an annual rainfall of nearly 400 in.; it is known to have an endemism rate of roughly 25%. It is also one of the poorest regions of a poor and war-torn country (Myers 1988).

3.4.4 Neotropical Andes

The Western Amazonian region is considered to be possibly the most biodiverse area in the entire world (Myers 1988), although it is so relatively unexplored that firm figures are unavailable. The western rim of this region is the most diverse geographically, as the tropical forest climbs into the highlands of the eastern Andes; it is also one of the more threatened Amazonian regions, with an east–west paved highway scheduled for completion in 2009. Several large biopreserves exist in the countries along the eastern slope of the Andes and are known as the “string of pearls”, and these host countries face the need to preserve their rich biological heritage while developing wealth. Peru provides an example of some of the dilemmas faced by these parks. While its commitment to preserving large swaths of their eastern region is highly commendable, it is unable to devote much money to maintaining this region or enforcing conservation laws (see footnote 1). Illegal gold mining and other activities are a serious problem; however, these problems are balanced by a growing private conservation movement and recent cooperation between local indigenous groups and conservation groups.

4 How Did the Biodiversity Hotspots Get So Hot?

Human nature craves order; as we accumulate more and more biodiversity facts, we long to weave this mass of factual information into a tapestry of meaning. Charles Darwin, in the face of specimen collections numbering in the tens of thousands, developed a theory of evolution by natural selection and contributed ideas that when combined with later geologic discoveries would lead to the theory of continental drift. Modern scientists, faced with specimen collections in the millions and unique bioregions in the thousands, have a similar urge to make it all mean something. Why are some places more biodiverse than others? What does the earth's biodiversity say about its history, the history of life, and perhaps most importantly, the future of life on this planet?

That the earth's abundance of species changes as we go from higher to lower latitudes has been noted by innumerable explorers and travelers; also, species abundance generally decreases with altitude. The land surrounding Homer, Alaska, has far fewer species than the land surrounding Panama City; likewise, the windswept peaks of the Peruvian Andes are barren, while the rainforest of the Amazon Basin, just a few miles to the east, is one of the most species-rich areas on earth. However, the reasons for these patterns are still the subject of hot debate. One of the spurs to this debate is that most investigators have traditionally been determined to find a single cause for biodiversity distribution patterns, and expend much ink and energy in trying to rule out competing theories. In reality, of course, there is unlikely to be a single cause of species richness, and recent research has been more comprehensive and willing to combine putative causes into more global explanations.

The study of biodiversity distribution is largely a science of the mid-twentieth century; its rise coincided with the solidification of the theory of plate tectonics and continental drift. The leading biodiversity researchers hail from a wide span of disciplines, from paleobiology to botany to ecology to evolution; at their forefront were two young hotshots from the East Coast, Robert MacArthur and E.O. Wilson. These two ecologists burned up the biological scene by thinking about the world as an organic whole, rather than as a series of separate taxa with separate histories: their theory of island biogeography stated that the number of species on an island was directly related to the island's size and distance from other landforms (MacArthur and Wilson 1963, 1967). This theory held true regardless of what these species were or where the island was located and had the heady certainty of a law of physics. Like Darwin before them, Wilson, MacArthur, and their young cohorts were unwilling to limit themselves to the cataloging of mere facts.

The first publications on this subject came from the University of Southern California paleobiologist Alfred G. Fischer. He hypothesized that regions that had experienced a longer period of sustained stability, such as the tropical regions at the equator, had a lower rate of extinction and thus had accumulated more species (Fischer 1960). Thus, the Amazon Basin has such a spectacular glut of species because it has never experienced glaciation and has relatively little volcanic activity. This theory has come to be known as the “historical perturbation” theory and is still thought to have merit. However, it does have a logical flaw: it assumes that any spot on earth can attain immense biodiversity given enough time for species to accumulate (Brown and Gibson 1983).

Other researchers were quick to jump on the weak points of the historical perturbation idea: was it really plausible that a rocky alpine meadow, however long it might be stable, could ever have the abundance and diversity of a tropical river basin? Furthermore, certain areas on earth have presumably been extremely stable, yet have a low species count – the bottom of the ocean floor, for example, is very stable compared with most terrestrial environments. Yet relatively few organisms live there. Several researchers argued that the productivity of an environment (or the harshness of its conditions) was more important to biodiversity than stability, including Robert MacArthur (1965, 1972), his PhD advisor G. Evelyn Hutchinson (1959), and the ecologists Joseph Connell and Eduardo Orias (1964). Likewise, environments that provided a greater diversity of habitats nearly always had more species – thus favoring a complex rainforest, with its hundreds of gradations in light, temperature, and water availability as you moved from the forest floor to the tree canopy. Areas dominated by 6-in.-tall alpine plants just could not compete. Robert MacArthur and his colleague John MacArthur were the first to document this relationship for birds of various habitats in the eastern United States (MacArthur and MacArthur 1961).

As the study of biodiversity distribution patterns stretched into its second and third decades, researchers began noticing more complex relationships that appeared to be influencing these patterns. It began to appear that competition, predation, dispersal mechanisms, and other basic survival strategies also affected how plants and animals were distributed, as well as how many of them a particular environment could support. While it is difficult to prove that any of these factors cause biodiversity, rather than merely being products of a great number of species existing in a given space, such studies have helped define how biodiversity operates and have come to be as much a part of the discipline’s landscape as island biogeography or evolution.

One of the essential paradigms of biodiversity patterns was provided in the early 1970s by two independent researchers. Dan Janzen, a young professor of conservation biology working in Costa Rica, began to notice that adult trees appeared to be regularly spaced throughout the forest, rather than clumping around an originating parent tree. He determined that this pattern was consistent enough to be stated as one of the basic, indisputable facts about tropical lowland forests: compared with temperate forests, they have a large number of species, a low concentration of adult representatives of each species, and these adults are spaced regularly throughout the forest, rather than in clumps (Janzen 1970). He discussed his observation with his colleague Joseph Connell at the University of California Santa Barbara and discovered that Connell had observed a similar phenomenon during his work with the tropical rainforests of Australia and in fact was preparing to publish his observations (Connell 1971). Janzen rushed to submit his work to *The American Naturalist*, where it was published in the last issue of 1970. He was careful to acknowledge his conversation with Connell in his introduction, and the hypothesis they devised has come to be known as the Janzen–Connell hypothesis. It states that the more seeds and seedlings there are scattered about on the ground, the more seeds are going to be eaten. Since the number of seeds and seedlings is highest at the base of the parent tree (which may also be attracting seed eaters through a variety of cues), a seed's chance of survival increases the farther it gets from the parent tree. This relationship is particularly strong in the tropics, where many more seed eaters are able to eat only one sort of seed and has actually caused the trees of the tropics to regularly space themselves throughout the forest (which also leaves room for other tree species to grow up between them, leading to a higher level of tree species diversity).

From his work on Australian rainforests and coral reefs, Connell soon came up with another important contribution to biodiversity work: the Intermediate Disturbance Hypothesis. It says that the most biodiverse areas are those that are subject to frequent, low-intensity disturbances that keep an area from achieving a stable climax forest (except in isolated pockets) and yet do not subject it to frequent all-encompassing disasters that wipe out everything.

Taking the Intermediate Disturbance Hypothesis one step further, Steven Warren and colleagues (Warren et al. 2007) have recently proposed the "Heterogeneous Disturbance Hypothesis". Warren's experience working as an ecologist on military training areas throughout the world caused him to notice that military training areas contain disproportionately high biodiversity. The reason for the high numbers of taxa on military training areas can be attributed to a matrix of intact native ecosystems interspersed with a wide variety of disturbances (shapes, sizes, duration, intensity, etc.) resulting from military training. The result of such heterogeneous disturbances is an impressive assemblage of diverse species in the various habitat conditions.

Other factors are also likely to play a role in biodiversity. Darwin's famous finches are the classic example of evolution: when he visited the Galapagos Islands, he found that on each island there was a slightly different species of finch, each specialized to eat a different size of seed. All species were descended from a single species that had emigrated from the mainland; once isolated from the main population, each had changed over time to become a different species. Similarly, it has long been a commonplace of biodiversity studies that land masses that are isolated tend to have a more distinct flora and fauna – think Madagascar, New Zealand, and Australia. The larger the island, the more species it can support overall, and the longer it has been isolated, the weirder that species assemblage tends to be. Isolation crops up in explaining the biodiversity of many regions, not all of them islands. The Amazonian tropics were demonstrated to have undergone multiple cooling periods in which pockets of rainforest were isolated from each other, making a series of rainforest "islands". These areas, known as refugia, have now been demonstrated to harbor unusually high numbers of endemic birds and lizards (Haffer 1969). The refugia hypothesis has since been applied to isolated patches of habitat all over the world, from mountain peaks within the American Southwest's Great Basin region (Brown 1971) to current conservation biology work arguing that the world's biological reserves are becoming islands.

Biodiversity research began in the 1960s, the same decade that brought us Earth Day, the Wilderness Act, *Silent Spring*, and other landmarks in the drive to conserve our natural resources. The default position during these years was that humans were bad for the environment. Similarly, most researchers during the second half of the twentieth century assumed that once a landform was colonized by humans, its biodiversity would steadily decrease. There seemed to be much empirical evidence in support of this theory, from the loss of the Pleistocene megafauna of North America (wooly mammoths, ground sloths, saber-toothed tigers, etc.) to the extinction of several large prehistoric species from New Zealand, Madagascar, and other islands around the world. The largest and most damning piece of evidence has been the widespread worldwide extinction following modern human industrial development. Recently, however, some researchers have begun to question whether humans have always been so bad for biodiversity; in some cases and some places, they argue that human activity has actually increased the number of plant and animal species (Heckenberger et al. 2003, 2008).

Recent revelations by archeologists working in the Amazon basin have shattered the notion of the Amazon as an untouched wilderness. Starting with work by Anna Roosevelt in the 1980s (Roosevelt 1991), a new picture of the pre-Columbian Amazon as a region where huge numbers of indigenous peoples lived and actively managed the land around their cities has emerged. Roosevelt and other contemporary Amazon archeologists like Michael Heckenberger and his colleagues have argued that far from being primeval forest from time immemorial, the Amazon Basin was recently home to numerous large and thriving civilizations (Heckenberger et al. 2003). The recent popular book *1491: New Revelations of the Americas Before Columbus* by Charles Mann has further popularized this notion. Mann portrays a densely populated Amazon region where human inhabitants built thriving cities, transformed the land and forests around them, and cleverly harvested its bounty. Its current lightly populated state is due to European disease and persecution by colonizers; the indigenous populations that we know from the Amazon today are merely deprived refugees who fled the carnage (Mann 2005). By contrast, in 1491 the Amazon was a vast, highly populated and variegated parkland characterized by interlinked villages, well-planned networks of roads, causeways, bridges, canals, and dams. This civilization supported itself through the growing of a diverse array of crops, including manioc, several kinds of fruit trees, and fish farming. While these villages did have zones of "acute human influence", such as that found in village centers and on roads, they were notable for their ability to sustain large numbers of people in an ecologically sustainable way (Heckenberger et al. 2003, 2008). Furthermore, the areas where these agricultural complexes used to exist are now zones of increased biodiversity, a fact that he attributes to centuries of careful anthropogenic management (Heckenberger et al. 2003). The incredible biodiversity that we see in the Amazon today may thus be partially attributed to the heterogeneous disturbances caused by past human civilizations. We can no longer ignore the possible role of our own species not only in destroying biodiversity but also in creating it.

Recent research in the *Proceedings of the National Academy of Science* indicates that while introduced predators often lead to extinctions among native species, introduced competitors do so much less frequently – and can even increase biodiversity by encouraging the development of new traits and, potentially, new species (Sax and Gaines 2008). In their analysis of six islands facing massive biological invasion, from Hawaii to New Zealand, Dov Sax of Brown University and Steven Gaines of the University of California, Santa Barbara, found that all six islands had doubled their biological diversity in the 200 years following invasion and that extinctions were almost all due to being directly eaten by invaders, rather than being competed out of existence. This research of course is highly controversial and even proponents of looking objectively at biological invasions note that it is not merely quantity that counts; how the new species change how the native species interact is equally important (Zimmer 2008).

5 The Future of Biodiversity

Iconoclastic studies like that of Heckenberger and coworkers and Sax and Gaines may point the way toward the future of biodiversity research. Science has accepted and absorbed the theories of evolution, continental drift, and the general principles of biodiversity distribution. There is still important work to be done in these areas, but the field seems to cry out for new ways of looking at the facts. A new, more nuanced understanding of how human activity fits into the biodiversity picture is desperately needed – both a better grasp of how humans benefited biodiversity in the past and how modern humans can coexist with biodiversity in the future. Pioneers in this new vision include Michael Rosenzweig from the University of Arizona. In his 2003 book, *Win-win Ecology*, Rosenzweig lays out his vision of a future planet earth where biodiversity coexists among human enterprise. At the heart of Rosenzweig's vision is Reconciliation Ecology, which he defines as "the science of inventing, establishing and maintaining new habitats to conserve species diversity in places where people live, work or play" (Rosenzweig 2003).

Critical to this new vision is a more thorough understanding of how the different components of a biome work together. While large-scale patterns, such as which birds are attracted to which fruit and which flowers are dependent on which beetles, are still being worked out, the small-scale patterns that underlie this biodiversity need to be better documented, in both wildlands and anthropogenic landscapes. This is where chemical ecology comes in. To date, most chemical ecology studies are on single pairs or isolated assortments of taxa. The monarch butterfly incorporates toxins from the milkweed plant to defend itself; a tobacco plant emits a certain type of chemical when attacked by a tobacco budworm, thus attracting wasps that parasitize the budworms. These relationships are important to document. However, what patterns of chemical interactions emerge when whole communities are studied? Are there general principles which seem to govern the development of interactions, such as those proposed by MacArthur, Wilson, and others when explaining patterns of biodiversity distribution? Do these interactions change in response to human development or climate change – even when the species assemblages themselves appear to stay the same? If they do change, what import does that have for the rest of us?

The 15 years after the crash of Theodore Parker and Alwyn Gentry's plane in Ecuador have been mixed for the world of conservation. The fledgling RAP program has continued to thrive, making regular assessments of biodiversity needs from all over the world, and has led to the preservation of several key areas in Bolivia, Peru, and other South American countries. Hundreds of biodiversity preserves and partnerships have been developed globally, and many new species have been identified and studied. However, millions of acres of forest have been lost to an expanding human footprint, hundreds of species have been lost (some of them without ever having been known to science), and the climactic predictions postulated since at least the 1970s have come to pass. More than ever, the world is in need of a new vision of biodiversity. We hope this volume can contribute to this vision.

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Chemical Ecology: Definition and Famous Examples

Emily Wortman-Wunder and Jorge M. Vivanco

Abstract This chapter examines the history of the field of chemical ecology and along the way provides concepts and interesting scientific stories. The chemical ecology examples involve several kingdoms involving positive and negative interactions. The scientific instruments and techniques that have facilitated the advance of this field are portrayed. The information presented in this chapter provides an introduction to the concepts that will be depicted throughout the book.

1 Introduction

In the early 1940s, Adolf Butenandt, a German scientist already famous for his Nobel prize-winning discovery of several human sex hormones (Karlson 1995), began to be interested in the problem of the silkworm female sex attractant chemical. It had been known for years that a single female moth could attract dozens of males simply by choosing a good spot and exposing the glands at the tip of her abdomen. A French naturalist described a typical "invasion" of giant emperor moths after he trapped a newly hatched female moth under a cover of wire gauze. The bat-sized moths filled his study room, his son's bedroom, the kitchen, and every room where a window had been left ajar; activity was most frenetic in the room with the virgin moth: "With a soft *flic-flac* the great night-moths were flying round the wire-gauze cover, alighting, taking flight, returning, mounting to the ceiling, redescending" (Fabre 1920).

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As early as 1882 Lintner, the state entomologist of New York, hypothesized that if it were possible to somehow harness this "irresistible and far-reaching force", an economic way to control problematic moth species could be devised (Lintner 1882). But all attempts to do this had failed: the moths could not be "milked" for whatever substance they were emitting, and baiting a trap with severed female moth abdomens did not work either (Young 1997). Until the sex attractant chemical could be isolated and identified, this idea could not be put into practice.

In the summer of 1953, Butenandt and his colleagues attacked the problem with gusto, buying up every silkworm cocoon they could find. This effort yielded 500 kg of starting material: from this massive haul, they were able to isolate 25 mg of a crystalline hormone responsible for the male molt and only trace amounts of the female sex attractant chemical, far too little to attempt an identification (Karlson 1995). This task took an additional 500,000 female silkworm moth sex glands, extracted in Japan and sent to Germany via airmail (Karlson and Butenandt 1959). Once purified, the elucidation of the chemical's structure took only a year. Butenandt and his colleagues called the substance bombykol in honor of the silkworm moth (*Bombyx mori*). They coined the term pheromone (in place of the confusing term "ectohormone", or "externally acting self-stimulator") to emphasize the momentousness of what they had found: for the first time a chemical produced by one individual was shown to be "designed" to produce a certain effect in another individual.

The identification of bombykol is usually seen as the birth of the discipline of chemical ecology. Several elements came together to make the discovery possible: fluency and research experience in multiple disciplines (in this case biochemistry, physiology, and entomology), some basic naturalist knowledge (Butenandt had been an amateur butterfly collector in his youth), and advances in laboratory techniques that made purification and identification of unknown chemicals possible. From its very beginning, chemical ecology has been dependent on these elements and has developed in tandem with developments in biology, chemistry, genetics, and other fields.

Chemical ecology is generally defined as the study of how various organisms interact with each other and with their environment on a chemical level, and how these interactions determine individual and population dispersal and distribution. Its early decades were spent "cracking the code" of chemical communication between various organisms, from ants to humans, and this aspect of chemical ecology is still alive and well. Recent years have taken advantage of developments in mass spectroscopy (MS), liquid chromatography (LC), gas chromatography (GC), genomics, and molecular technology to examine ever-more-minute interactions. Where Adolf Butenandt in 1959 had to collect 500,000 sex glands to purify enough material to work with, new NMR technologies can use as little as 5 μl of material to determine the chemical structure of a compound (Dossey et al. 2006). Likewise, increasing attention has been directed toward the level "below" traditional chemical ecology, the molecular mechanisms underlying the production and reception of chemicals, and also to the level "above", where chemical exchange influences populations and community.

2 Famous Examples

2.1 Insects

Chemical ecology began with insects, and several watershed chemical ecology studies have concerned insects. This entomology bias has several causes: insects are typically easy to collect and study; their behavioral responses to chemicals are fairly straightforward to demonstrate; many are eye catching and capture the popular imagination; and perhaps most significantly, insects' relationship with plants is essential to the global food supply. The classic studies of chemical ecology have probed sex attractant pheromones in a wide range of insects, the chemical basis of insect defenses, how colonial insects maintain social networks, and how some insects mark routes to food sources. Finally, no discussion of basic chemical ecology is complete without a mention of Reichstein et al.'s demonstration of the Monarch butterfly's ability to harness the toxins of milkweed for its own protection.

Blister Beetles and Insect Defense. People have long been aware of the role of chemicals in insect defense. In sprays, stinks, secretions, and stings, insects have sought to protect themselves from animal predation in a variety of chemical ways. There are too many examples here to count; this volume alone addresses recent advances in several species (see chapters "Defensive Behaviors in Leaf Beetles: From the Unusual to the Weird" and "Microbes: A New Frontier in Tropical Chemical Biology"). Perhaps one of the most typical examples is the aptly named blister beetle family (Order Coleoptera, Family Meloidae). Infamous for centuries for their poisonous properties, this beetle was the subject of pathbreaking work by chemical ecology pioneer Thomas Eisner. In the 1960s and 1970s, Eisner and colleagues identified blister beetles' signature chemical (cantharidin) through a series of chemical and behavioral studies (Carrel and Eisner 1974). Blister beetles reflexively secrete cantharidin when touched; this chemical causes blisters and swelling when applied to human skin and has the happy effect of making spiders and ants drop the beetle and walk away. Cantharidin appears to be synthesized only by males; females receive a cantharidin "booster shot" during mating which then serves to protect them and their eggs for the remainder of their lifespan (Eisner et al. 2005). Furthermore, there exist other insect species of various orders that are attracted to blister beetles solely for the purpose of ingesting cantharidin, which they then use for mating and defense.

Ants and the Olfactory Sense. The role of chemicals in the life of social insects has been another essential area of study for chemical ecology. Ants, in particular, have long been known to communicate information to each other through chemical cues. In 1748, Benjamin Franklin described this experiment to a friend: he tied an earthen pot of "treacle" to the ceiling with a piece of string. A single ant left inside the pot eventually found its way up to the ceiling and from there to its nest; within half an hour scores of ants had followed its trail back to the pot and were eating happily. Thus, he was "inclined to believe that these little insects could by some means communicate their thoughts or desires to each other" (Kalm 1959); scent trails (i.e., chemical trails) were suspected. Chemical ecology has allowed us to break this code and "listen" to the ants. Harvester ants, for example, have 14 different external glands for the secretion of various chemicals, as well as several organs for reception. They use chemicals to communicate information on a wide range of subjects, from mapping food sources to distinguishing nest mates from strangers (Gordon 1999). They have also been widely studied and "ant literature" is a perennially popular and growing subcategory of nature writing.

In his description of one of the most famous examples of ant olfaction, Wilson described how he smeared a live ant with oleic acid, the chemical ants use to determine whether another ant is dead or alive. The ant's nest mates refused to believe it was alive and carried it off, wriggling wildly, to the trash pile; they would keep returning it to the trash pile until the substance had worn off (Wilson 1963). Wilson was so impressed by ants' ability to ignore other cues (such as the live ant's wriggling) that he described ants as being "dominated" by their olfactory sense.

Monarch Butterflies. The landmark tritrophic plant–insect–predator interaction was identified early in the era of chemical ecology. In 1964, a young scientist by the name of Miriam Rothschild, who had been casting about for several months to find a senior scientist to help her study the well-settled but so far unprovable hypothesis that Monarch butterflies were able to incorporate the poisons of the milkweed plant to protect themselves from potential predators, contacted Tadeus Reichenstein, the Swiss scientist who had won the Nobel prize for his work with the hormones of the adrenal cortex (Rothschild 1999). It had been noted for over a hundred years that most birds would not eat Monarch butterflies; those that do typically vomit them up immediately. It was also known that Monarch larvae feed solely on the milkweed plant, which was also known to be poisonous; several observers, therefore, had reached the conclusion that the Monarch butterfly somehow incorporated the toxins of the milkweed plant to protect themselves from predation. Rothschild, Reichenstein, and their colleagues set out to secure proof for this observation by conducting a carefully controlled study. At a field station in Trinidad, their associates reared hundreds of Monarch butterflies from eggs and fed them on a local milkweed, *Asclepias curassavica*. Since Trinidad lacked the facilities to examine them on site, the pupae were preserved and airlifted to laboratories in London and Switzerland. The toxins responsible for their toxicity were determined to be cardiac glycosides, identical to those present in the milkweed; furthermore, these were molecules that butterflies were thought to be incapable of synthesizing themselves (Reichstein et al. 1968). The insects were found to store the toxins throughout their bodies, rather than sequestering them into particular glands, and each adult butterfly contained enough toxin to kill a cat. This chemical ecology example shares several key traits with Butenandt's work: both relied on substantial collections of specimens (1,540 butterflies), and both had to expend considerable effort and expense to overcome the logistical hurdles of bringing nature into the laboratory.

2.2 *Plants*

Plants make chemicals: this is one of the central facets of life on earth. Plants make chemicals and animals respond to these chemicals; chemical ecology has simply made us more aware of the depth and complexity of these interactions. We have always known that plants communicate with animals, particularly insects, using chemicals. However, plants have now been shown to communicate with bacteria, fungi, and other plants using chemicals. Some of this communication is startlingly specific, and much of it appears to have been developed over many years of coevolution. Research into information exchange between plants has benefited greatly from new molecular and genetic techniques and is still very much in progress: while there are some areas of plant chemical ecology that are now considered basic tenets, many of the most fascinating hypotheses, such as interplant alarm systems and allelopathy, have yet to be satisfactorily demonstrated.

2.2.1 Communication in Plants

That plants exchange information, with each other and with organisms in other phyla, is generally accepted. Plants communicate to defend themselves against predators and to facilitate pollination, seed dispersal, and other critical processes. Much of this information is chemical in nature: plants release volatiles and other secondary metabolites from a range of tissues, from intact leaves, roots, and flowers to diseased, crushed, or otherwise damaged tissue. Until recently some or all of this release of volatiles was thought to be passive (a rose flowers, therefore it smells); now most of these processes have been shown to be active and deliberate. For example, a recent study indicated that root exudation, one way plants have of delivering chemical signals to the outside world, is an active process controlled in part by cellular transporters (Loyola-Vargas et al. 2007; Badri et al. 2008).

More surprisingly, plants can be extremely specific in this communication. For example, when attacked by the tobacco budworm (*Heliothis virescens*), tobacco, cotton, and maize plants can emit a particular blend of chemicals that attracts the parasitic wasp *Cardiochiles nigriceps*, which lays its eggs in the budworm larvae. If attacked by the closely related *Heliothis zea*, the plants emit a different chemical cocktail, and the parasitic wasps are not attracted (DeMoraes et al. 1998). Finally, one of the most interesting positive interactions involving plants is the legume–rhizobium interaction.

2.2.2 Plants and Microbes

Legume roots release flavonoids into the soil, which attract rhizobial bacteria; these bacteria then release certain chemicals that cause the legume root hairs to curl and initiate the nodulation process by which the rhizobia help legumes capture nitrogen from the environment. Recent studies have shown that some legumes can expand the range from which they attract bacteria by releasing volatiles that attract larger organisms, such as nematodes; these organisms bring the rhizobium (food for the nematodes) with them in the folds of their skin (Horiuchi et al. 2005).

While demonstrating the importance of chemical signals to plant processes has been straightforward in some cases, in other cases research into chemical communication has raised more questions than it has answered. These are typically situations in which it is not sufficient to simply identify a chemical produced by a plant and its effect on another plant, but the actual operation of this interaction in nature must be demonstrated, as well as a subsequent ecological or evolutionary consequence. Furthermore, many of the plant processes occur in locations that are hard to analyze under natural conditions, such as the soil. While the gas chromatograph has been a wondrous instrument for the examination of airborne plant emissions in the field, this instrument has not had much success in demonstrating what is happening underground. Nor has it been straightforward to prove that interactions that may happen in the laboratory actually occur in any significant way in nature. Thus, many of the plant communication hypotheses discussed below are still controversial.

2.2.3 Allelopathy: Negative Plant Talk

While the basic idea of negative allelopathy – that some plants compete against other plants by poisoning the soil – has been around for millennia, the road to conclusive proof of this phenomenon has been a rocky one. The third-century Roman author Theophrastus described a well-known agricultural conundrum: some plants, particularly walnut trees, somehow poisoned the soil in which they were grown, keeping away competing seedlings but also making it impossible to plant new plants in an area where walnuts had been grown. While the idea was extensively explored in relation to crops, no more significant work on allelopathy in nature was done until Cornelius Muller hypothesized in 1964 that the spacing of purple sage shrubs (*Salvia leucophylla*) in the California chaparral was due to toxic leachates (Muller et al. 1964). When 10 years later one of his graduate students demonstrated that most of the purple sage spacing was due to rabbit herbivory and other factors (Halligan 1976), the science of allelopathy appeared to have been nipped in the bud. Numerous scientists have applied themselves to the problem, and they keep hitting up against the same obstacles: they can prove that plants produce toxins in nature or that these toxins are effective against likely neighbor plants or that these toxins are present in the environment, but not all three in combination.

2.2.4 A Different Plant Story: The Plant as Paul Revere

It is likely that plants can communicate in a competitive, hostile way, but can plants communicate positively with each other, sending out alarm signals that enable fellow plants to ready their own defenses against a particular predator? The idea has been around since the early 1980s, when University of Washington's David F. Rhoades presented a paper at an American Chemical Society conference suggesting that willow trees under tent caterpillar attack could induce the production of anti-insect compounds in neighboring trees. Researchers criticized this study, and a similar one conducted by a team at Dartmouth College, for lacking sufficient controls (Chen 1990; Wilkinson 2001); however, many researchers have shown that plants can communicate via airborne volatiles in a controlled setting (Arimura et al. 2000; Kessler and Baldwin 2001, 2002; Horiuchi et al. 2007). Both ethylene and methyl jasmonate have been shown to influence neighboring plants of different species under laboratory conditions; furthermore, increasing evidence shows that plants can positively influence neighboring plants through root secretions (Prithiviraj et al. 2007). In the end, the obstacles to this sort of research have to do with the nature of the study subject as much as with the technology used to study it. It is easy to show that an insect is influenced by a chemical: expose it to the substance, and record its behavior. Plant behavior is much harder to record. For several decades, researchers tried to demonstrate response as a change in the chemicals produced within or secreted by leaves or roots; this was found to be problematic on many fronts. A new method is to show a response on the level of gene expression: this is a promising new technology, but one that still carries the weight of controversy. Nevertheless, many prominent researchers are beginning to believe that interspecies plant communication, both positive and negative, occurs in plants.

2.2.5 Plant Neurobiology: A Contradiction in Terms or a Vision of the Future?

A recent article in the *New York Times* (Yoon 2008) highlighted a controversial new development in plant biology: the idea that plants can "behave" in much the same way as primitive animals. The science of plant neurobiology (as defined by the founders of the Society of Plant Neurobiology) is based on several articles published in well-respected journals over the past decade or so. Although these articles have been peer reviewed, they nearly always appear to be accompanied by a storm of controversy, from Dudley and File's description of *Cakile edentata*'s ability to recognize genetically similar plants (Dudley and File 2007, 2008; Klemens 2008). Much of the problem stems from the language plant neurobiologists choose to employ, from that loaded word "neurobiology" in organisms that so far have not been demonstrated to actually possess nerve cells to the idea of classifying the release and absorption of certain chemicals as "behavior" and "communication". Acceptance of these terms is growing, however, and many defenders of plant neurobiology like to point out that a century ago the idea that plants had "physiology" was equally ridiculed – and now most major universities have a department exclusively devoted to its study. Similarly, it is likely that many of the outlier ideas of plant chemical ecology, from interplant alarm systems to allelopathy, will one day be accepted and understood.

2.3 Vertebrates

Chemical ecology exists in vertebrates as well, in taxa from fish to primates. Scent is well suited to territorial vertebrates needing to communicate information over relatively large distances in space and time, as well as to solitary animals needing to reconnoiter at key moments to reproduce the species. Fish use pheromones to find mates, maintain hierarchical social structures, and distinguish friend from foe; amphibians are infamous for their noxious secretions, and wide-ranging birds such as the albatross and various scavenger species use scent to hone in on food. There are even examples of defensive chemicals in bird species from areas as far flung as the Philippines and the Peruvian Amazon. Virtually all mammals use scent in some way, from defense (skunks) to territorial marking (the canids), to mate attraction. Even the relatively scent-illiterate *Homo sapiens* has been shown to choose mates and regulate ovulation via pheromones, at least in some settings (McClintock 1971; Hays 2003).

While most vertebrate chemical ecology involves airborne chemicals, one of the most famous examples involves chemicals communicated by contact: the poison dart frogs of South America. Like Monarch butterflies, these frogs are aposematic (they present vivid coloration meant to warn predators of their toxicity); also like Monarch butterflies, they incorporate their toxins from the food they eat, a fact exploited by people who raise the strikingly patterned frogs at home. As early as the mid-nineteenth century, explorers reported how the Emberá Indians of Colombia’s Pacific coast milked poison from certain frogs to coat the tips of their blowgun darts. Although the Emberá had ceased to exploit this technology by the 1960s (Tayler 1996), young chemical ecologists eager to combine adventure with research set off in search of the chemical keys to this interaction. John Daly of National Institutes of Health describes how he embarked in 1963 on “what was considered dangerous field work” to collect specimens of *Dendrobatidae* frogs (Daly 1995). He and his colleagues soon found that if these frogs were raised in captivity they did not possess the toxic alkaloids for which they were famous; he later discovered that these toxins were sequestered from prey, including ants and mites (Daly 1995). In his review of his life’s work, Daly reiterates the importance of technology: technical advances in the 1990s made possible chemical revelations related to Dendrobatidae life history that would have been impossible 15 years before (Daly 1998).

2.4 Microbes

While many of the interactive chemicals produced by microbes have been known and exploited for years, microbes represent the final frontier of chemical ecology. Only in the last decade have chemistry methods become sophisticated enough to measure the chemicals emitted by fungi and bacteria; likewise, the wide-ranging impacts of these chemicals is just beginning to be understood. While we have known or intuited that chemicals play an important role in the interaction of bacteria or fungi and their potential predators (toxins produced by botulism or the deadly amanita mushroom spring to mind), only recently have we begun to realize the complexity and sophistication of microbial communication.

For instance, many bacterial species use a process known as quorum sensing to coordinate various aspects of their behavior, including gene expression and colonization of a particular surface. Quorum sensing, in which bacterial cells release a chemical compound that effects specific changes once a particular concentration is reached, allows some bacteria to aggregate and to respond to various stimuli as a coordinated group.

Other examples include chemicals produced and secreted by pathogenic bacteria to dampen the defense responses of their potential hosts and to facilitate infection; these chemicals are known as virulence factors and are present in plant, human, and animal pathogenic bacteria and fungi. Beneficial fungi and bacteria have also evolved close relationships with their hosts; for example, the microbial flora of humans tends to be quite specific, varying from individual to individual (Eckburg et al. 2005). Similarly, the roots of plants have adapted to their specific soil microbes through years of coevolution; this relationship is initiated and maintained through the release of chemicals by roots. Interestingly, when a plant is moved to a new soil environment, the plant tends to eliminate the soil microbes present in their immediate rhizosphere, possibly due to the lack of specific signals or carbon sources needed by the microbes present in those new environments or by the production of antimicrobial toxins to which the microbes are not familiar (Broeckling et al. 2008).

3 The Machines that Make Chemical Ecology Possible

The story of chemical ecology is largely the story of technology (Meinwald and Eisner 2008; Takken and Dicke 2006). Many of the greatest stories in the discipline – the sex attractant hormones, the unique relationship between tobacco and parasitic wasps, the interrelationship between Monarch butterflies and milkweed – are the studies that proved what naturalists had guessed for years. A quick perusal of the early chemical ecology studies demonstrates how eager scientists were able to break through the final barriers preventing them from examining the chemical relationships between organisms; they also reveal how arduous those barriers were, from Adolf Butenandt’s team assembling a viable study sample by importing half a million silkworm moth pupae from Japan to Tadeus Reichstein’s dedicated colleagues, hauling several thousand butterfly pupae from Trinidad to the only labs that could process the information they needed. Thus, no understanding of the field is complete without a survey of the key pieces of equipment and methodologies that got it where it is today.

Gas Chromatograph. A basic instrument of analytical chemistry used to separate and roughly identify different volatile chemicals in a complex sample. This instrument first started to be used for entomology (and hence chemical ecology) in the early 1960s and was at that time so primitive that most scientists preferred other methods (Rothschild 1999). Now it is a standard piece of chemical ecology equipment. New versions of the gas chromatograph are smaller and more sensitive, so that they may be taken into the field and a plant's or animal's chemical emissions can be measured in situ. The gas chromatograph is usually used in combination with the mass spectrometer.

Mass Spectrometer. Another basic instrument of analytical chemistry used to determine the mass (and hence the identity) of unknown compounds. It ionizes sample compounds and then projects the particles at a magnetic plate. The place at which the particles bounce off the plate indicates their mass; this information is compared against libraries of chemical compounds to determine a possible match. This instrument is typically used in combination with other instruments, such as the gas chromatograph or various liquid chromatographs.

High Performance Liquid Chromatographer (*HPLC*). In recent decades, this has been the standby in chemical ecology, as it is relatively affordable and easy to use. It can separate, identify, and quantify compounds that could have various levels of solubility in a liquid solution.

Nuclear Magnetic Resonance (*NMR*). This technology exploits the discovery, in 1938, that different atoms within a molecule resonate at different frequencies and is used to determine the structure of molecules. This instrument is used to determine the structure of new compounds or to confirm a possible structure obtained by mass spectroscopy.

Molecular Genetics/Genomics. This branch of study attempts to harness the rapidly increasing body of knowledge about genes and put it to work to discover how various organisms respond to stimuli at a whole genome level. These studies are revolutionizing how we think about genes.

cDNA Libraries. Not all organisms' genomes can be sequenced. In cases where the species' genome is not available for comparison, the RNA produced in a plant in response to a particular treatment is extracted and converted to DNA in the laboratory. This process is called creating a c (complementary) DNA library. These libraries can then be sequenced to identify the specific genes produced or expressed following a treatment to determine the biological activity of certain proteins.

Model Organisms. Model organisms are those for which whole genome information is known and for which other tools exist such as mutant lines in particular genes, "knockout" lines (where a particular gene has been selectively removed), or lines with the ability to be transformed. The main model organisms are *Arabidopsis thaliana* (plant), *Caenorhabditis elegans* (nematode), and mice, although new plants and animals are being added to this list.

Increasingly, researchers are trying to develop instruments that help bridge the gap between the laboratory and the field – taking advantage of the laboratory's ability to control variables while producing research that reflects the actual world and gives us information that is meaningful to nature. One important recent development in the field of chemical ecology is the portable gas chromatograph, which allows scientists to measure organisms' chemical emissions in the field. Furthermore, increasingly scientists are going to extra lengths to bring together the expertise of divergent fields. One such example is the workshop that produced this volume, the 2008 Pan-American Advanced Studies Institute, “Interdisciplinary Studies in Chemical Biology”. This workshop brought together chemists, geneticists, ecologists, entomologists, and others in a remote location to exchange ideas, swap techniques, and explore the rainforest, a place likely to have a myriad of chemical ecological interactions.

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Chemical Defenses of Insects: A Rich Resource for Chemical Biology in the Tropics

Aaron T. Dossey

Abstract Insects make up the largest and most diverse group of organisms on earth, contributing to as much as 80–90% of the world's biodiversity. Approximately 950,000 species of insects have been described; some estimate there are 4,000,000+ species in total. Over 70% of drugs on the market are derived from natural compounds. However, insects are one of the least explored groups in drug discovery. The world adds about 70 million people each year. In this chapter you will find: (1) an introduction to the topic of arthropod chemical biodiversity and chemical defense; (2) a brief discussion on various uses of insect chemistry by various cultures; (3) an overview of insect venoms and other chemical defense studies, with a case study on methods utilized to analyze ant venoms; (4) a short discussion on the importance of preserving tropical habitats for bioprospecting; (5) a review of research on stick insect (Order Phasmatodea) chemical defenses, stick insects as a model for biosynthesis studies and my personal experiences with the editors of this book and 2008 PASI workshop in Peru which resulted in this chapter; (6) an overview of examples from the literature of insect-derived substances with medicinally relevant biological properties such as toxins and antibiotics; (7) a brief description of the importance of studying biosynthetic pathways in insects and other organisms from whence valuable natural products are identified and (8) a list of recommended literature which I expect would be of particular interest to the readers of this chapter.

1 Introduction

Up the trunk of a giant Brazil nut tree crawled an intriguing and majestic creature: a walkingstick insect. As it made its way across, another fellow citizen of the jungle approaches – a lizard. This is not good news for the insect, as most lizards will make

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a meal or light snack of an insect such as this without batting an eye. The lizard approaches stealthily and attacks with the utmost precision. With the insect nearly within the munching distance of the lizard's mouth, suddenly the mission is aborted! In an instant the lizard retreats as if it had encountered some sort of invisible force field. Capitalizing on this opportunity the insect makes its escape: quickly falls to the ground, spreads its bright yellow wings, and takes flight into the deeply shaded darkness of the Peruvian rainforest of the Tambopata National Reserve.

Stories like this are played out many times every day in the wild (Fig. 1). This masterful escape by the walkingstick insect is just one of thousands of examples in the insect world. The lizard was an unfortunate victim of that insect's most potent defense mechanism, its chemical weaponry. Many species of walkingsticks, as well as hundreds of thousands of other insect species (beetles, true bugs, ants, etc.), produce defensive sprays, secretions, or venoms that help protect them from predation.

Fig. 1 *Anisomorpha buprestoides* deploying its chemical spray and repelling a brown Cuban Anole (*Anolis sagrei sagrei*). (**a**) Black and white Ocala National Forest color form of *A. buprestoides* mating pair (females are larger than males) deploying its chemical defense spray. (**b**) Same type of insect as in (**a**) successfully repelling a brown Cuban Anole (*Anolis sagrei sagrei*), a non-native invasive species in Florida. Photograph by Rod C. Clarke and Adam Scott, British Broadcasting Corporation (BBC) Natural History Unit. Still image taken from high-speed video for the future natural history series "Life" (Fall 2009)

2 Background

It is impossible to cover every published example of insect chemical defenses in a single chapter, or even in a single book. However, I will provide some classic and intriguing examples of insects and the chemical compounds they produce to protect themselves from attack. To provide a sense of the vastness of the chemical biodiversity which exists among insect defense mechanisms, one can look at Pherobase (http://www.pherobase.net), a resource aimed at cataloging insect semiochemicals. Semiochemicals are chemical substances used by organisms for communication (pheromones, kairomones, etc.) or defense, which is arguably a form of communication. This database consists of species, taxonomic designations, literature references, and most importantly chemical structures linked to the species which produce them and their function. In Pherobase alone, there are a total of 623 chemical substances identified as "defense substances" (as of July 22, 2008). This number includes other invertebrate taxa, but the vast majority of compounds represented in this database come from insects. Even as large and impressive a database as Pherobase fails to encompass the entire diversity of reported chemical defense substances from insects.

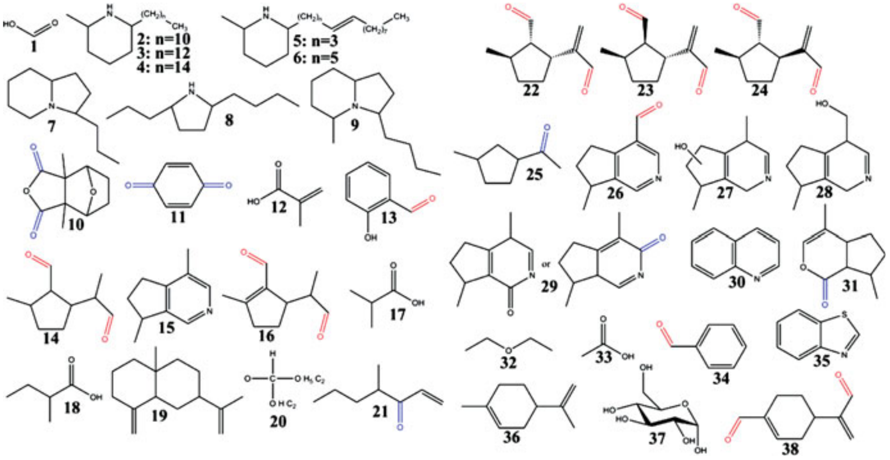
Insects make up 80–90% of the total biodiversity on earth (Hellmann and Sanders 2007), and there are approximately 950,000 described species of insects (Berenbaum and Eisner 2008). It is estimated that 4,000,000 insect species exist on earth (May 2000), but the vast majority is yet to be discovered or described. In addition to their abundance and diversity, insects are of critical importance to mankind. They perform such vital functions as decomposition of dead plant matter and animal waste, they add nutrients to the soil, keep the populations of plants and other animals in check, pollinate at least 177 crop species worldwide, and provide food for many other animals and humans (Hellmann and Sanders 2007). Beyond these examples, there is a vast amount of scientific and technological discovery yet to be explored in insects. In fact little is known about the biosynthetic mechanisms or greater ecological significance of the vast majority of insect defensive chemicals and there remains a vast wealth of chemistry, biochemistry, and chemical ecology yet to be explored. Thus, it is imperative that we preserve and protect the natural environment upon which we depend for our existence, especially the insects with which we share it.

In this chapter, I will separate arthropod chemical defenses into two groups (1) *venoms*, which will be defined as chemicals injected directly into the body tissue of the recipient and (2) *sprays and secretions*, which includes substances sprayed, secreted, oozed, bled, or otherwise exuded from their respective arthropod sources – which encompasses nearly all nonvenom chemical defense strategies utilized by arthropods. In chapter "Defensive Behaviors in Leaf Beetles: From the Unusual to the Weird", another fascinating defensive strategy, fecal shields, used by insects of a particular family of beetles, the Chrysomelidae (leaf beetles), will be described. It is as yet uncertain, however, if chemistry plays a significant role in that particular case of insect self-defense.

3 Chemical Weapons in Insects: History and Examples

3.1 Ethnoentomology

A large portion of animals on earth, particularly insects, utilize chemicals as their primary tools of warfare and defense (Scheme 1). Their study has been the topic of many publications over the past few decades (Blum 1981; Eisner 2003; Eisner et al.



Scheme 1 Molecular structures for chemical compound used by various insect species for defense. *Lines* indicate chemical bonds, ends of lines and intersections are carbon atoms unless otherwise noted by "O" (oxygen atom), "H" (hydrogen atom), or "N" (nitrogen atom). Characteristic ketone moieties are *blue* and aldehyde groups are *red*. (**1**) Formic acid, (**2**) solenopsin A, (**3**) solenopsin B, (**4**) solenopsin C, (**5**) dehydrosolenopsin B, (**6**) dehydrosolenopsin C [**2**–**6** are from venom of the ant *Solenopsis invicta* (Lofgren et al. 1975; MacConnell et al. 1971)], (**7**) 3-propylindolizidine, (**8**) 2-butyl-5-propylpyrrolidine, and (**9**) 3-butyl-5-methylindolizidine (**7**–**9** are from the ant *Myrmicaria melanogaster* (Jones et al. 2007), (**10**) cantharidin from blister beetles (Family Meloidae), (**11**) benzoquinone from darkling beetles (Order Coleoptera, Family Tenebrionidae), millipedes (Class Diplopoda), and various other arthropods, (**12**) methacrylic acid, (**13**) salicylaldehyde (compounds **12**–**13** from beetles in the genus *Calosoma* (Family Carabidae) (McCullough 1969), (**14**) iridodial from various insect species and some plants, (**15**) actinidine from various insects and some plants, (**16**) chrysomelidial from the larvae of leaf beetles (Family Chrysomelidae) (Meinwald et al. 1977), (**17**) isobutyric acid, (**18**) 2-methylbutyric acid, and (**19**) selinene (**17**–**19** from butterfly caterpillars in the Family Papilionidae), (**20**) compound hypothesized from *Agathemera crassa* in 1934 (Schneider 1934), (**21**) 4-methyl-1-hepten-3-one produced by *Agathemera elegans* (Schmeda-Hirschmann 2006), (**22**) dolichodial, (**23**) anisomorphal, (**24**) peruphasmal [compounds **22**–**24** produced by *Anisomorpha buprestoides* and *Peruphasma schultei* (Dossey et al. 2006, 2008)], (**25**) 1-acetyl-3-methylcyclopentane from *Megacrania* sp., (**26**–**29**) analogs of actinidine (compound **17**) produced by *Megacrania* sp. (Ho and Chow 1993), (**30**) quinoline produced by *Oreophoetes peruana* (Eisner et al. 1997), (**31**) nepetalactone, produced by the walkingstick species *Graeffea crouani* (along with iridodial, Scheme 1, Compound **14**) (Smith et al. 1979) and other insects and plants, (**32**) diethyl ether, (**33**) acetic acid, (**34**) benzaldehyde, (**35**) benzothiazole, (**36**) limonene (compounds **32**–**36** from *Sipyloidea sipylus* (Bouchard et al. 1997), (**37**) glucose, and (**38**) parectadial from *Parectatosoma mocquerysi* (Dossey et al. 2007)

2005), aided tremendously by modern technologies in analytical chemistry (Dossey et al. 2006; Brey et al. 2006). However, various properties of insect chemical defenses have been known to mankind for much of our history.

In one case, the Satere-Mawe people from the Amazonian basin of Brazil have used bullet ants (*Paraponera clavata*) in rite of passage rituals for manhood and social status (Haddad et al. 2005; Bailey et al. 2007; Tremaine 2004). In this gruesome ritual, a hundred or so of these bullet ants are first mixed into an herbal brew until they are fully anesthetized. Next, the ants are woven into a glove made of palm leaves with the stingers on their abdomens pointing toward the inside. This glove is then placed inside a more ornate ceremonial glove. Young men in the tribe must place their hands into the glove for a full 10 min and do this a total of 20 times in order to be respected as men by their elders and to be eligible to hold leadership positions. The excruciating pain from just one of these stings can last several hours.

Another example of ancient use of insect defense chemicals by man is the defensive secretion from blister beetles (Family Meloidae). Cantharidin (Scheme 1, compound **10**), the active substance in blister beetle defensive secretions, causes irritation of the urinary tract that gives a false impression of sexual stimulation (Sandroni 2001). This substance was used by both the ancient Chinese and Greeks for a wide variety of medicinal purposes, from removing warts to enhancing sexual libido (Moed et al. 2001). Examples of its use by European royalty include Livia Caesar of Rome (wife of Augustus Ceasar) (James and Thorpe 1994) and King Henry IV of England. However, the use of this chemical, typically referred to as “Spanish Fly”, is illegal in the USA except for the prescribed treatment of warts or for use in animal husbandry (Gottlieb 1993).

The famous poison dart frog toxins used by natives of South America are also insect-derived chemicals that are passed to the frogs through diet. Poison dart frogs are used by South American natives to create poison darts and spears used for hunting. These frogs, when kept in captivity, tend to lose their toxicity over time (Daly et al. 2005). In nature, frogs largely feed on arthropods and it has been shown in recent years that their alkaloid toxins are largely sequestered from arthropods, particularly insects and millipedes, that they ingest (Clark et al. 2005; Daly et al. 2005). Since many insect chemical weapons are intended for use against vertebrate assailants, it is also clear that insect chemical defenses represent a large reservoir of potentially medically relevant substances. Indeed some insect defensive substances have been studied for their potential use as medicines.

3.2 Venoms

Probably the best known examples of arthropod chemical defenses are the venoms used in the bites and stings of such creatures as spiders (Class Arachnida), scorpions (Class Arachnida), ants, bees, and wasps (Class Insecta, Order Hymenoptera). The chemical weapon payloads of these creatures are directly injected into the victim. Many of these venoms contain proteins and peptides as their active components.

The most famous example of proteinaceous insect venom is mellitin found in the sting of the European honeybee (Api's mellifera). The venom of the previously mentioned bullet ants contains a neurotoxic protein called poneratoxin refs.

Ants are also known to use a variety of small molecules such as formic acid (Scheme 1, compound **1**) and alkaloids to defend themselves. Some examples of these compounds are given in Scheme 1. One of the better known alkaloid venoms in the USA comes from the tropical red imported fire ant, *Solenopsis invicta*, and other ants in this genus. This invasive species (not native to the USA) was introduced into the USA (Mobile, AL, or Pensacola, FL) from cargo ships coming from Brazil between 1933 and 1945 and has spread into at least 18 states since (Collins and Scheffrahn 2001–2008). The venoms of these ants are mostly made of piperidine alkaloid compounds called solenopsins (Scheme 1, compounds **2–6**) (Lofgren et al. 1975; MacConnell et al. 1970, 1971). Later in this chapter, I will use this example to give a basic explanation of some of the tools and methods used by chemists to study such natural substances as venom chemicals.

Fire ants characteristically attack in numbers by congregating onto a victim, possibly an unfortunate person standing in a fire ant mound (Fig. 2), and, in response to an alarm pheromone they produce, will all basically sting at the same time. To inject their venom, they bite the victim with their mandibles (jaws) and then pierce his or her skin with the stinger found at the end of their abdomen (back end). The solinopsins in their venom cause a small area of necrosis (cell dying) about 1–2 mm in diameter over a few days. In many cases, swelling and itching will also occur, but this response is actually due to proteins found in the venom (Tankersley 2008).

Besides the solenopsins from fire ants, other species of ants make a variety of alkaloids. A good and recently published example of the variety of alkaloids produced by ants comes from the ant species *Myrmicaria melanogaster* of Brunei (Jones et al. 2007). This single species of ant was found to contain 14 different alkaloids – three generic structures of several of those shown in Scheme 1 (compounds **7–9**). In addition to alkaloids, ants have been shown to use other organic compounds as chemical defense such as iridodial (Scheme 1, compound **14**), dolichodial (Scheme 1, compound **22**), and actinidine (Scheme 1, compound **15**), among others.

3.2.1 Studying Venoms

The venom of fire ants is a classic and well-studied example of insect chemical defense. Thus, they make a very useful model with which to explain some of the tools and methods used in insect chemical ecology. To start any analysis of venom, it is necessary to have or develop an efficient and robust method of collecting the desired chemical components. Such a method should be performed as to be sure that the substances analyzed are indeed from the venom glands of the organism and not from some other part of the body. Often ant venom compounds are analyzed from samples of ants simply rinsed in methanol. While this is a very efficient method, one cannot be sure that the compounds identified are all venom components. Other

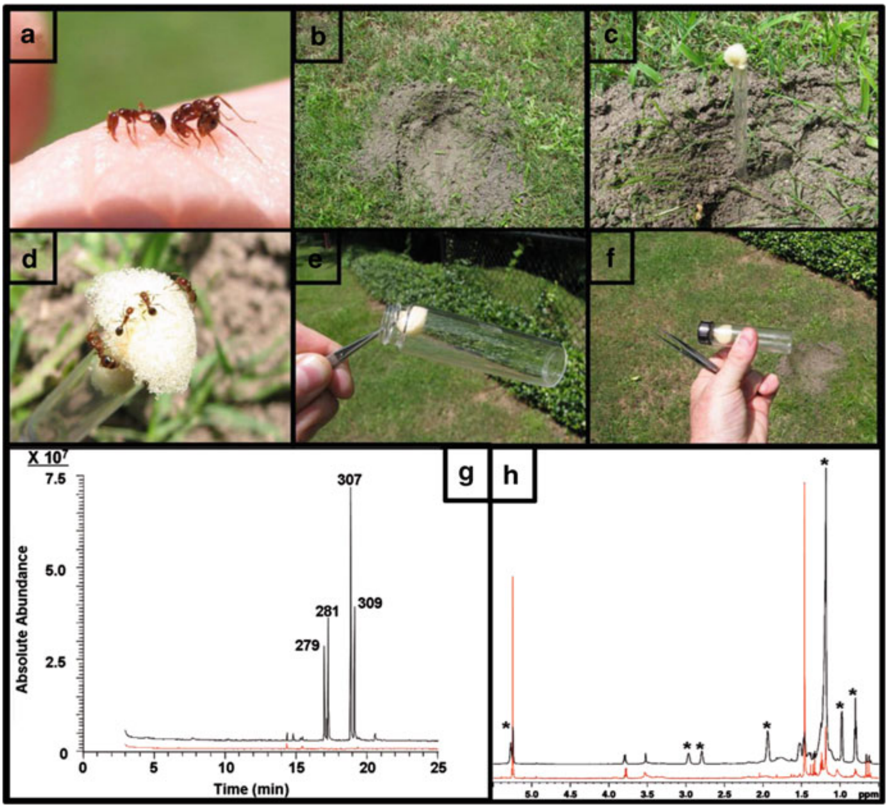


Fig. 2 Stinging behavior of and venom collection from the red imported fire ant: *Solenopsis invicta*. (**a**) Major and minor workers stinging Dr. Aaron T. Dossey's hand in their typical stinging posture, (**b**) ant mound with a footprint to disturb the ants, (**c**) closeup of glass pipette stuck in mound in (**b**), (**d**) ants stinging foam in glass pipette, (**e**) foam being placed into glass vial with clean forceps for venom extraction, and (**f**) glass vial and forceps in (**e**) with vial shown capped with a Teflon® coated cap, (**g**) one-dimensional ^{1}H NMR spectrum of venom alkaloids collected from *S. invicta*. The foam is extracted with CH_2Cl_2, dried by blowing nitrogen gas over it, then re-dissolved in about 15 μL of deuterated methylene chloride (CD_2Cl_2) for NMR. The black spectrum is the venom alkaloid extract and the red spectrum is a blank. *Asterisks* show the peaks which correspond to those previously reported for *S. invicta* venom alkaloids (MacConnell et al., 1970), (**h**) gas chromatograph of *S. invicta* venom alkaloids. The *black trace* is the venom alkaloid extract and the *red trace* is a blank. Four peaks are noted with the masses of the compounds they represent. These masses, qualitative relative retention times, and mass spectra (data not shown) correspond to those of published data for known *S. invicta* venom alkaloids (MacConnell et al., 1971). Photographs by Dr. Aaron T. Dossey

researchers have developed a variety of ways to isolate ant venom by (1) electrocuting the ants so that they eject their venom components onto some inert surface, (2) dissecting the venom glands and removing the contents with a glass capillary, or even getting the ants to sting an inert porous material for further extraction (Piek 1986). To do this is rather simple, but this requires a sufficiently aggressive ant species and a good number of ants. Venoms are certainly a diverse and rich source of chemical substances with very clear hypothesis-generating biological activities. However, it is often challenging to obtain sufficient quantities for analysis.

Once a crude natural substance is collected, the chemicals of interest must be separated and subjected to various analytical chemistry techniques to determine what the substance is made of. Various methods of extraction and types of chromatography are used to separate the different substances. The chemist will then use a variety of spectroscopic and spectrometric techniques (such as NMR and mass spectrometry), to determine the type, connectivity, and arrangement of atoms in those molecules – these being important aspects of their molecular structure.

Nuclear magnetic resonance (NMR) utilizes giant superconducting magnets with magnetic fields typically 200,000–400,000 times that of the earth to study the molecular structure of chemical compounds. NMR uses some convenient properties of atoms to achieve this (1) in the presence of a magnetic field, atomic nuclei precess (like a toy top tilting as it spins) at frequencies depending on what element it is (hydrogen, carbon, etc.) and (2) the rate (or frequency) of this precession is affected by the amount of electrons around those atoms. In NMR, radio frequencies are sent into a chemical sample placed inside the large magnetic field. The atoms in the sample will then give off a characteristic set of radio frequencies of their own. These frequencies and how those nuclei interact with one another give the chemist much important information about the molecular structure of the chemicals in the sample.

Mass spectrometry (mass spec) is another technique often essential to determining the identity and molecular structures of chemicals in a sample. Mass spec uses chemistry or high-energy beams to break molecules into parts. The mass of these parts can be measured based on the time it takes them to fly through a vacuum chamber. The masses of the fragments, like pieces of a puzzle, can be used to reconstruct the original arrangement of atoms in the molecules. Probably the best known use of mass spec, Gas Chomatography coupled mass spectrometry, on GC-MS, is sometimes even used by police detectives in forensic analysis of crime scene evidence. Mass spec databases are often used to make very rapid identifications of commonly known chemical substances – but study of unknown substances usually takes much longer.

As described earlier, there are multiple methods one can use to obtain ant venom. I have personally put the “ant stinging porous material” method to the test (Fig. 2). First, I mounted three precleaned pieces of foam culture flask stopper on the top of clean glass Pasteur pipettes with forceps by inserting about 1/3 of each foam piece into the large end of the pipette. Then, I located a red imported fire ant (*Solenopsis* invicta) mound, stepped on it to agitate the ants, and stuck the three pipettes into the area where the emerging ants were in highest density. After 10 min, I removed the foam pieces with forceps and flicked the remaining ants off the material. One of the first indications that venom alkaloids were present on the foam piece was that, when rubbed onto pH indicator paper, the paper would turn blue, which indicates presence of an alkaline (basic) substance. The foam was extracted with methylene chloride (CH_2Cl_2) and the resulting residue was analyzed by NMR and mass spec. Indeed NMR and mass spec signals corresponding to previously published values for fire ant venom alkaloids were observed (MacConnell et al. 1970, 1971) – along with few other minor signals (Fig. 2). Thus, the method was a success!

3.3 Sprays and Secretions

As stated earlier, the vast majority of animals on earth are insects. Beetles alone make up the group (Order Coleoptera) with the largest number of described species of any other group of animals on earth. There are approximately 400,000 named species so far, comprising about 25% of all named species of organisms (Hammond 1992). The plethora of chemical compounds they produce for defense is similarly as vast and diverse (Blum 1981; Dettner 1987; Eisner 2003; Eisner et al. 2005). Aldehydes and ketones are common functional groups used by insects for chemical defense (Scheme 1). Aldehydes are a portion of a molecule consisting of a carbon atom double bonded to an oxygen atom and single bonded to a hydrogen atom and to another carbon atom (aldehydes are highlighted in red in Scheme 1 and Fig. 3). Aldehyde containing compounds tend to cause a burning sensation. Ketones are portions of a molecule consisting of a carbon atom double bonded to an oxygen atom and single bonded to two other carbon atoms (ketones are highlighted in blue in Scheme 1 and Fig. 3). Carbon atoms almost always have exactly four chemical bonds attached to them. Beetles, as well as many other insects and even plants, also tend to produce monoterpenes as active components of their chemical weapons. For example, chrysomelidial (Scheme 1, compound **16**) produced by leaf beetles (Family Chrysomelidae) is an isomer of anisomorphal (Scheme 1, compound **23**) – with the only difference being placement of the double bond (Meinwald et al. 1977). Dolichodial, anisomorphal, and peruphasmal (Scheme 1, compounds **22**, **23**, and **24**, respectively) are diastereomers (compounds with the same chemical formula, but different configurations at one or more of the functional groups, resulting in compounds that are not mirror images) and are the active components of several walkingstick insect defensive sprays (Dossey et al. 2006, 2008; Eisner 1965; Meinwald et al. 1962), as well as some ants (Cavill and Hinterberger 1961; Cavill et al. 1976; Cavill and Whitfield 1964; Pagnoni et al. 1976). Probably the best-known beetle defensive substance is cantharidin (Scheme 1, compound **10**), a compound from the blister beetles (Family Meloidae) that has a long history of medicinal use as discussed previously. Blister beetles have a very interesting way of deploying this chemical weapon. They can spontaneously bleed through the joints in their legs a blood concoction enriched in cantharidin (Eisner 2003; Eisner et al. 2005) (Fig. 3a). In some types of beetles, such as "fire-colored beetles" (Family Neophyrochroa), cantharidin is actually passed from males to females during mating (Eisner et al. 2005).

One of the most famous chemical defense mechanisms used by a beetle is that of the Bombardier beetle, characterized by the work of Eisner and Meinwald (Eisner 2003; Eisner et al. 2005) (Fig. 3b). The rather spectacular spray given off

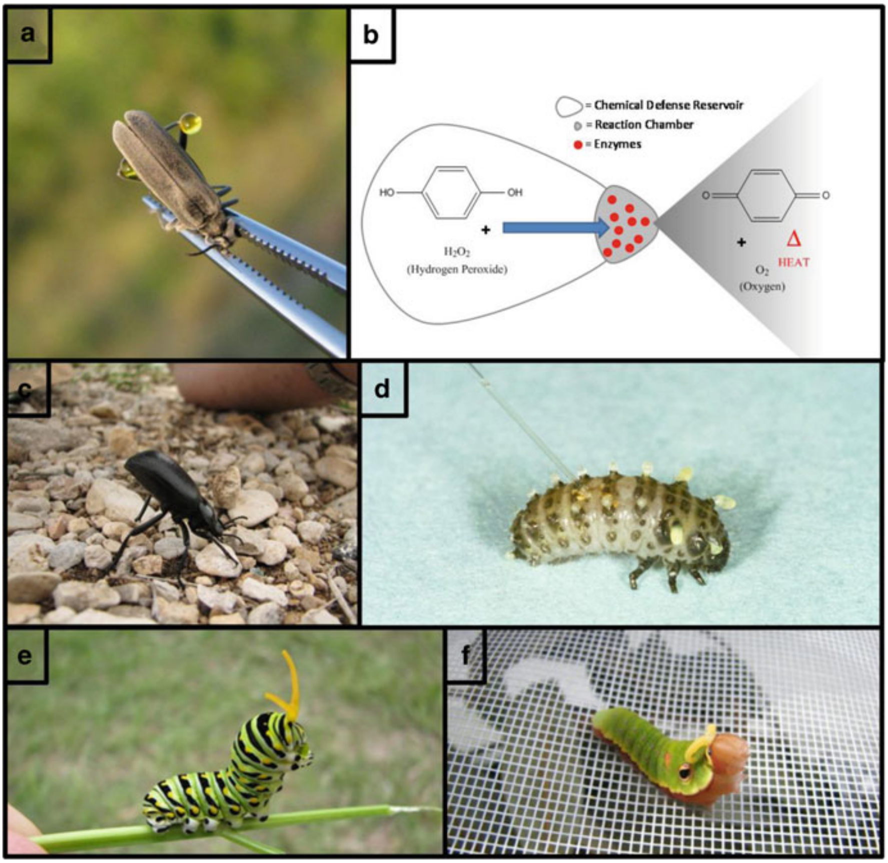


Fig. 3 Insects and their chemical defenses. (**a**) A blister beetle (*Epicauda* sp., Family Meloidae) deploying its typical defensive bleeding of cantharidin (Scheme 1, Compound **10**), (**b**) Bombardier beetle chemical defense mechanism (*Brachinus* sp., Family Carabidae, Order Coleoptera). (Figure adapted from diagrams in (Eisner, 2003) and (Eisner et al., 2005). This apparatus is in the posterior (rear) end of the beetle. In this diagram the rear end of the beetle is to the right, (**c**) darkling beetle (Genus *Eleodes*, Family Tenebrionidae) in its typical defensive posture – Carlsbad Caverns, New Mexico, USA, August 15, 2006, (**d**) Larva of leaf beetle species (*Phaedon cochleariae*, Family Chrysomelidae, Order Coleoptera) shown with drops of defensive secretion on protruded glands after being agitated. Glass capillary is being used to milk the secretion, (**e–f**) swallowtail butterfly caterpillars shown with chemical defense glands (osmeteria) extruded after having been agitated: (**e**) Eastern Black Swallowtail (*Papilio polyxenes*) and (**f**) Spicebush Swallowtail (*Papilio troilus*). Photographs in (**a**), (**b**), (**e**), and (**f**) by Dr. Aaron T. Dossey. Photograph in (**c**) by Nhu Nguyen. Photograph in (**d**) by Dr. Michael Hoscovec. Caterpillars are from Shady Oak Butterfly Farm in Brooker, FL, USA

by bombardier beetles when attacked is the result of a violent chemical reaction, which occurs upon the mixture of reactants and enzymes in the animal's defensive apparatus (Fig. 3b). Specifically, hydroquinone and hydrogen peroxide, stored ready and waiting in the beetle's defensive reservoirs, are brought together by enzymes in a high-energy oxidation/reduction reaction to form benzoquinone (Scheme 1, compound **11**), water, and heat. The reaction creates temperatures up to 100°C (212°F). During the reaction, pressure builds up in the defense gland reservoir until the substance can no longer be contained. At that point a rapid-fire series of pulsed sprays of hot toxic chemicals are deployed directly at the offending stimulus. Thus, it is the boiling of the mixture of these components and water which builds pressure and causes the explosion of toxins in the face or mouth of a predator unlucky enough to select a bombardier beetle as their next meal – or simply an unfortunate passerby who gets too close for the beetle's comfort.

The bombardier beetle is a member of the group known as ground beetles (Family Carabidae). Other examples of chemically armed ground beetles are species in the genus *Calosoma*. In the USA, probably the most familiar is *Calosoma scrutator*, also known as the Fiery Searcher or Caterpillar Killer because of its predatory nature. These beetles very often emit an indescribably nasty smelling concoction, which persists on one's skin even after hand washings and the passing of several hours. The main ingredients in the chemical defense sprays of *Calosoma* are methacrylic acid (Scheme 1, compound **12**) and salicylaldehyde (Scheme 1, compound **13**), which is emitted from the end of their abdomens. In fact, most adult beetles spray their defensive secretions from the end of their abdomen (the back end). To collect these secretions for analysis, one can simply cause the beetle to spray into a glass vial. However, since their abdomens often point downward, an alternative is to get the beetle to spray onto an inert surface such as a glass slide and collect the secretion off of that. In the case of the blister beetles or other insects that produce more of an ooze than a spray, a glass capillary is a very useful tool for collecting their secretions.

Another interesting example of beetle chemical defense occurs in darkling beetles (Family Tenebrionidae), which is the fifth largest family of beetles. These beetles are very common in the more arid regions of the southwestern USA, and as both adults and larvae they are scavengers, feeding on both live and decaying plant matter. Some of the larger darkling beetles, such as those in the genus *Eleodes*, are well known for their chemical defense. They can spray a large amount of benzoquinones when disturbed, similar to other beetles. However, many darkling beetles have a warning behavior associated with this defense – before launching their chemical spray, they will raise the rear end of their abdomen and stand on their head (Fig. 3c). This is a warning to back off before you get sprayed, equivalent to the coiling and rattling of a rattlesnake before it strikes. In fact, the chemical weaponry of benzoquinone-weilding darkling beetles is so successful that beetles in the genus *Moneilema* mimic their defensive head-standing posture when disturbed (Eisner 2003; Eisner et al. 2005; Evans and Bellamy 2000). This mimicry is pure deception because *Moneilema* spp. have no chemical defense spray of their own. For some beetles, it is the chemical defense of the larval stage for which the beetle is best known. The life cycle of beetles, as well as butterflies and moths (Order Lepidoptera), is called holometabolous (complete metamorphosis). This means that they have larval stages that look very different from the adults.

The larvae later become a pupa, an immobile stage like the cocoon of a moth or the chrystalis of a butterfly, which subsequently morphs into the adult. Other types of insects, such as walkingsticks (Order Phasmatodea) and grasshoppers (Order Orthoptera), are hemimetabolous (incomplete metamorphosis). This means that instead of a larval stage they have a nymph stage which resembles the adult, and lack a pupal stage. The larvae of many leaf beetles (Chrysomelidae) have pores along their body, which secrete droplets of liquid (Fig. 3d) containing repellant compounds such as chrysomelidial (Scheme 1, compound **16**) when the insect is disturbed. Leaf beetle species that make chrysomelidial have been shown to obtain this substance one of two ways (1) make it themselves using sugars or other metabolites that they consume or (2) access chemical precursors directly from the plants they feed on (Kunert et al. 2008). They control their resources so efficiently that, in the event of a false alarm, they can bring the liquid back in for later use.

There are thousands of other insects besides ants, beetles, and walkingsticks that make impressive and potent defensive chemicals and their methods of deployment are as diverse as the chemicals they make. For example, some termites, such as *Nasutitermes exitiosus* from Australia, will surround an offending creature and spray a sticky adhesive – a glue – at their opponent (Eisner 2003; Eisner et al. 2005). Often the termites will work as a team, especially for larger opponents, spraying this glue to immobilize their attacker. Butterflies (Order Lepidoptera, which also includes moths), particularly their larval stage (caterpillars), can also wield chemical weapons to keep from being eaten. Swallowtails (Family Papilionidae) have a particularly spectacular display associated with their chemical defense; a brightly colored defensive gland just behind their head (Fig. 3e, f). Unlike many other insects, swallowtail caterpillars do not spray their defensive chemicals. Rather, these caterpillars will rear back and wipe their pungent smelling defensive gland, wet with defensive secretion, onto the offending stimulus or predator. The most spectacular aspect of this defensive mechanism is the gland itself. When the insect is disturbed, the gland shoots out of the area behind their head, seemingly out of nowhere. The glands of these caterpillars are often covered with small pungent short-chain fatty acids, such as isobutyric acid and 2-methylbutyric acid and (Scheme 1, compounds **17** and **18**), whose smells have been described as rancid butter or gym sock. These acids are produced by several North American species such as the Eastern Black Swallowtail (*Papilio polyxenes*) (Fig. 2e), Spicebush Swallowtail (*Papilio troilus*) (Fig. 2f), and the Giant Swallowtail (*Papilio cresphontes*). The gland of the Giant Swallowtail is deep red and can be ejected particularly violently and extends past the rear of the body. However, other species of swallowtails, such as the more tropical Polydamas swallowtail (*Battus polydamas*), which occur in the southern USA (Florida and Texas) and through Central and South America to Argentina, make very different compounds – sesqueterpenes, such as selinene (Scheme 1, compound **19**) (Eisner et al. 1970, 1971, 2005; Eisner 2003). Indeed, even among these closely related butterfly species, this impressive example of chemical biodiversity exists.

3.4 Significance of Tropical Species for Chemical Biology Exploration

Most chemical biology and our overall understanding of the natural world come from studies done in temperate zones, and many of the examples represented in this chapter are describing the defenses of temperate insects. This is because historically most of the scientifically advanced nations occur in the temperate zone (Europe, North America, Central and Eastern Asia), while tropical regions are mainly in countries that lack the scientific infrastructure necessary to study the vast wealth of chemical biodiversity which exists within their borders. Thus, chemical ecology and other scientific studies of temperate species are disproportionately represented. The word disproportionate is particularly applicable, because most of the world's diversity and species richness is found in the tropics (Erwin 1997; Groombridge and Jenkins 2002; Hester and Harrison 2007). Accordingly, predation pressures and the need to fend off attack have led to added diversity of chemical strategies in insects (Eisner 2003; Eisner et al. 1995, 2005; Meinwald and Eisner 1995). In fact, there are very few nontropical species of walkingstick insects that produce any sort of defensive secretion. There is much to be explored and discovered in the tropics, especially in the realm of insect chemical biology.

3.4.1 Defensive Chemical Ecology of Walkingstick Insects (Order Phasmatodea)

Walkingstick insects (also called "phasmids") are best known for their nonchemical defensive mechanism, camouflage (Brock 1999). Their common name, walkingstick, indicates one of the ways in which some species blend into their environment, by mimicking sticks, twigs, or leaves to avoid predators. However, many species of phasmids also emit irritating chemical substances, which they deploy against potential attackers (Bedford 1978; Bouchard et al. 1997; Carlberg 1981, 1985a, b, 1986, 1987; Chow and Lin 1986; Dossey et al. 2006, 2007, 2008; Dossey 2009; Eisner 1965; Eisner et al. 1997; Ho and Chow 1993; Meinwald et al. 1962; Schmeda-Hirschmann 2006; Smith et al. 1979). In fact, many species of walkingstick insects do not blend into their background; they display bright colors as a warning to predators, a defense mechanism known as aposematism. Most walkingsticks deploy their chemical weapons from the front, in contrast to beetles, many of which spray from the back end. However, males of at least one type of phasmid, the New Guinea Spiny Stick (Genus *Eurycantha*), have a defense gland at the rear end of their abdomen. They use this gland in an elaborate defensive behavior – when threatened, the males will raise their abdomen in the air, repeatedly expose their scent gland, which gives off a skunk-like odor, and prepare their spiny rear legs to jab at the offending stimulus (Bedford 1975, 1978; Brock 1999). These insects are large, strong, robust creatures and their spines can inflict minor cuts on human skin. However, *Eurycantha* are exceptional in this mode of defense. Most species of phasmids produce their noxious defensive spray in a pair of laterally symmetric glands in their prothorax which has openings on each side just behind the head (Chow and Lin 1986; Dossey 2009, 2010; Eisner et al. 1997; Eisner 1965; Happ et al. 1966). All the examples of walkingstick sprays given in this chapter are tropical species, with the exception of *Anisomorpha buprestoides*, which ranges from subtropical to temperate USA. In fact, only two temperate species of phasmids have been reported to make defensive sprays, *A. buprestoides* and *A. ferruginea* (both from the USA).

4 Early Work on Phasmid Defensive Secretions

Some of the earliest work examining insect defensive sprays was conducted using walkingsticks (Meinwald et al. 1962; Schneider 1934) (Fig. 4a–c). The earliest chemical analysis on walkingstick defensive chemistry was conducted in 1934 on *Agathemera crassa* (formerly *Paradoxomorpha crassa*) from Chile (Schneider 1934). Phasmids in the genus *Agathemera* are known locally in Chile by the names "chinchemoyo" (Schneider 1934), "chinchemolle", "chinchimol", or "tabolango" (Schmeda-Hirschmann 2006). The 1934 publication on *A. crassa*, published in Spanish by Chilean researchers, utilized the technology available at that time (before NMR and mass spec had been invented), and the chemical structure reported is rather unusual (Scheme 1, compound **20**) and likely incorrect. Later, in 2006, analysis of the defensive chemistry from a closely related species, *A. elegans* (also from Chile), revealed a more likely chemical structure (Scheme 1, compound **21**) (Schmeda-Hirschmann 2006), showing a substance that is extremely persistent and often retained for years in dead and dried specimens.

It was not until the pioneering work of Eisner and Meinwald in 1962 that another walkingstick insect defensive secretion was analyzed and published. Meinwald et al. collected thousands of milkings from hundreds of individuals of *Anisomorpha buprestoides*, the "Southern Two-Striped Walkingstick Insect" from the southeastern USA, extracted an oily pungent substance, and analyzed it using various analytical techniques available at the time including NMR and mass spec. From these data they were able to determine the structure of the active component, which they named anisomorphal (Scheme 1, compound **23**). Shortly after the discovery of anisomorphal, Eisner demonstrated experimentally that the chemical spray of *A. buprestoides* was actually quite potent in repelling predators such as beetles, ants, and bluejays (Eisner 1965). Later, Ulf Carlberg performed quantitative experiments demonstrating the effectiveness of *A. buprestoides* chemical defense against Norwegian rats (*Rattus norvegicus*) (Carlberg 1985a). However, *A. buprestoides* is not always able to keep from being eaten. Black bears in Florida have been known to make a meal of these insects (Eisner et al. 2005; Roof 1997) – perhaps they even enjoy the spicy flavor of the spray.

The next species of phasmid whose defensive chemistry was published was the coconut stick insect, *Graeffea crouani*, from the South Pacific region (Smith et al. 1979). At the time this insect was reported to be a major pest of coconut palms. The work by Smith et al. determined that the secretion of these species contains at least

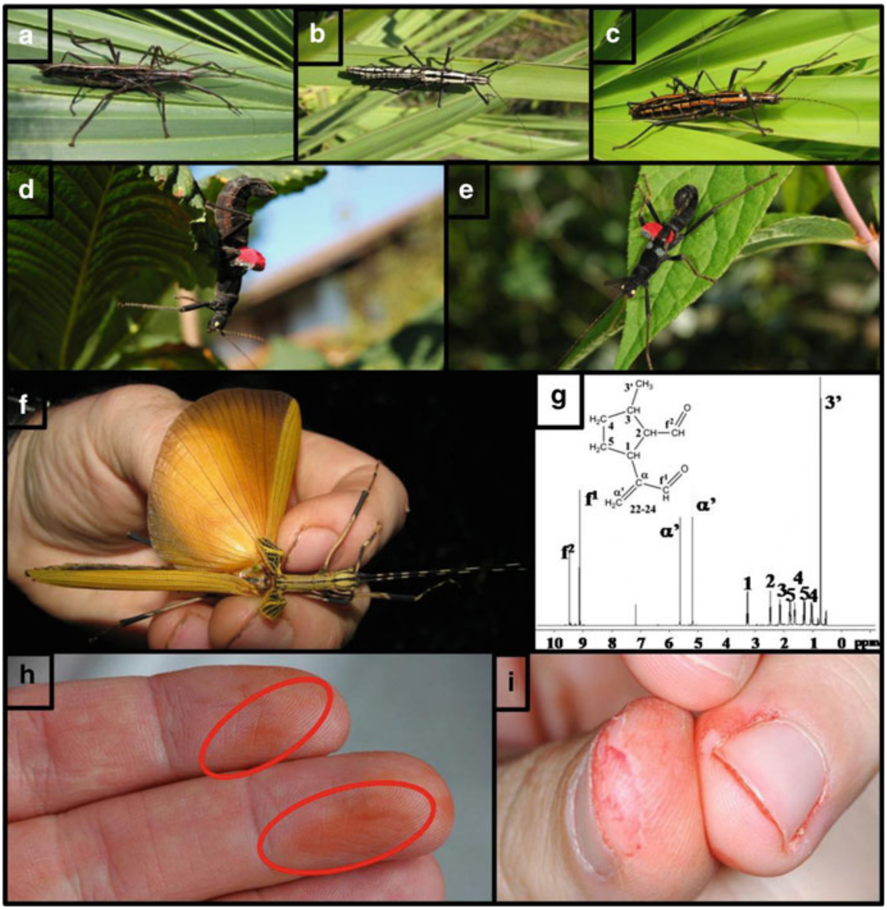


Fig. 4 Walkingstick insects and their chemical weapons. (**a–c**) *Anisomorpha buprestoides*, (**a**) Brown form (Gainesville, FL), (b) Black and White form [“Skeleton Stick” (McMonogle and Dossey, 2007), Ocala National Forest, FL], (**c**) Black and Orange form (Archbold Research Station, FL), (**d**) *Peruphasma schultei* female, (e) *P. schultei* male (captive reared in Germany by Oskar V. Conle), (**f**) *Pseudophasma annulipes* adult female found in Tambopata National Reserve, Peru (12°50′32″ S, 60°17′ 18″ W), December 7, 2007. Species identified by Oskar V. Conle, (**g**) one-dimensional ^{1}H NMR spectrum of *Pseudophasma annulipes* defensive spray dissolved in benzene-d6 and structure of anisomorphal. Numbered hydrogens in the structure correspond to the numbered peaks in the NMR spectrum, (**h–i**) effect of defensive spray from *Parectatosoma mocquerysi* on the hand of Oskar V. Conle (**h**) minutes and (**i**) several hours after sprayed by *P. mocquerysi*. *Red circles* indicate affected areas. Photographs (**a**), (**b**), (**c**), and (**f**) by Dr. Aaron T. Dossey. Photographs (**d**), (**e**), (**h**), and (**i**) by Oskar V. Conle

two isomers of iridodial (Scheme 1, compound **14**) and nepetalactone (Scheme 1, compound **31**). Interestingly, nepetalactone (McElvain et al. 1941; McElvain and Eisenbraun 1955) is also the compound in catnip (*Nepeta cataria*) responsible for the behavioral effects on cats (Eisner 1964). A few years after the coconut stick insect's spray was studied, other researchers began analyzing and studying the chemical defense of *Megacrania tsudai* (Chow and Lin 1986; Ho and Chow 1993) from Taiwan. In the Chow and Lin publication, *M. tsudai* was erroneously identified as *M. alpheus* (*M. tsudai* is the only species in this genus present in Taiwan) (Hsiung 2007). In general, members of the genus *Megacrania* (Hsiung 2007) are referred to as "peppermint stick insects" due to the characteristic odor of their defensive spray. In the studies by Ho and Chow, it was first determined that actinidine (Scheme 1, compound **15**) was the major component of *M. tsudai* defensive spray. In that same paper, they demonstrated that experimental nymphs of *M. tsudai* whose defense glands had been emptied by milking were less likely to survive in the wild than control nymphs that had not been milked. In addition to actinidine, the 1993 paper by Ho and Chow also identified several minor components in the spray, which are illustrated in Scheme 1 (compounds **25–29**). Notice that most of those compounds are very similar to actinidine (Scheme 1, compound **15**), and that actinidine and nepetalactone (Scheme 1, compound **31**) have similar structures. It turns out that some plants containing actinidine have also been shown to elicit similar behavioral responses in cats to that induced by nepetalactone (Tucker and Tucker 1988).

Another phasmid species to have the chemical composition of its defensive spray identified was *Sipyloidea sipylus* (the "pink-winged stick insect") (Bouchard et al. 1997). The chemical spray of this species contains a rather complicated mixture; probably the most chemically diverse phasmid chemical spray analyzed to date (Scheme 1, compounds **32–36**). Among the various components are acetic acid (Scheme 1, compound **33**) and benzaldehyde (Scheme 1, compound **34**). Both of these can be rather effective irritants in high doses. Benzaldehyde and acetic acid are found in the chemical defenses of various other insect species as well. Interestingly, the most abundant compound in *S. sipylus* spray as reported by Bourchard et al. was diethyl ether (Scheme 1, compound **32**) (about 70% of the total volatiles measured), a chemical commonly used as a laboratory solvent. Bouchard et al. also examined the effectiveness of the five identified compounds from *S. sipylus* defensive spray (Scheme 1, compounds **32–36**) as pest control against rats (*R. norvegicus*), and found that a test mixture of these substances was effective in repelling the rats. Earlier experiments performed by Ulf Carlberg using live *S. sipylus* insects also demonstrated the effectiveness of their chemical defense against predation by the same rat species (Carlberg 1986). Based on experimental comparisons between *S. sipylus*, *Anisomorpha buprestoides*, and *Extatosoma tiratum*, the chemical defense of *S. sipylus* was the most effective of the three.

Oreophoetes peruana from Peru also has a defensive secretion that was characterized by Eisner et al. They discovered that the major nonaqueous component of *O. peruana* defensive spray was quinoline (Scheme 1, compound **30**) (Eisner et al. 1997). Various derivatives of quinoline have been found in several other insect species; however, this was a very unusual finding because quinoline itself occurs very rarely in nature. In fact, *O. peruana* was the first animal species ever shown to produce quinoline (Eisner et al. 1997). Once quinoline was shown to be the chemical component in *O. peruana* defensive spray, Eisner et al. was able to demonstrate that it is indeed effective against a variety of potential predators such as ants, spiders, cockroaches, and frogs. Interestingly, quinoline is very similar to the active insect-repelling component of moth balls, naphthalene. A collaborator on this study was Randy Morgan from the Cincinnati Zoo. This is important to point out because live insect collections such as at zoos and insectariums are very valuable resources for scientific research. Often permits to transport exotic insects can be difficult for researchers to obtain, but many exotic animals are already legally being reared at zoos. It is also important to acknowledge the contributions of nonchemists to the field of chemical biology and of amateur entomologists to science. In addition to the work by Eisner et al., the actual spraying mechanism of *O. peruana* was recently analyzed by high-speed photography and structure of the gland was analyzed by electron microscopy (Bein and Greven 2006).

Both male and female Australian Spiny Sticks (*Extatosoma turatum* – also known as the Australian Stick or Macleay's Specter) produce a secretion which smells a bit like toffee (Bedford 1978). Carlberg, who has probably done the most extensive studies of the effectiveness of phasmid chemical defense of any researcher, tested whether the secretions of *E. turatum* would repel frogs (*Xenopus laevis* and *Rana esculenta*) or rats (*R. norvegicus*). According to his study, the chemical defense of this species was rather poor against these predators (Carlberg 1985b). Interestingly, the *X. laevis* frogs refused to eat the abdomens of the insects that did not contain the defense glands. Although *E. tiratum* clearly emits a chemical secretion when disturbed, the small amount and pleasing odor of this substance seems to suggest it may have some function besides warding off predators for which it is better suited. Additionally Dossey et al. has also found that the leaf insect species phyllium westwoodii produces only a tiny amount of spray when disturbed (Dossey 2009). This spray was found to contain dimethyl alkyl pyrazines, which not only seem ineffective as innitants, but are common components in the odor/escence of chocolate, coffee and roasted food (Dossey 2009). In fact, it has been postulated that the chemical defenses of some walkingsticks may function as pheromones, yet this hypothesis has not yet been tested (Dossey et al. 2008; Tilgner 2002).

5 Continuing the Tradition of Discovery Using New Technologies

My studies of phasmid defensive chemistry were inspired by my life-long hobby and passion for studying insects. In 2005–2006 I was keeping a few *A. buprestoides* in captivity. When I first read the work by Meinwald (Meinwald et al. 1962) and Eisner (Eisner et al. 1997) on *A. buprestoides,* I was intrigued by this report that the defensive secretion contained only a single compound. It is not often that nature produces single substances so pure and in such large quantity. At the same time, the laboratory I worked in as a PhD graduate student had just received and installed a new cryogenically cooled micro NMR probe which was reported to be the most sensitive in the world (Brey et al. 2006) – being able to detect very small quantities of organic substances in timeframes shorter than possible using other NMR probes. In February 2006, the *A. buprestoides* I was keeping were small, about an inch or so long. I thought, considering the reported purity and apparent quantity of spray produced by these animals, I would use them to put this new NMR probe to the test. Because the original studies on the defensive chemistry of *A. buprestoides* and other phasmids were done using organic solvent extracts, I decided to use deuterated water to determine if there were any additional aqueous components in the secretion (Dossey et al. 2006; Amato 2006; Dossey 2006), which turned out to be a fruitful endeavor. In addition to the previously known anisomorphal (Scheme 1, compound **23**), I discovered that the defensive sprays of young *A. buprestoides* can also contain dolichodial (Scheme 1, compound **22**), peruphasmal (Scheme 1, compound **24**), and, because I used water rather than organic solvent, I also observed glucose (Scheme 1, compound **37**) in the mixture.

At the same time as I was studying *A. buprestoides* defensive spray, Oskar Conle had just described a new species of walkingstick insect, which he named *Peruphasma schultei* (after Rainer Schulte, a German herpetologist in northern Peru) (Fig. 4d, e) (Conle and Hennemann 2005). When I read about this new species I emailed Oskar and asked for a sample of the secretion of *P. schultei*. It turned out that *P. schultei* spray also contained glucose and an isomer of dolichodial that was differed from the anisomorphal or dolichodial found in young *A. buprestoides* defensive spray. Following the tradition of compound nomenclature established by Eisner and Meinwald of incorporating the genus of the walkingstick insect in the chemical name, I named this new compound peruphasmal (after the genus *Peruphasma*). Peruphasmal was originally found in ants but was referred to only as an isomer of dolichodial (Cavill et al. 1976) rather than given its own name. Since that first analysis of defensive spray from *P. schultei* adults, I have found that samples from young *P. schultei*, unlike those of young *A. buprestoides*, also contain only glucose and peruphasmal, and not anisomorphal or dolichodial.

Some specific variables in *A. buprestoides* ecology correspond to the dolichodial isomeric variability in their defensive spray. Specifically (1) different populations produce different isomers in different ratios, and (2) at different life stages, the young of some populations produce a varying ratio of isomers in their defensive spray which switches drastically when they reach sexual maturity (Dossey et al. 2008). By systematically analyzing single milkings of individual insects, wild caught from various locations around the state of Florida and captive-reared individuals at various life stages through sexual maturity, I found that populations of the black and white and the black and orange color forms produce only anisomorphal as adults in the wild, whereas populations of the brown color form in Gainesville, Sanibel Island, and Gulf Hammock produced only anisomorphal, only peruphasmal, or some combination of the two. The young examined at different life stages were progeny of a peruphasmal-producing population in Gainesville. As young, they produced variable mixtures of anisomorphal and dolichodial and only traces of peruphasmal. However, upon reaching sexual maturity, all animals studied switched to producing only peruphasmal, as had their parents (Dossey et al. 2008).

These studies on the defensive sprays of *A. buprestoides* and *P. schultei*, my first in insect chemistry, demonstrated several important points. First, the work was aided tremendously by modern high-sensitivity NMR technology (Amato 2006; Brey et al. 2006; Dossey et al. 2006). Being able to analyze material from single young insects allowed the comparisons between individuals and within an individual over time possible. In general, modern analytical chemistry technology and instrumentation has improved greatly in recent years, making much more of nature's chemistry amenable to analysis and, thus, potentially useful to humans. Second, simple and direct sampling of substances such as defensive secretions and venoms is very beneficial in these sorts of studies. When the substance comes directly from the gland of the animal that produces it and it is not chemically manipulated or modified, there is greater confidence that the observations made are biologically relevant. Third, it is important to consider all variables and sources of material in natural products chemistry. In looking at the aqueous phase of phasmid defensive secretions, I was able to find glucose, which was previously not known to exist in these substances. I was also able to find proteins in *A. buprestoides* defensive spray. It is important to look everywhere and use a variety of techniques and experimental conditions in bioprospecting to surveying the chemical complexities of a natural substance. In studying different insects on different days, I was able to determine that, in fact, the defensive secretions varied in their chemical composition (Amato 2006; Dossey 2006; Dossey et al. 2006, 2008). Finally, it is important to not be afraid to pursue new ideas, even if it appears that everything is already known about a particular system. In *A. buprestoides* defensive spray, in addition to the presence of glucose, I was able to determine that in fact there are three possible isomers of dolichodial produced (Amato 2006; Dossey 2006; Dossey et al. 2006, 2008) rather than only one (anisomorphal) as previously reported (Eisner et al. 1997; Meinwald et al. 1962). As technologies improve or new minds approach questions in science, new discoveries are very likely just around the corner.

In December 2007, I had the opportunity to travel outside of the USA for the first time. I spent some time in Lima, Peru, and then headed off for several days at Explorer's Inn, an eco-lodge in Peru's Tambopata National Reserve (TNR). Immediately upon arrival, the immense level of biodiversity in this area became clear. Over ten species of butterflies were clustered at the edge of the Tambopata River drinking from run-off puddles. Walking toward the lodge from the boat I saw many millipedes, beetles, butterflies, and ants galore. Ants are very much an omnipresent feature of the forest; they can be found on any tree or plant or few square yards of forest floor. This was very exciting, but I was there mostly focused on collecting defensive secretions from insects. Among my primary targets: walkingstick insects.

On the first day of hiking, I saw several walkingsticks; however, these were different than walkingsticks in the USA. They had wings and were strong fliers so that one could hardly get a few feet from them without them fluttering off into the dark forest undergrowth. From a photograph I sent him shortly after the trip, a colleague Oskar Conle was able to identify this insect as *Pseudophasma annulipes*

(Fig. 4f). This is the first published record of this species occurring in Peru, although it was previously reported from Bolivia. During the same trip, several males and females of this species were observed flying in the daytime within about 20 ft of ground level. One mating pair was also observed resting on a fern. A defensive spray sample collected was from the female in Fig. 4f which I was finally able to catch landing on the ground where I could successfully milk her. My milking method requires that I put a slight pressure against the insect with a vial to keep it firmly on the gland opening to collect a maximum amount of spray. A couple of months later I received the sample I had collected in the mail from our collaborators in perv. Immediately I extracted a small portion of it in deuterated benzene and acquired an NMR spectrum (Fig. 4g), which matched exactly to that of anisomorphal (Scheme 1, compound **23**). Gas chromatography and mass spectrometry experiments also verified that the substance was anisomorphal. This is the first study on the defensive secretion chemistry of this species and the first report that it contains anisomorphal. It demonstrates a continuing trend of dolichodial-like isomers as chemical defenses in North and South American walkingsticks closely related to the genus *Anisomorpha*.

One of the most exciting discoveries for me in my walkingstick defensive chemistry project was a novel compound, parectadial (Scheme 1, compound **38**), which I discovered from the species *Parectatosoma mocquerysi* of Madagascar (Burks 2007; Dossey et al. 2007). That discovery began as part of my continuing collaboration with Oskar Conle whom, as you may recall, I began collaborating with on the paper which included the analysis of *P. schultei*. Subsequent to that publication I asked Oskar which would be the next most exciting walkingstick species defensive secretion to analyze. That is when he told me about this interesting secretion from *P. mocquerysi* that causes reddening and peeling of the skin (Fig. 4h, i). As soon as I got the sample in the mail from Oskar I was able to analyze it by NMR and mass spec. First I examined the water soluble components, which again contained glucose. There appeared to be only a small number of other peaks besides glucose. The mass spec analysis confirmed that indeed there was only one other component. Upon further analysis of the NMR data, I was able to easily determine the structure of this component. It also turned out to be a compound completely new to science. Thus, in the aforementioned tradition of naming walkingstick defensive compounds, I named this new compound parectadial after the genus *Parectatosoma* from which it was isolated (Burks 2007; Dossey et al. 2007). For the publication of parectadial's discovery and structure. I was awarded the Jack L. Beal award for best paper of the year in the Journal of Natural products for 2007 (Kinghorn, 2008).

The effects parectadial has on skin suggest it may have value as a medicinal compound for conditions such as cancer or psoriasis. Additionally, parectadial is very similar in structure to perillyl alcohol (POH) and perillaldehyde, which have both been explored for use against cancer in a number of studies (Elegbede et al. 2003; Fernandes et al. 2005; Yeruva et al. 2007). POH has even been the subject of several clinical trials for treatment of cancer (da Fonseca et al. 2008; Ripple et al. 1998). Thus, it is logical to believe that parectadial may also have significant efficacy against cancer. However, further investigation is needed to test such a hypothesis.

6 Biosynthesis: New Discoveries for Pathway Elucidation

Phasmids have several advantages which make them useful for biosynthesis studies, foremost among them is their large size, and for chemical biology and biosynthesis bigger is often better. Other advantages are that they are easily cultured in the laboratory and are long lived, with many species living well over a year. Some species are also parthenogenic, which facilitates genetic studies. Finally, they are a very diverse group of insects that produce a wide variety of chemicals for defense and other purposes.

In my first insect chemistry paper I discovered that glucose, the most fundamental nutrient and carbon source for all living systems (Scheme 1, compound **37**), was present in the defensive secretions of *A. buprestoides* and *P. schultei* (Dossey et al. 2006). Since that paper, the defensive sprays of nearly every phasmid (as well as those of other insects) I have investigated have contained glucose (Dossey et al. 2007, 2008, 2009, 2010). One other group has also found glucose in the defense spray of yet another phasmid species, *megacrania nigrosulfurea* (Prescott 2009). This observation turns out to provide possible clues into how the defensive compounds of walkingstick insects are made and transported to their glands. As mentioned earlier, larval beetles in the family Chrysomelidae make similar compounds for chemical defense, and the research teams of Profs Wilhelm Boland and Jacques Pasteels have done extensive work characterizing the biosynthesis of chrysomelid defensive chemicals (Kunert et al. 2008), including the use of glucose. They have shown that glucose-conjugated precursors are transported into the glands of these beetles (Kuhn et al. 2007). Once inside the gland reservoir, the glucose is removed and various other chemical steps, such as oxidations and cyclizations (Veith et al. 1996), are performed on the precursor to give rise to the final product which is secreted or sprayed by the animal upon being disturbed or attacked (Oldham et al. 1996; Feld et al. 2001). This means that all of the required machinery to make these chemical transformations exists in the gland reservoir and are quite possibly released in the defensive secretion itself. In fact, it has been shown in some chrysomelid beetles that components of this biosynthetic machinery can be found in their defensive secretions (Feld et al. 2001; Kunert et al. 2008; Oldham et al. 1996).

For biosynthesis studies, the goal is to understand how the chemical compounds of interest are made by the organism(s). The primary tools by which chemistry is done in nature are enzymes. One of the important discoveries I made recently for biosynthesis in phasmids is that there are several proteins present in the defensive spray of *A. buprestoide*. The proteins were extracted individually and analyzed by mass spec. This will provide information on their sequences. Since all protein sequences are coded for by genes (DNA), such information will aid in cloning the genes from which those proteins came. In a process known as recombinant

DNA technology, these genes can be put into and expressed in various types of cells such as bacteria, insect cells, or even live plants (Voet and Voet 1995). Theoretically, if those genes are expressed in those new cells properly, the compounds made by those genes will also be made. The genes can possibly even be mixed and matched to make new compounds other than the actual ones made by the insects! Since proteins are the direct products of genes, the previously mentioned benefit of parthenogenic walkingstick insects is also an attribute, which makes some species beneficial to biosynthesis studies.

7 The Future of Insect Chemistry: From Biosynthesis to Drugs

By now it should be clear that insects are among the most important organisms on earth for many aspects of science and human existence – from biosynthesis to agriculture to disease (Dossey 2010). In these later sections, a discussion is given of some specific examples of how natural compounds from insects and other invertebrates can be important for technological advances, particularly in drug discovery. While reading this section, use your imagination and consider the even broader implications of insects and discovery.

8 Insects as a Source of Drugs

8.1 Toxins

Some of the most commonly sought types of natural substances are toxins (Fox and Serrano 2007). Natural products in general are attractive sources of material for drug discovery. Over 70% of drugs on the market are derived from or based on natural compounds (Newman and Cragg 2007). Compounds produced by nature are optimized for their functions, such as binding to specific target proteins in cells (Ortholand and Ganesan 2004). There are two major types of toxins that are attractive as drug lead compounds, those that can kill cells and those that function as neurotoxins. Cell killing or cytotoxic substances are generally sought for use as anticancer chemotherapeutics (Lodish 2000). For example, cantharidin (Scheme 1, compound **10**) from blister beetles and its chemical derivatives have already been explored for use as anticancer therapeutics (Sakoff et al. 2002; Sagawa et al. 2008). It is also often used in the treatment of warts (Moed et al. 2001). Cantharidin causes blistering of the skin, a common property of compounds known as vesicants. Blistering is the result of cell dying, thus being a property suggestive of usefulness as an anticancer compound. Venoms of stinging insects and other arthropods often possess properties that give them great potential as lead compounds for drug discovery. The previously mentioned solenopsins from red imported fire ants have also been pursued for a variety of medicinally relevant applications due to their ability to illicit necrosis in human tissue. Solenopsin, an alkaloid found in the venom of fire ants (Brand et al. 1973; MacConnell et al. 1970, 1971), has been investigated for its ability to inhibit angiogenesis (Arbiser et al. 2007), as an inhibitor of nitric oxide production (Yi et al. 2003), for its effect on the nervous system and on cardiosuppression in humans (Howell et al. 2005). In addition to venoms, other substances from insects can prove to be potent toxins useful for fighting cancer. For example, in 2005 three novel antineoplastic agents were isolated from preserved grasshopper specimens from Texas (*Brachystola magna*) (Pettit et al. 2005). It is fascinating to imagine that even an old jar of grasshoppers could potentially hold the cure for cancer.

Neurotoxins are often studied for their use as pain killers. In fact, one pain medication from a sea snail, Prialt® (Ziconotide) derived from the species *Conus magus*, has been approved for use by the USA Food and Drug Administration (FDA) and is already on the market as an alternative to morphine to treat chronic pain (Amstutz et al. 1998; Justice et al. 1994; FDA and UFADA 2006). This compound was discovered by an undergraduate researcher, in the laboratory of Baldomero Olivera (McIntosh et al. 1982; Machalek 2002), one of the most famous cone snail toxin laboratories in the world. Arthropods also produce a vast array of neurotoxic substances that merit study as possible drugs. Spider venoms in particular are the most common arthropods that have been studied for a variety of reasons oops. Most spider venoms contain neurotoxins. This is because they are most often used to paralyze prey rather than for defense. In insects, venoms can also contain neurotoxic substances of interest. For example, the venom of the bullet ant previously mentioned contains a protein called Poneratoxin. This protein blocks voltage-dependent ion channels in insects. It has even been proposed as a potential insecticide if expressed in an insect virus (Szolajska et al. 2004). There is no medical application yet for this toxin, so it could represent yet another opportunity for discovery in medical science.

8.2 Antibiotics

With the emergence of various antibiotic-resistant microbes, the search for new and novel antibiotics is a particularly important goal of modern drug discovery. Insects, as with many organisms, are susceptible to infection by microorganisms. In fact, many insects have dormant immobile stages (pupae), which are particularly vulnerable to a number of harmful elements. However, insect pupae can often sit dormant and immobile in such environments as soil or rotten logs without becoming infected. This protection against infection is partly due to antimicrobial substances. They are often peptides, but in some cases antimicrobial secondary metabolites have been found (Bexfield et al. 2004; Huberman et al. 2007b; Meylaers et al. 2004) (Dossey 2010). These substances are found in the hemolymph (blood) of the insects rather than sprayed or secreted. Nonetheless, they are a form of chemical defense against microbial attack. For example, two families of antimicrobial peptides have been discovered in the pupae of cecropia moths (*Hyalophora cecropia*) from North America: the cecropins and the attacins (Boman et al. 1991; Boman and Hultmark 1987; Hultmark et al. 1980, 1983). Cecropins and defensins have subsequently been found in a number of other insect species (Cociancich et al. 1994), as well as other organisms (Pillai et al. 2005).

Additionally, fly larvae have been successfully used in hospitals to remove dead tissue from wounds quickly simultaneously protecting against infection (Whitaker et al. 2007). In relatively recent studies, antibiotic substances have been discovered in the larvae of flies ranging from small organic metabolites such as lipids to antibacterial peptides and thus far unidentified compounds (Bexfield et al. 2004; Huberman et al. 2007a, b; Meylaers et al. 2004; Natori 1994; Whitaker et al. 2007). In fact, substances from larvae of one species of diptera used in wound healing medicine, *Lucilia sericata*, have already been shown to be effective against methicillin-resistant *Staphylococcus aureus* popularly known as "MRSA" (Bexfield et al. 2004). Thus, flesh eating flies provide a potential source of chemical substances which are likely beneficial to humans in use as antibiotics. However, these compounds are used out of their natural context in treating human ailment. Flesh flies usually feed on dead animals in the wild. Thus, they may not be defensive chemicals per se – I am unaware of any study demonstrating what benefit they provide to the fly or fly larvae. It could be defensive against microbial pathogens, selective for certain microbes to help the larvae digest the flesh, or even in slowing the flesh rotting process as to give the larvae sufficient time to feed on the carcass.

Insects themselves are only the tip of the iceberg as far as the biodiversity they represent. Many species of insects also harbor symbiotic fungi and bacteria, which aid them in a variety of functions such as food digestion and warding off pathogens. A very recent study demonstrated chemical warfare that occurs between fungi on the body of the beetle, *Dendroctonus frontalis*, a common pest of pine trees in the USA (Berenbaum and Eisner 2008; Scott et al. 2008). The larvae of the beetle depend on two species of pine-dwelling fungi for food, *Entomocorticum* sp. and *Ceratocystiopsis ranaculosus*. A third species of fungus, *Ophiostoma minus*, can help protect the beetles against the tree's chemical defenses but can also inhibit the growth of *Entomocorticum* sp., which the beetle larvae depend on for food. Scott et al. found that a fourth species, an actinomycete, produces an antibiotic compound new to science called mycangimycin. This antibiotic inhibits the growth of *O. minus*, thus allowing the beetle's larval food bacteria to thrive in a fascinating example of multispecies interaction. In addition to insects, their associated microbes or fungi can also prove to be a rich source of promising toxins. For example, two novel cyclopeptides called hirsutatins were discovered in 2005 from the insect pathogenic fungus *Hirsutella nivea*. These peptides showed activity in killing the microbial (protozoan) parasite *Plasmodium falciparum* and the causative agent of tuberculosis (*Mycobacterium tuberculosis*) (Isaka et al. 2005). These examples demonstrate that not only insects, but also their associated microbes (symbiotic, parasitic, etc.) have a vast potential in the future of new antibiotic discovery.

9 Biosynthesis: Toward New Methods of Chemical Production

Recent interest in useful chemical substances from natural sources has created a need to understand how these compounds are made by their originating organisms (Ajikumar et al. 2008). Thus, it is important to be aware of opportunities to characterize biosynthetic enzymes and pathways of natural products as they arise. With my recent findings of chemical biodiversity in walkingstick insects, as well as the presence of proteins in their defensive secretions, it is clear that these organisms represent a good model system for discovery of biosynthetic mechanisms of a variety of substances. The proteins found in walkingstick defensive secretions are likely to have valuable use as components in bioengineering combinatorial approaches in the overall biosynthesis of many sorts of compounds, even ones not produced by walkingsticks or possibly not produced by nature at all. In general, insects and other invertebrates represent one of the greatest resources available for the broad field of chemical biology that exists in the natural world. Mankind would be well advised to simultaneously protect and capitalize on our access to this rich resource.

10 Suggested Reading and Viewing

Here is a list of books and television programs, most of which were referred to earlier in this chapter, for suggested reading. These will be very useful and enjoyable for anyone interested in, involved with, or simply intrigued by the natural world, chemical defense, or the fascinating creatures we call insects:

- *For the Love of Insects* – Thomas Eisner, 2003
- *Secret Weapons: Defenses of Insects, Spiders, Scorpions, and Other Many-Legged Creatures* – Thomas Eisner, 2005
- *Life in the Undergrowth* – David Attenborough, a television series by the BBC Natural History Unit, also available on DVD.
- *Chemical Ecology: The Chemistry of Biotic Interaction* – Thomas Eisner and Jerrold Meinwald (Editors), 1995
- *World Atlas of Biodiversity: Earth's Living Resources in the 21st Century*, Brian Groombridge and Martin D. Jenkins, 2008
- *Biodiversity Under Threat*, R. E. Hester and R. M. Harrison, 2007
- *Biodiversity II*, Marjorie L. Reaka-Kudla, Don E. Wilson, and Edward O. Wilson, 1997
- *Chemical Defenses of Arthropods* – Murray Sheldon Blum, 1981
- *The Amazing World of Stick and Leaf-Insects* – Paul D. Brock, 1999
- *Stick Insects of the Continental United States and Canada* – Chad Arment, 2006
- *An Inordinate Fondness for Beetles* – Arthur V. Evans and Charles L. Bellamy, 2000

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Defensive Behaviors in Leaf Beetles: From the Unusual to the Weird

Caroline S. Chaboo

Abstract Chrysomelid leaf beetles are a geologically ancient group of primarily herbivorous insects. As herbivores, they are important ecologically, in food chains, and economically, as pests or as bio-controls in agriculture. This paper reviews some of the interesting defenses they show in relation to living exposed on plants. Gregariousness and subsocial behaviors (maternal guarding) in two groups of chrysomelids help individuals to survive predators and parasites. Larvae, and sometimes eggs, may be covered with feces to avoid detection or to deter attacks. Sequestering noxious chemicals from host plants is another strategy for survival. Some chrysomelids maintain some chemicals that are so toxic, that in Bushmen tribes in southern Africa use the beetles as a source of poisons for their hunting arrows.

1 Introduction

Insects are the most common animals on Earth, accounting for about 1 million of the known 1.6 million named species (Grimaldi and Engel 2005). Because of this remarkable diversity in species and biomass, insects play a fundamental role in ecosystem structure and function. Leaf beetles, known scientifically as the family Chrysomelidae, are a particular group of beetles that specialize in eating and living on plants (Crowson 1981). Over 40,000 species of leaf beetles have been described and these use more than 210 families of plants as hosts (Jolivet and Hawkeswood 1995). Leaf beetles provide many excellent models for illustrating how individuals survive and how species can interact in food chains, communities, and ecosystems.

As their name suggests, leaf beetles are herbivores. Their typical life cycle involves the eggs, larvae, pupae, and adults occurring mostly on their host plant, although some have become highly specialized as soil-dwelling detritivores, or as myrmecophiles (living with ants), or as termitophiles (living with termites). Leaf beetle adults and larvae are the main feeding stages and may use all plant

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parts – roots, stems, leaves, fruits, and flowers. Aside from general biological interest in how their extraordinary diversity and specialization have evolved, there is much agricultural interest in leaf beetles that have become pests of important food crops and ornamental plants, from rice to corn to orchids (Jolivet and Hawkeswood 1995). A life on plants may be advantageous in many ways because plants are a dominant life form on Earth and have enjoyed a long evolutionary history. The success and diversity of many nonplant species can be attributed to the evolution and dominance of plants. However, plant specialists face many dangers through exposure to abiotic (e.g., temperature, humidity, insulation) and biotic dangers (e.g., predators, prey), and by being restricted to the habitat and food resources offered by their hosts. Chrysomelids appear to face great pressure of attacks from predators (mainly Heteroptera, true bugs) and parasites and parasitoids (Diptera and Hymenoptera) (Cox 1994, 1996). Parasites live with but do not kill their hosts; however, parasitoids do ultimately kill their hosts (Eggleton and Belshaw 1992). Adult beetles can escape attacks by flying away or jumping off the plant, but the immature stages are particularly vulnerable since they are soft bodied and move far more slowly by walking on the surface. Here I explore some of the varied, interesting, unusual, and even weird behaviors exhibited in various life stages of leaf beetles.

2 Sociality

One of the most remarkable insect behavioral defenses is that of sociality and maternal care (Wilson 1971). While sociality in ants, bees, and wasps is better studied and known, sociality in other insects is less obvious to many people. Several groups within Chrysomelidae show sociality that arises when females lay clutches of eggs together which then hatch and pave the way for groups of larvae staying together, feeding, and eventually pupating together. This kind of gregarious behavior has several advantages over solitary behavior. For example, by living in a herd, individuals may find more protection from predators and prey. This advantage is more obvious for a herd of cattle, but the principle applies similarly to tiny insects. Group living may also help individuals conquer their host and take advantage of food. A vertebrate analog might be the ease of a pack of wolves over a solitary hunter in taking down a large mammal. In similar fashion, some leaf beetles minimize the effects of highly toxic plants by feeding in groups and each individual shares a small amount of the toxin thus reducing the effect of a single concentrated dose on a single individual.

3 Maternal Care

Some leaf beetles have gone a step further in the evolutionary pathway from solitary to gregarious behavior, as they have become subsocial where the mother stays and cares for her offspring (Costa 2006). True sociality, eusociality, is defined as having overlapping generations so that older offspring helps the parent care for the younger offspring (Wilson 1971). This behavior has evolved multiple independent times within two subgroups of Chrysomelidae, the subfamily Cassidinae (Fig. 1a) and the subfamily Chrysomelinae (Fig. 2a) (Chaboo 2007), a pattern that suggests similar

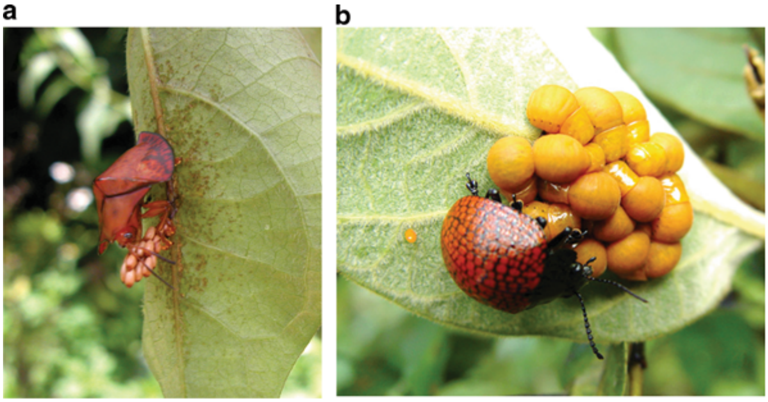

Fig. 1 Maternal care in Chrysomelid beetles. (**a**) Female cassidine, *Acromis spinifex*, guarding her egg clutch in Trinidad (photo, F. Merino). (**b**) Female chrysomeline, *Doryphora* sp., guarding her larvae in Brazil (photo, F. Frieiro-Costa)

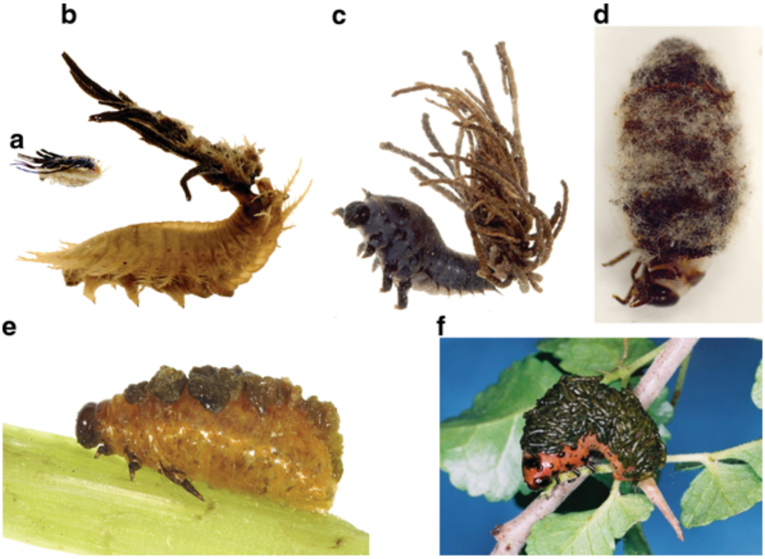

Fig. 2 Defensive shields and cases of Chrysomelid beetle larvae. (**a**) A larva from Panama holding the shield flat and protecting its back, and (**b**) holding the shield vertically, ready to strike, USA. (**c**) A larva with ornate shield, Uganda. (**d**) A larva inside its fecal case coated with trichomes from the host plant, USA. (**e**) A larva with a wet fecal coat directly on its back, USA. (**f**) A larva with a wet fecal coat on its back, South Africa

ecological forces may be driving this complex behavior. All the cassidine (tortoise beetle) maternal care species have been discovered in the Neotropics, from Costa Rica to Peru. Chrysomeline species with maternal care have been found in Costa Rica, Brazil, and Japan. Members in these two subfamilies are not directly related, but they exhibit common traits such as having the eggs and larvae living on leaves where they are very exposed (Frieiro-Costa and Vasconcellos-Neto 2003). The leaf beetle mother may coat her egg cluster with offensive chemicals (Hilker 1994) or build layers of protective membranes that insulate the eggs and make it harder for a predator or parasitoid to reach the eggs (Hinton 1981). The larvae feed together with the mother always hovering nearby. When a predator or parasitoid approaches, the mother pushes her offspring together to form a tight cluster and then she sits on them, prepared to physically attack the intruder. If the intruder persists, she may lead or push her brood to a new location on the plant (Upton 1996). Apart from physically touching the young, we suspect that other signals (e.g., sound) may aid in the communication between chrysomelid mothers and their offspring, as has been demonstrated in other subsocial insects, e.g., bugs (Crocroft 1996). A cassidine mother will guard her young through their sedentary pupation phase and will only depart when the last young adult has emerged (Chaboo 2001). Chrysomeline females depart earlier as the final larval stages migrate down the plant to pupate solitarily in the soil (Kudo and Ishibashi 1995; Kudo et al. 1995; Kudo and Hasegawa 2004).

4 Defensive Constructions

One of the most bizarre behaviors exhibited by animals exists in Chrysomelidae. Several kinds of the leaf beetles have larvae with an elaborate shield held over the body or a hard portable case worn like a top hat by individuals as they move around the plant. Other animals that construct domiciles, nurseries, or fortresses may produce their own materials (e.g., silk) or use materials from the environment, e.g., the logs of a beaver lodge. Some even use a mix of different materials from multiple sources, e.g., a bird's nest of twigs may be held together by stolen spider silk (von Frisch 1974; Hansell 2005). Animal architecture is a fascinating area of study because it involves so many aspects of an organism's morphology and physiology interacting with its ecology. Chrysomelids can be viewed as miniature builders dealing with similar issues of protection in a harsh environment.

5 Fececology

In a group as large and diverse as leaf beetles, there are many kinds of defense constructions (Fig. 2). In all these various leaf beetle constructions, the material for constructing is most astonishing – the feces of the animal. The subfamily Cassidinae has ~3,000 species whose larvae carry a mobile shield made of dried feces, attached to paired processes at their hind end, and held over the body like an umbrella (Fig. 2a, b) (Chaboo et al. 2007). This shield may be held flat on the dorsum or elevated to hit an attacker. In two other leaf beetle subgroups, the subfamily Criocerinae (~1,400 species) (Vencl and Morton 1999) and in some members of the subfamily Galerucinae [~14,000 species (Chaboo et al. 2007)], the fecal material is simply piled directly onto the back of the animals, with some falling off as the animal moves around but regularly replenished to maintain coverage of the exposed dorsal surface (Fig. 2b, c). In Cryptocephalinae leaf beetles (~4,000 species), the mothers take time to build a fecal case entirely around every single egg (Brown and Funk 2005; Chaboo et al. 2008). When the larva hatches, it steps out of the case and flips the case over its body, and thus spends the rest of its life wearing this case like a top hat (Fig. 2d). The case is expanded as the larva grows to accommodate the increasing size. The feces emerge as a semisolid material but harden by exposure to air and thus form an adobe-like hard defense.

Feces are not an obvious building material for many animals but it has the advantage that the animal is always producing it (Olmstead 1994, 1996). Chrysomelids further enhance the effectiveness of their shields and cases using offensive chemicals sequestered from their host plants (Gómez et al. 1999; Vencl and Morton 1998; Vencl et al. 2005; Nogueira-de-Sá and Trigo 2005).

6 Defensive Chemicals

Chrysomelids possess a range of glandular and hemolymph defensive chemicals. It is very interesting in an evolutionary sense that plants produce chemicals to protect themselves from being eaten by herbivores. In the long history of evolution, some herbivores have become specialized to detoxify these chemicals and even to co-opt them for their own development and survival (Blum 1994). Chrysomelids can sequester plant chemicals in all life stages, in the body fluids or in special glands. Some chrysomelids even display warning coloration (aposematism) of bright or contrast colors, e.g., red, yellow, and black, to signal to other animals that they are offensive and not worth eating.

7 Tritrophic Interactions

Up until now, I have discussed species interactions between two tiers, plants and herbivores. But these are only two of the tiers that can connect longer chains of species interactions. Globally, complex trophic relationships have been documented between a few plant families, chrysomelid herbivores, and their carabid beetle parasitoids (Table 1).

Carabidae (ground beetles) is another speciose beetle family with ~40,000 species. Adults and larvae are commonly generalist predators of insects, but some subgroups have become specialized predators and parasitoids of chrysomelids (Erwin 1979;

Table 1 Tritrophic interactions between plants, chrysomelid beetle herbivores, and carabid beetle parasitoids

Plant	Solanaceae *Solanum*	Burseraceae *Commiphora*	Anacardiaceae *Sclerocarya*	Salicaceae *Salix*	Apocynaceae *Apocynum*
Herbivore	*Leptinotarsa*	*Diamphidia*	*Polyclada*	*Salix*	*Apocynum*
Parasitoid	*Lebia*	*Lebistina*	*Lebistina*	*Lebia*	*Lebia*
		African Poison Arrow Beetles			

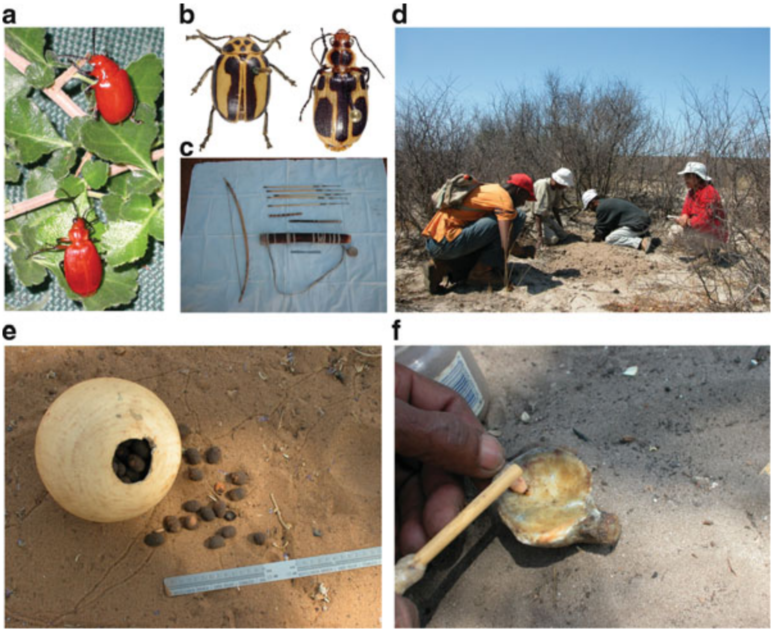

Fig. 3 (**a**) A live Lebistina adult (*bottom*) mimics the color and form of its prey, Diamphidia adult (*top*) on its host plant, Commiphora in South Africa (photo, K. Ober). (**b**) A dead Diamphidia adult (*right*) and its Lebistina parasitoid (*left*). (**c**) A San bushman's hunting equipment, with poisoned arrows. (**d**) Collecting poison beetles in the Tsumkwe Conservancy, Namibia (the *author in red shirt*). (**e**) An ostrich egg full of poisonous beetles. (**f**) Squeezing a poison beetle to apply its juice (hemolymph) to an arrow

Weber et al. 2006). Relationships in the plants–chrysomelid herbivores–carabid parasitoids associations have not been studied in detail but display many interesting biological phenomena including plant–insect interactions, beetle life history evolution (free-living to parasitoid), mimicry complexes (carabid parasitoids resemble their colorful aposematic chrysomelid hosts) (Fig. 3a, b), and defensive chemistry (sequestration and de novo synthesis). Some compounds are already known to be so highly toxic that southern African San tribes use crushed chrysomelids to poison the tips of arrows to kill large warm-blooded prey, such as antelopes and giraffes.

8 Arrow Poison Beetles

The San, also known as Ju/’hoansi or !Kung, are among the original inhabitants of southern Africa and have a distinctive clicking language. They are probably most familiar to the public as the central characters in the 1980 comedy film “The Gods must be crazy” and its four sequels (Uys 1980). About 80,000 San are left today, living mainly in Namibia and Botswana (Lee 2003; Lee and DeVore 1998). San hunters collect chrysomelid beetles in the genera *Diamphidia* and *Polyclada* throughout the year to use as poison on their hunting arrows (Breyer-Brandwijk 1937; Mebs et al. 1982). The beetles are not confined to southern Africa, but range from South Africa to the Sahara and into Zanzibar, and are found by locating their host plants, *Sclerocarya* (Anacardiaceae) and *Commiphora* (Burseraceae) (Fig. 3b).

Members of both the Anacardiaceae and Burseraceae have highly interesting chemistry. *Commiphoras* are well known as the sources of frankincense and myrrh, which were once so important medically in ancient times that they were mentioned in the New Testament as the choice of the precious gifts for the baby Jesus. Familiar members of the Anacardiaceae are avocadoes, mangoes, sumac, and poison ivy; these plants produce white or yellow latex that can have irritating properties.

Adult *Diamphidia* and *Polyclada* lay eggs on *Commiphora* and *Sclerocarya* and the hatched larvae go through several developmental stages before migrating underground where they build a protective cocoon. While underground, they develop through the pupal stage and emerge as adults. Larvae of the parasitoid carabid beetles seek out these underground pupae and attach themselves to the latter. The parasitoid feeds on a single host, consumes it completely, then pupates and emerges at the surface as an adult.

The chrysomelid poison arrow beetles are very effective, slowly paralyzing and eventually killing large mammal prey. It is unclear how old this San hunting practice is, but it appears that poison arrows originated in many cultures and in many places (Maingard 1932). For example, the use of poison dart frogs by Chocó Indians in Colombia is a well-known hunting practice (Myers et al. 1978).

Chemical relations between host plants, herbivores, and parasitoids are an interesting theme in this system. Resins of *Commiphora* and *Sclerocarya* have been commercially harvested for use as incense for over 3,000 years. Chrysomelid and carabid beetles are generally known as remarkable chemical factories, with some being lethally poisonous. Chemical analyses of *Diamphidia* pupae isolated a toxic protein, diamphotoxin (De la Harpe and Dowdle 1980; De la Harpe et al. 1983). Coincidentally, a similar molecule, leptinotarsin, has been identified in several species of the North American chrysomelid, *Leptinotarsa*, and its carabid parasitoid, *Lebia* (Hsiao 1978; Crosland et al. 1984). Both diamphotoxin and leptinotarsin act as slow-paralyzing agents and cause death within hours of injection in tested animals. A really interesting question to explore in the future is the reaction of the carabid parasitoid to the chrysomelid toxin – they may not react, or may require the toxin for their own development, or they might even sequester the toxins to use in their own defense. The latter scenario might explain the carabid's own aposematic and mimetic colors that makes it difficult to distinguish their host chrysomelids (Fig. 3a, b).

The San people have tremendous respect for these poisonous beetles – only the chief hunter is allowed to collect the live beetles and store them in ostrich egg shells (Fig. 3c), to be used sparingly or to be exchanged with hunters from other family units scattered throughout the Kalahari region. About ten beetles are crushed into a thick paste that is applied to each arrow (Fig. 3d). Unfortunately, a variety of modern political factors are forcing the San to become sedentary, give up their hunter-gatherer form of life, and stop hunting with poison arrows (Dieckmann 2007). Old indigenous practices such as these are disappearing around the world, but there is still much to understand and learn from such ancient cultures.

The global tritrophic association between host plants, chrysomelid herbivores, and their carabid parasitoids is an undeniably complicated one. The critical first steps in studying the evolution of this association are to systematically document individual relationships and develop evolutionary hypotheses for the taxa involved. Such data will identify the specificity of relationships and possible coevolutionary scenarios, and suggest close relatives that may share similar properties. Thus, evolutionary patterns can guide researchers to expand their database of knowledge and discover larger patterns in nature. While toxic beetles such as *Diamphidia* and *Polyclada* have not yet been discovered in the New World, one can predict that the chrysomelids used for San poison arrows probably represent a tip of the iceberg of diversity. This framework has proven to be a useful guide to discovering New World relatives of the African species that show similar patterns of plant associations in Peru and the Dominican Republic (Chaboo unpublished data). The next step is to examine the underlying chemical patterns that can explain the close association of this group of beetles with particular plant families.

9 Conclusions

After a long evolutionary history and intimate association with plants, dating to the Cretaceous, chrysomelids are extremely diverse in their ecology and behavior today. Elucidating the mode and tempo of their evolution and their relationships at the genetic, chemical, ecological, and evolutionary levels can shed light on how individuals live and survive, form communities and food chains, and interconnect into ecosystems. This chapter discusses just a few remarkable biological patterns in Chrysomelidae.

We remain uncertain about the exact number and distributions of solitary, gregarious, and subsocial chrysomelid species. The present list of known species can frame future field studies with the aim of discovering additional species, unveiling their reproductive biologies, and unraveling the ecological factors that drive social evolution. Similarly, the accumulated data on defensive behaviors for a small fraction of species enable detection of diverse patterns of behavioral, chemical, and physical defenses in all life stages. Now, research must transition to a new, more analytical phase by proposing specific hypotheses to discover additional species and better explain the defense patterns.

The chemistry of chrysomelids remains largely descriptive, identifying the molecules involved. Future research should change focus and explore the metabolic pathways of their creation, which may be due to sequestration directly from the host plant, manipulation of molecular structures, or de novo synthesis. Diamphotoxin and leptinotarsin are still to be compared in detail. It seems unusual, though not improbable over the long course of evolutionary time, for two similar-acting toxic molecules to appear in a clade of 40,000+ species. Phylogenetic connections at the levels of gene, species, and branches of the tree of life must be assessed to determine their relatedness. Their medical significance also awaits exploration – any natural molecule that can act with such toxic precision has potential in drug treatment or drug delivery. Research on the San arrow poison beetles is a race against time; indeed, indigenous tribes in very different geopolitical areas of the world are facing rapid extinction, along with their languages and ancient knowledge of nature.

Chrysomelid leaf beetles are a model system for biological research due to their species diversity, host plant relations, ancient lineage, and diverse biologies. A new generation of interdisciplinary chrysomelid biologists focusing on hyperdiverse tropical areas could greatly contribute to enhancing and expanding fundamental theories and models of life histories, mimicry complexes, chemistry, and species associations.

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Microbes: A New Frontier in Tropical Chemical Biology

Tiffany Weir, Daniel Manter, and Waltraud Kofer

1 Introduction

Upon his first encounter with a neotropical rainforest, Alexander von Humboldt provided this description . . . *the treetops, hung about with lianas, and crowned with great bushes of flowers, spread out like a great carpet, the dark green of which seemed to gleam in contrast to the light. . .. But more beautiful still than all the wonders individually is the impression conveyed by the whole of this vigorous, luxuriant and yet light, cheering and mild nature in its entirety* (Kritcher 1999). Indeed, the first Europeans to encounter these forests must have been agape with wonder at the massive buttressed strangler figs, kapoks, and Brazil nut trees adorned with winding, snakelike lianas. While this diversity is easily noted and appreciated, an even greater wealth of organisms dominated by fungi, bacteria, algae, and protozoa remains largely unexplored. These organisms collectively referred to as microbes make up more than 50% of the earth's biomass and as the oldest life forms on our planet have evolved an incredible array of diversity suitable for survival in every habitat imaginable.

Microbial diversity in soil ecosystems exceeds that of the more readily visible and appreciated eukaryotic organisms such as plants and animals. It has been estimated that 1 g of soil may support up to ten billion microbes comprised of

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thousands of different species, although less than 1% of these are amenable to laboratory study by culturing and growing under controlled conditions. In light of our inability to culture the overwhelming majority of microorganisms, several culture-independent techniques have been developed. The most popular of these is the analysis of rRNA gene sequences, based on the premise that these genes represent an evolutionary chronometer (Lane et al. 1985; Woese 1987; Stahl et al. 1985). While the original purpose of this technique was to elucidate evolutionary relationships between organisms, it has been successfully applied to the study of diversity as well. More recently, new methods such as pyrosequencing (Ronaghi et al. 1998) combined with high-throughput parallel platforms like 454 technology can simultaneously sequence up to 400,000 DNA molecules (e.g., rRNA gene fragments) yielding approximately 100 million base pairs of DNA sequence in a single day. To date, this technology has primarily been used as a rapid, low-cost, and high-quality method for sequencing entire genomes, although it has also been successfully used in metagenomic studies to examine microbial phylogenetic and functional diversity (Roesch et al. 2007; Sogin et al. 2006; Edwards et al. 2006). For example, in one environmental sample, Sogin et al. (2006) performed 18,000 pyrosequencing reads and found more than 3,000 unique rRNA molecules or operational taxonomic units (OTUs). By their estimation, the detection of low abundance OTUs could increase total estimates of marine microbial diversity by as much as three orders of magnitude.

Unfortunately, the revolution in culture-independent analysis techniques has not yet led to an explosion of microbial diversity studies in the Neotropics, and catalogs of microbial diversity are still dwarfed by those of plants and animals. A recent continent-wide study of soil bacterial diversity in North and South America included several Neotropical locations and had the surprising result that tropical soils contained the lowest biodiversity and species richness of any of the tested soils; concluding that the lack of diversity was correlated with low soil pH (Fierer and Jackson 2006). This result is intriguing and, although no direct relationship between aboveground plant diversity and soil microbial diversity has yet been shown, strong evidence suggests that each plant species shapes the underlying soil microbial community. Thus, one would conclude that the plant diversity of the tropics should support a diverse array of soil microbes. For example, Broeckling et al. (2008) used two model plant species (*Arabidopsis thaliana* and *Medicago truncatula*) and found that each cultivated its own unique soil microbial community and was able to modify, through its root exudates, the microbial community from foreign soils. Another study (Broz et al. 2007) showed a similar observation but under field conditions. This study found that soils collected in Montana from high-density stands of the Eurasian invasive plant, spotted knapweed, had significant declines in fungal community composition and diversity compared with soil collected from low-density stands of the weed. The fact that Eurasian invasive weeds decrease the diversity and relative numbers of microbes (Broz et al. 2007) in the soil strengthens the hypothesis that there is a coevolutionary link between roots and soil microbes that is mediated by the release of root exudates. As a result, one might expect a high microbial

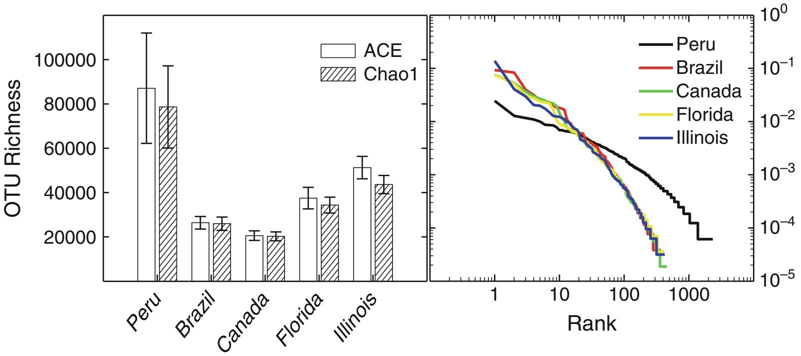

Fig. 1 Estimated number of total OTU richness (**a**) and rank–abundance curves (**b**) for the soil bacterial community at five sites. Brazil, Canada, Florida, and Illinois data is from Roesch et al. (2007)

diversity in the tropics consistent with the diverse plant community; however, it has gone undetected in previous studies (Fierer and Jackson 2006; Rousk et al. 2010) due to incomplete sampling, high spatial variability, and a low relative abundance of any given microbial species.

In a recent study, we assessed microbial species richness at three sites with varying overstory complexity (Manter et al. unpublished). By analyzing multiple soil samples from each site, we found that the soil microbial community in the rainforest of Tambopata is species rich (Chao estimated total richness = 22,157 ± 1097), extremely heterogeneous (Fig. 1) and typified by microbes with a low relative abundance, and as such could not be detected using standard soil sampling procedures. In this study, nine soil samples (0.5g) were collected around the base of three different Brazil nut trees and each tree supported a significantly different microbial community, which is consistent with the unique assemblage of plants surrounding each tree. Although the final assessment of total microbial diversity in the tropics awaits a more comprehensive analysis, it is clear that a diverse, but spatially heterogeneous, array of microbes is intricately involved with a healthy tropical rainforest, providing an equally diverse array of functions necessary to support the aboveground macrobiota.

2 Role of Microbes in Tropical Ecosystems

Microbes are responsible for numerous processes that keep ecosystems functioning properly. They have many of the same functions in tropical forests as in temperate ones, but because of the tight nutrient recycling required to sustain the high productivity of tropical rainforests, a disruption in the functional diversity of microbial communities may have more pronounced consequences. In the following sections, we highlight the roles of microbes in decomposition and nutrient cycling, as well as in biogeochemical processes.

2.1 Decomposition

It is little surprise that many early explorers of the tropical rainforest assumed that the lavish macrobiotic diversity and growth was supported by a fertile soil, leading to disastrous attempts at European-style agriculture in the tropics. In reality, most tropical rainforests are “wet deserts”, typified by nutrient poor, acidic soils. The rapid and complete decomposition of decaying organic material leaves little in the way of a humus layer, and high levels of rainfall wash away much of the essential nutrients from the thin layer of topsoil. These acidic soils are often high in aluminum and iron oxides that are toxic at high levels. The majority of the nutrients that are retained in the system are tied up in living plant material, thus a tight recycling of these nutrients is required for the proper functioning of tropical forest ecosystems. A consortium of microbes is the key element in this recycling effort. They are largely responsible for decomposition of litter on the forest floor, increasing the biological availability of some soil nutrients and the translocation of these nutrients back to actively growing plants. Without microbes, the illusion of tropical forest fertility would be shattered.

The rate that microbes can decompose matter is primarily thought to be a function of temperature and moisture; however, the possibility that microbes in the tropics produce more efficient enzymes for degradation is also worth exploring, particularly as the world is racing to develop improved plant-based fuels. Although termites, some invertebrates, bacteria, and fungi all contribute to decomposition, fungi are thought to be the most important decomposers of plant structural material, such as lignin and cellulose, in terrestrial ecosystems. However, we have conducted studies on lignin degradation using microbial slurries from decaying log samples collected in the Tambopata National Reserve in Peru and found that bacterial communities may be equally important in lignin degradation (Fig. 2). Termites, another important degrader, particularly in the tropics, also enlist the help of microbes in breaking down lignocellulosic material from plants. The termites “pretreat” wood by breaking it down into smaller particles providing better access for a community of more than 200 different species of microbes to break down the lignocellulose polymers into hydrogen and simple sugars. In the termite, a different set of microbial enzymes utilizes these degradation products to produce acetate, the energy source consumed by the termite (Breznak and Brune 1994; Warnecke et al. 2007). The United State Department of Energy is currently funding studies to sequence the genome of this microbial consortium to identify enzymes involved in hydrogen production that could be used in bioreactors to generate hydrogen fuel from wood products (Warnecke et al. 2007).

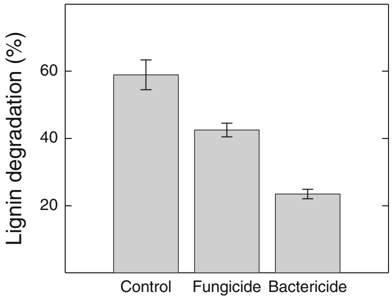

Fig. 2 A study using a microbial pool from soil samples obtained in the Tambopata National Reserve, Madre de Dios, Peru, shows that removal of either the fungal or bacterial component reduces the rate of lignin degradation. The addition of a fungicide (100 μg ml^{-1} cycloheximide) or bactericide (100 μg ml^{-1} streptomycin, 100 μg ml^{-1} penicillin) reduces the rate of lignin decay. From this study, it appears that both taxonomic groups are required for the efficient and rapid degradation of lignin. It is also interesting that the bactericide treatment caused a significant reduction in lignin degradation suggesting a significant role for the bacteria in this process – a contribution that is frequently overlooked

2.2 Nutrient Uptake

While some populations of microbes are occupied with releasing nutrients that are tied up in organic matter, other populations are busy returning them to inhabitants residing above the forest floor. Most plants form beneficial associations with fungi and bacteria to help them obtain and utilize essential elements, such as nitrogen and phosphorus. Perhaps the most ubiquitous and best understood of these symbioses are the associations formed between plants and mycorrhizal fungi. Roughly 92% of all plant families, or 80% of all plant species, form mycorrhizal associations (Wang and Qiu 2006). The fungi form associations either through direct penetration of the plant cell membrane (endomycorrhizae) or by sheathing the root tips with hyphae (ectomycorrhizae) (Fig. 3). In either case, they have direct access to carbon sources that are produced by the plant through photosynthesis and then translocated from the leaves to the roots. In return, the massive hyphal network is both physically and chemically more suitable for obtaining water and nutrients, particularly inorganic phosphates, and this direct connection with plant roots greatly extends the root system of the plant. These associations are particularly important in nutrient poor tropical soils. In addition, this tangled network of root and hyphae forms a dense mat on the forest floor that helps to stabilize and anchor the plants and helps to minimize loss of soil during the frequent and heavy rainfalls.

The chemical signaling events involved in recognition between plants and mycorrhizae are still being worked out. There is a large body of evidence suggesting that plant root exudates, particularly flavonoids, which have been implicated as signals in other plant–microbe associations, have growth-stimulating effects on compatible mycorrhizae (Tsai and Phillips 1991; Poulin et al. 1997; Akiyama et al. 2002). However, despite stimulating the growth of some mycorrhizal fungi, there is

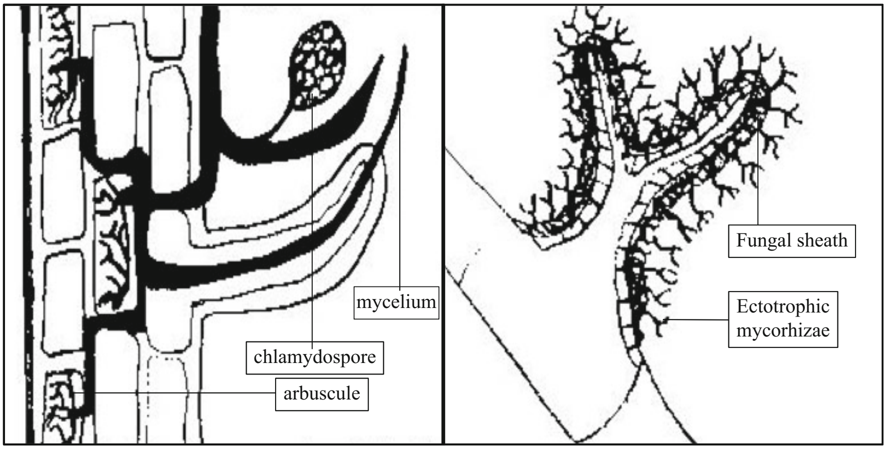

Fig. 3 The *first panel* shows an endomycorrhizal association with a root. The hyphae of endomycorrhizae penetrate the walls of root cortical cells, where they form vesicles and arbuscules. Approximately 85% of all plant species form this type of association with mycorrhizal fungi. By contrast, ectomycorrhizae are much less prevalent with only about 10% of plant species form these associations. They do not penetrate the cortex, but instead form a fungal sheath around the root tip and an inward growing Hartig net, which surrounds the root cortical cells. Ectomycorrhizal associations are generally restricted to short lateral roots

little evidence to suggest that flavonoids are directly involved in host–symbiont recognition. A breakthrough in identifying an early chemical signal involved in mycorrhizal colonization occurred during a study with a model legume plant, *Lotus japonicus*. Prior to plant root penetration, the fungal hyphae will undergo extensive branching when within the vicinity of a host root and a component of root secretions from *L. japonicus*, strigolactone, was identified as the chemical that causes this branching to occur (Akiyama et al. 2002). Strigolactones are widely produced in the plant kingdom and their secretion by some plant roots is reportedly enhanced under conditions of low inorganic phosphate (Yoneyama et al. 2001), making this a plausible candidate for a host–symbiont recognition signal. It is interesting to note that these compounds also stimulate seed germination of some parasitic plant species, suggesting the possibility that these parasites have evolved the ability to eavesdrop on plants recruiting beneficial fungi and have seized that opportunity to infect host plants (Cook et al. 1966).

Many bacteria are also useful in assisting plants to obtain nutrients from their environment, with the nitrogen-fixing rhizobial bacteria being the most commonly cited example. The symbiosis occurs when chemical signaling between the plant and bacteria results in the formation of a symbiotic structure, a nodule, on the plant root (Fig. 4). The bacteria inhabit this structure and alter their metabolism to be able to fix atmospheric nitrogen to a form usable by the plant. This symbiosis is unique between members of the legume family, Fabaceae, and a diverse group of bacteria that are primarily classified in the order Rhizobiales. However, only a small percentage of nodule-forming bacteria have been characterized, and it was

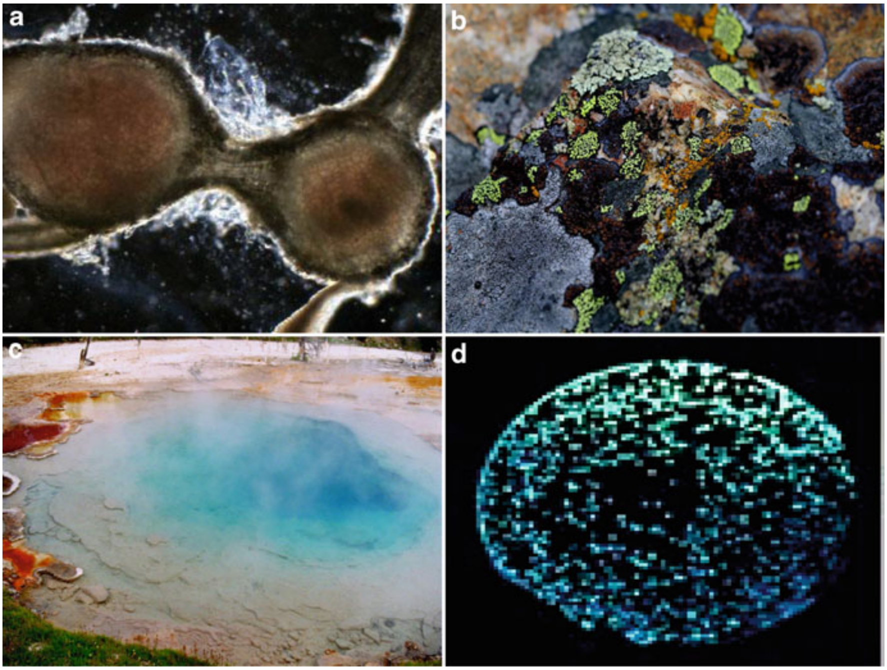

Fig. 4 (**a**) Nodules, housing the symbiotic bacteria *Sinorhizobium meliloti*, in the roots of alfalfa (photo provided by Clelia de la Peña). (**b**) Lichen are multi-organism communities that assist in the process of rock weathering. (**c**) A thermal pool at Yellowstone National Park houses numerous extremophiles, microbes capable of surviving in extreme environments. (**d**) Colonies of bacteria that utilize quorum-sensing to emit light. (Photos provided by T. Weir)

discovered that several tropical legumes form this symbiosis with distantly related members of the order Burkholderiales, suggesting that a much more diverse group of bacteria is capable of nodulation and that much of this diversity may exist in tropical environments (Moulin et al. 2001). Like in mycorrhizal associations, the secretion of flavonoids from plant roots is an important chemical signal. These plant chemicals stimulate the secretion of bacterial proteins that are in turn recognized by the plant, resulting in biochemical and physiological changes in the plants that facilitate the formation of nodules and allow infection by the bacteria. This symbiosis is agriculturally important as nitrogen tends to be deficient in soils and addition of synthetic nitrogen fertilizers is costly and damaging to the environment. As a result, many crop legume seeds can be purchased with a rhizobia seed coat to facilitate the symbiosis and ensure the presence of the bacteria in a particular soil and legumes are often important components of crop rotation schedules and intercropping systems.

Other bacteria are also capable of forming symbioses that provide nitrogen for use by plants, but their contribution in tropical rainforest ecosystems is far less than the rhizobia–legume interaction. The actinomycete, *Frankia*, functions similarly to

Rhizobia but interacts with several different plant families to form nitrogen-fixing nodules and is more prevalent in colder climates, although it may be important in some high elevation tropical forests. Cyanobacteria can also fix atmospheric nitrogen and will form associations with tropical species such as bryophytes that grow as epiphylls on the leaf surface of other plants and with cycads. There are also numerous free-living bacteria that fix nitrogen in the soil, but in general their contribution of available nitrogen generated is much less than obtained through symbioses.

Finally, there is a group of soil-dwelling bacteria that have been designated generically as plant growth-promoting rhizobacteria (or PGPRs), including any microbe bacteria that has direct or indirect positive effects on plant growth and yield. The PGPRs are usually studied in the context of agriculture, and indeed their use in some tropical agricultural systems is fairly widespread. In Mexico, more than 350,000 ha. of maize is inoculated with the PGPR *Azospirillum brasilense*, while the inoculation of soybean with *Bradyrhizobium japonicus* is common in Brazil and Bolivia (Castro-Sowinski et al. 2007). The mechanisms by which these microorganisms benefit plants are as varied as their taxonomic classifications. Many PGPRs act indirectly to benefit plants by producing antibiotics that kill potential pathogens or by producing chemicals that stimulate other beneficial organisms such as mycorrhizae. A strain of *Streptomyces* has been reported to simultaneously induce growth of the beneficial ectomycorrhizae *Aminita muscaria* by secreting auxofuran, while suppressing pathogenic fungi through the secretion of several antibiotics (Riedlinger et al. 2006). More direct mechanisms of PGPRs include the Rhizobia–legume symbioses described in an earlier section, competitive colonization of the roots thus providing a type of protective barrier against pathogens (Bais et al. 2004), and induction of a plant’s systemic defense responses (Domenech et al. 2007) rendering them more resistant to pathogen attacks. As mentioned, the inoculation of crop plants with these PGPRs is increasingly being used as a tool in integrated pest management and sustainable agriculture practices, but there is still little known about the importance of these bacteria in natural environments. However, there is a growing body of evidence suggesting that plant root secretions attract and maintain specific rhizosphere microbial communities (Broeckling et al. 2008; Broz et al. 2007), and as these studies progress it will likely shed new light on the importance of PGPRs outside of agroecosystems.

3 Biogeochemistry

3.1 Soil Formation

Over millions of years, water trickling down from the high Andean sierra and converging into the Colca River has traveled between the Nevado Ampato and Nevado Coropuna volcanoes creating the Colca Canyon, a geological rift over

3,500 m deep. This impressive and dramatic image typifies rock weathering processes and indeed the inorganic aspects of mineral weathering have been the best studied (White and Brantley 1995). However, one of the most important sources of rock weathering and erosion on Earth is microbes. They contribute to the formation of soils and the release of minerals that are essential for all forms of life. Microbes use various means, both mechanical and chemical to break down rock and solubilize their mineral components. Like drops of water that collect in small cracks and expand by freezing to create larger fissures, fingers of fungal hyphae invade microcracks in rock surfaces and can gradually generate enough stress to cause a fracture. Lichen, a composite organism comprised of a symbiosis between a fungus and a photosynthetic organism, typically an algae or cyanobacterium, penetrate rocks using thalli that absorb water and swell, generating additional stress. They also deposit organic and inorganic salts that can absorb water and expand (Fig. 3).

The most basic mechanism of chemical weathering used by microbes is the secretion of chemicals that improve the solubility of typically insoluble elements and minerals such as iron and aluminum (Blum 1988; Welch and Ullman 1993). Microbes do this by secreting acids, which lower the pH in their immediate vicinity increasing the solubility of particular elements. They also secrete chemicals such as chelators and siderophores that have a strong affinity for particular ions and directly weaken metal–oxygen bonds. Returning to the case of lichen, it has been suggested that the photosynthetic component of lichen communities provides carbon sources to drive the secretion of organic acids from fungal components, which in turn mobilize and transport mineral nutrients (Banfield et al. 1999). The role of carbon funneled to mycorrhizal fungi through plant root associations is also being investigated to determine its importance as a driver of chemical weathering processes that result in soil formation from bedrock (Kelly et al. 1998).

Another group of microorganisms responsible for rock weathering are the extremophiles, a group of organisms capable of surviving in environments where few if any other life forms can survive (Fig. 3). Among these extremophiles are microbes called chemolithotrophs, “rock eaters”, which consume electron-rich inorganic compounds for energy. For example, sulfur bacteria metabolize inorganic sulfur to sulfate, and nitrifying bacteria oxidize inorganic nitrogen compounds for energy, while iron bacteria derive their energy from oxidizing the ferrous ion, manganese, or aluminum. In all cases, acid end products are produced that further break down rocks. While these microbes are very helpful in breaking down inorganic materials on a small scale, on a larger scale they are often responsible for environmentally harmful phenomena such as acid mine drainage.

3.2 Atmospheric Contribution of Soil Microbes

Tropical soils play an important dualistic role in the mediation of climate. On the one hand, they are active sinks for many gases, and on the other hand they also emit greenhouse gases and add to global warming. The main actors are soil microbes, which control the flux of atmospheric trace gases, such as hydrogen gas (H_2), carbon dioxide (CO_2), methane (CH_4), carbonyl sulfide (COS), nitrous oxide (N_2O), and nitric oxide (NO). Greenhouse gas production in soil is complex and depends on environmental factors, such as climate, soil properties, vegetation, topography, and human activity, and also varies greatly by site and time, making it difficult to generate realistic estimations on a global scale. Furthermore, microbial diversity and its effect on trace gas fluxes has not been taken into consideration despite being an important aspect for estimating the influence of microbial metabolic processes of tropical forests on global trace gas budgets.

In general, soil redox potential, carbon source, and electron acceptors govern the microbial production or consumption of greenhouse gases in soils. For example, the availability of electron acceptors varies with soil aeration and is a major determinant of the quality and quantity of trace gases emitted or consumed. If soils are aerated, microbes utilize oxygen (O_2) as the dominant electron acceptor resulting in release of carbon dioxide (CO_2). When soils are irrigated or after major rainfalls, O_2 in the soil is decreased and CO_2 production also gradually decreases until soils are anaerobic and nitrate becomes the main electron acceptor resulting in the production of nitric oxide, nitrous oxide, and nitrogen gas. If anaerobic conditions persist, Mn^{4+}, Fe^{3+}, sulfate and finally hydrogen are used as electron acceptors. Hydrogen is used by methanogens, resulting in the production of methane.

Annual and seasonal changes in microbial-based soil emissions are natural and depend on soil microbial activity. Tropical soils are active emitters of nitrous oxide (N_2O), which is the third most active greenhouse gas, contributing about 6% to global warming and is responsible for the reduction of ozone in the stratosphere. In general, soil N_2O emissions are due to microbial consumption of NH^{4+} or NO^{2-}, which are utilized by nitrifying or denitrifying bacteria (Poth and Focht 1985). Nitrous oxide emissions of tropical rainforest soils have been estimated in the range of 1.3 Tg N/year. The N_2O budget in recent years has been greatly affected by changes in land use and climate, resulting in increased N_2O emissions from tropical soils.

The soil–atmosphere exchange of methane (CH_4) is also the result of simultaneously occurring production and consumption. Because of the differences in precipitation and temperature, the CH_4 sink/source budget of tropical soils varies diurnally and seasonally. Under anaerobic conditions, especially after continuous rainfalls, when soils are waterlogged and depleted of oxygen by decomposing bacteria, methanogens use hydrogen as an electron acceptor to produce methane. By contrast, under aerobic conditions, methanotrophs consume methane, using oxygen as their terminal electron acceptor (Topp and Pattey 1997). Thus, tropical upland forests mainly act as sinks for atmospheric CH_4 (Butterbach-Bahl et al. 2004; Kiese et al. 2003; Keller et al. 1986). Other factors playing key roles are soil pH and microbe substrate availability (Chan and Parkin 2001; Khalil and Baggs 2005; Smith et al. 2003).

Similar to N_2O and CH_4 emissions, variations in carbon dioxide (CO_2) emissions originating from primary rainforest soils are largely affected by changes in soil moisture, thus emission in the wet season and during rainy periods is markedly higher. The average carbon dioxide (CO_2) efflux from primary rainforest tropical soils is estimated to be between 60 and 88 mg $Cm^{-2}\ h^{-1}$ (Kiese and Butterbach-Bahl 2002; La Scala et al. 2000; Ishizuka et al. 2002; Raich 1998). Doff Sotta et al. (2004) described the mean annual efflux from the Amazon region to range between 44.6 and 76.3 mg $Cm^{-2}\ h^{-1}$ during the dry season and higher (>85 mg $Cm^{-2}\ h^{-1}$) during the wet season.

Carbonyl sulfide (COS) is normally produced as a byproduct of carbon disulfide (CS_2). It is thought that in the troposphere CS_2 is oxidized by a reaction with OH, producing COS and sulfur dioxide (SO_2). Carbonyl sulfide is one of the most abundant volatile sulfur compounds in the atmosphere with an average global concentration of about 500 ppt (Barnes et al. 1994); it has a long troposphere lifetime (2–7 years) and causes chemical ozone destruction (Lacis et al. 1992). Besides photolysis and reactions with oxygen-free radicals in the stratosphere, vegetation and soils are the main terrestrial sinks for COS. In general, forest soils act as sinks rather than sources of COS and CS_2 gases (Van Diest and Kesselmeier 2008; Kesselmeier et al. 1999). Major determining factors are soil water content, as diffusion is an important factor for COS uptake, and temperature which affects the activity of carbonic anhydrase, the main enzyme for COS uptake in soil microorganisms (Kesselmeier et al. 1999; Kuhn and Kesselmeier 2000). Unfortunately, there are few data on COS and CS_2 emissions from tropical soils rendering it very difficult to estimate global soil emissions of COS.

Driven by environmental concerns, recent monitoring techniques and GIS-coupled biochemical models are being designed as tools to simulate greenhouse gas emissions to estimate carbon sequestration, trace gas emissions, and denitrification–decomposition processes of high temporal resolution for various ecosystems and regions and under varying environmental conditions (Butterbach-Bahl et al. 2001; Kiese et al. 2005; Werner et al. 2006). It is very likely that these will be realistically applicable on a continental and global scale within the next few years. These models together with further cataloging of functional microbial diversity of tropical forest soils will help to greatly enhance our knowledge about the contribution of tropical forests to atmospheric trace gas budgets playing a role in climate regulation.

4 Cell-to-Cell Communication or “Microbial Chemical Ecology”

In the warm, shallow waters of the Pacific Ocean lives a curious sea creature, the bobtail squid, which remains hidden in sand by day and emerges to hunt at night. This clever creature has found a way to deter its nocturnal predators by producing a light organ that glows pale blue through the water, mimicking moonlight and preventing its own shadow from being cast on the sandy sea bottom. The light organ of the bobtailed squid, as well as several other squid and fish, is populated by the luminescent bacteria *Vibrio fischeri,* and it was studies using this tiny organism that revealed to the scientific world that bacteria could speak.

Chemicals that are exported by one bacterial cell and received by another mediate the language of bacteria, often referred to as "quorum sensing" or "cell-to-cell communication". Once the signal has been received, it results in the expression or repression of sets of genes that alter the behavior of the cell. Bacteria use this chemical language to coordinate group behavior in a bacterial population. These behaviors are as variable as the chemical signals used, ranging from the production of light-emitting proteins, the activation or suppression of virulence determinants, the ability to take up foreign DNA, and metabolic adaptations to adverse environmental conditions. In the case of *V. fischeri*, when the exported acylhomoserine lactone (AHL) signaling molecule is produced by a "quorum" of bacteria present in the light organ of a host squid, the molecules are perceived by receptor proteins in the bacteria, activating them. These activated receptors in turn bind to the DNA and initiate the expression of genes that lead to the production of the light-emitting protein luciferin. Thus, only when the bacteria have reached a threshold population is the light produced. This strategy prevents the bacteria from wasting energy in light production before they have reached sufficient numbers to create the desired effect.

The discovery of this chemical signaling system in *V. fischeri* was originally thought to be an interesting finding but of little scientific importance. It was only once chemical forms of cell-to-cell communication were found to be the norm in bacteria rather than the exception that the true magnitude of this discovery was realized. In the past two decades, cell-to-cell signaling systems have been identified in numerous bacterial species, fungi, and yeast. Perhaps some of the most controversial topics in this field today are whether different species can intercommunicate, what signals they use, and how they differentiate these signals. One prominent researcher from Princeton University has discovered that bacteria may use a signal molecule that she believes to be a universal language. The signals, generically termed AI-2, are a family of molecules derived from a single precursor that is capable of undergoing spontaneous rearrangement, allowing bacteria to respond to those that are produced by their own species but also to recognize and react to the molecules produced by other species (Xavier and Bassler 2005).

When the field of chemical ecology was conceived, it is unlikely that its founding fathers ever dreampt that the most prevalent form of chemical communication was occurring between microorganisms. However, as more microbial communication is decoded, we are beginning to learn not only how these signals affect the species that produce them, but also how they are incorporated in multispecies interactions and might influence the ecology of microbial communities. One elegant example of this is the *Pseudomonas* quinolone signal (PQS) produced by the ubiquitous and opportunistic pathogen *Pseudomonas aeruginosa*. This extremely hydrophobic signal is transported through aqueous environments packaged into vesicles. However, these tiny vesicle packages also contain other quinolones that act as antibiotics so if the signal is intercepted by another species of bacteria, the message received is fatal

(Mashburn and Whiteley 2005). This gives *P. aeruginosa* a competitive advantage in mixed species environments. *Bacillus* spp., Gram-positive bacteria that utilize short peptides as their primary signaling molecules, have also found an ingenious way to gain a competitive edge in mixed species interactions. These bacteria secrete a lactonase with broad specificity that allows them to degrade the AHL signaling molecules produced by many Gram-negative bacterial species. This effectively allows the *Bacillus* to carry on their own chemical communication while silencing that of their competitors (Teplitski et al. 2000; Dong et al. 2000).

Some eukaryotes have also developed their own chemical signals and other mechanisms that are capable of confounding bacterial quorum sensing. Numerous species of plants have been shown to secrete chemicals that mimic quorum sensing signals (Teplitski et al. 2000) potentially leading to the premature activation of quorum sensing pathways in pathogens and allowing the plant to detect and respond to them before there are sufficient numbers to result in an infection. The marine algae *Delisea pulchra* thwarts bacterial quorum sensing by producing structural analogs of the AHL signals, which competitively bind the AHL receptors and result in their degradation (Manefield et al. 2002). Finally, there is also some evidence that humans have evolved mechanisms against bacterial quorum sensing. A family of enzymes called paraoxonases (PON) contains at least one member that is capable of inactivating many different AHL signal molecules (Draganov et al. 2005).

5 Conclusions

The science of microbial ecology is in it's infancy, despite the numerous technical issues regarding sampling, terminology, and data evaluation that are still being refined. To complicate matters, many microbial ecologists have adopted terms used in traditional ecological studies, such as community and species, to describe molecular data derived from collected pools of environmentally isolated nucleic acids. This often leads to some level of confusion among taxonomists and ecologists. Despite these shortcomings, as the methods for detecting and identifying microbes in environmental samples has become more sophisticated, we are realizing just how few of them have been studied and characterized. This is particularly true in the humid tropics where many macroorganisms are still awaiting discovery. Although we have touched on only some of the more prominent roles of microorganisms in tropical rainforests, they also help to shape and influence the ecosystem in numerous other known, and unknown, ways that are too numerous to cover here. Each of these roles is defined by the chemistry of these organisms, making the area of tropical microbial chemical ecology an exciting and relatively untouched new field of research. Finally, just like the aboveground macrobiota that is part of the beauty and majesty of the more visible tropical rainforest, it is becoming increasingly clear that the same level of diversity exists for the largely “invisible” microbial community that is critical for its health and continued survival.

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Out on a Limb: True Confessions of a Bug Detective

Margaret D. Lowman

Abstract In a complex co-evolutionary dance, insects influence, and are influenced by plant chemistry and phenology. Although there is great controversy about the magnitude and setting of global species-richness on our planet, the bulk of terrestrial biodiversity reputably consists of tropical insects and plants. Tropical canopies are renowned not only for their high diversity of species, but also for their range of alkaloids, latex, and other secondary metabolites which in turn inspire a wealth of counter-adaptations by phytophagous insects. Herbivores in tropical tree canopies were literally unknown until canopy access was developed in the late 1970s. With the advent of single rope techniques to climb tropical trees in Australia, the world of canopy plant-insect interactions was discovered. Since then, it has become a hot-spot for biodiversity and for investigations about plants, insects, and the spatial and temporal attributes of their complex interactions.

1 Introduction

Canopy research is a mere 30 years old, making it one of the planet earth's most recent arenas of field exploration (Lowman and Rinker 2004). When pioneering biologists first gained access to tree canopies, little information about the biodiversity, processes, or critical interface between forests and atmosphere was understood. Through a great deal of perspiration, and perhaps a lesser amount of inspiration, canopy biologists discovered a new frontier high above the forest floor. As the first woman canopy ecologist working in Australian tropical and dry forests in the early 1980s, I welded my first slingshot from a length of iron pipe in the university repair shop, bought a length of rope, and sewed my first harness on a friend's industrial sewing machine (Lowman 1999). Armed with this simple

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equipment, I was fairly naïve about what towered beyond my reach in the tall *Nothofagus* and *Cedrela* trees of Australia's little-known forest remnants.

Through sheer determination and the good fortune to develop a deadeye with my slingshot, I amassed new knowledge about rain forest canopy leaves – their longevity, the existence of leaf cohorts defined by different spatial and temporal factors, herbivory levels, and the chemical warfare between foliage and insects. Although we scientists loathe admitting, some of our best discoveries are made quite by accident, and I am no exception to that rule. Other breakthroughs come from patient, long-term observations while living and working in the forests, in my case as a lone woman graduate student. This chapter summarizes some of my stories that led to discoveries about insect–plant interactions in the canopies of different forests – a lifetime of suspension "out on a limb".

2 The Pioneer Spirit: Tools, Methods, Sweat, and Toil

In the 1970s, tropical rain forests were considered a biological black box (translation: a big, dark region full of unknown phenomena). How many species existed in these complex forests? What mechanisms led to the coexistence of so many creatures in one place? How can ecologists begin to understand the intricate relationships between animals and plants in tropical forests before they are all destroyed by logging, burning, and clearing? As a young botanist and aspiring Indiana Jones, I wanted to confront the mysteries of these poorly understood ecosystems.

My career has not been conventional: I climb trees for a living. The realization that science had overlooked exploration into the tops of trees first transpired in the 1970s, when a few unlikely ecologists on different sides of the world began to look upwards, wondering what existed in those dark pockets of dense canopy foliage. As a graduate student in Australia, I chose to climb trees quite by accident. Only much later in my career have I recognized that many wonderful and creative discoveries in science actually happened quite by accident. In the case of canopy biology, it reads almost like a fairy tale (Lowman 2004).

I had selected Australia as my graduate research destination for several reasons. It was English speaking, and perhaps the only developed country in the world with true tropical rain forest. Yet its forests remained some of the least studied from a global perspective and also some of the most threatened by human activities. By the late 1970s, over three quarters of Australia's primary tropical and subtropical rain forests had been logged or cleared for agriculture, leaving only small patches of remnant vegetation, usually in inaccessible gullies where timber-getters could not go. The Queensland Government intended to just keep on cutting, reflecting the popular Aussie expression, "If it moves, shoot it; it if grows, chop it down". Australia boasted superb ecological gradients from cool temperate rain forests on the mountaintops and in Tasmania, to lowland rain forests in the humid valleys of Queensland, with subtropical patches in between. In addition, expanses of dry forest dotted the west-facing slopes of the Great Divide. In addition to the notion of unexplored rain forest, what aspiring ecologist is not fascinated by an island continent abounding with such unique creatures as koalas, wallabies, and cassowaries, to name but a few? And so I chose the “lucky country” for my first foray into field ecology, little knowing how unlucky I might be in facing the challenges that Australia offered: a white male-dominated society, devoid of other rain forest scientists for collaboration, a dearth of maps or species distributions, relatively little consensus on ecological attributes or even the names of many species, little respect by timber-getters for the boundaries of the national parks, the aggressive wait-a-while vine capable of holding back even the most intrepid rainforest explorer, and an incredible abundance of poisonous snakes underfoot!

In an evolutionary sense, Australia is unique because it represents the interface of two groups of plants: tropical flora from Indonesia and a temperate element originating in Antarctica/New Zealand. This overlap provides a relatively high diversity within one island continent and associations of plants found nowhere else on the planet. As a naïve, young graduate student at Sydney University, I followed the instructions of my supervisor who advised me to “drive north until it turns green, and then study it. Return to Sydney in three years with your results”. So my first introduction to Australian rain forests was the challenge of trying to recognize them, locate appropriate and safe sites, and then to select questions worthy of a PhD thesis. Fortunately, in this country of 7,682,300 km^2, the majority of vegetation was blue-gray (not tropical green) due to the coloration of the dry sclerophyll vegetation. So ultimately, finding areas of “green canopy” was indeed the best way to identify a patch of rain forest. I drove many thousands of kilometers during my first few weeks of exploration to determine where, how, and what would become the basis of my doctoral research.

Prior to the 1970s, most ecological work on tropical rain forests had been descriptive rather than experimental (e.g., Richards 1952; Jacobs 1981). Many tropical biologists in the late twentieth century grew up in the temperate zones, creating a bias in their approach and expectations of forests. Leaf longevity was one of those notions; as a product of a temperate upbringing in the New England region of North America, I only knew about forests where the leaves regularly abscised each year to avoid the rigors of winter. My first glimpse of the splendors of a lush, green tropical canopy and the notion that this ecosystem remained green year-round prompted the obvious question: What were the growth patterns, or phenology, of Australian rain forest leaves? This became the initial basis of my research: to monitor and track leaf longevity. But to quantify leaf longevity and the factors that affected a leaf’s lifespan, I needed to confront the leaves face-to-face, and the majority of them grew many meters above ground. I did not intend to climb trees as a career. In fact, I tried desperately to seek alternatives – Train a monkey? Utilize remote cameras on pulleys? Lean off cliff edges into adjacent tree crowns? Utilize satellite imagery? Alas, nothing quite sufficed for just being there. And to permanently mark and monitor canopy foliage, I simply had to figure out how to reach them. So I become an Arbornaut, or treetop explorer (Lowman 2004)!

Hearing about the Speleological (caving) Club at Sydney University, I befriended a crazy group of students (mostly male) with a passion for descending on ropes into caves. I explained my counter-intuitive desire to ascend on ropes into the tops of trees, rather than underground. They chuckled, and obviously thought I was quite insane. But they enthusiastically offered to train me in the skills required to master the hardware of single rope techniques (SRT). Australia had no REI catalog, L.L. Bean or other recreational outfitters, so I sewed my first harness using an industrial sewing machine and seat belt webbing. My canopy fashion garment was bright orange and terribly uncomfortable by today's professional arborist standards, but it worked. I purchased some rope and hardware from my caving colleagues, and crafted a slingshot in the Sydney University workshop, by simply heating up a length of iron and bending it into an appropriate shape. Some strips of rubber tubing provided a sling, and some lead fishing weights offered an excellent shot to complete my deadeye device.

My first climb into the crown of a coachwood (*Ceratopetalum apetalum*) was fraught with flailing, upside-down maneuvers as I struggled to center my body weight in a position conducive to grabbing foliage and studying it. The correct movement for an experienced tree climber is more or less similar to becoming a human inch-worm,

Fig. 1 Canopy access using SRT (single rope techniques) that launched treetop research

bending the knees and then standing up, over and over and over, while inching up the tree as the mechanical jumars grip the rope in the process of ascent. Watching an experienced tree climber is almost akin to appreciating fine ballet – the motions are graceful and elegant when properly executed. Despite many sore muscles the following day, the sensation of scaling my first tree was superb. I never looked back… or down… after that, and the forest canopy became my professional playground in which to ask exciting and unanswered questions about how forests function (Fig. 1).

2.1 Herbivores Rule*: Specialists, Generalists, and an Apothecary in the Sky*

My first field site was called Mt. Keira Reserve near Wollongong, New South Wales, where a lush subtropical forest boasted some enormous giant stinging trees. The gympie gympie or stinging tree (*Dendrocnide excelsa*) became a focal research species, due to its alleged toxic leaves that reputedly boasted both chemical and physical protection. The giant stinging tree leaves were densely covered with chemical and physical-stinging hairs, and very few creatures appeared adapted to eat its foliage. Upon close encounters in the canopy, however, I observed that the stinger leaves were bespotted with a characteristic lacework of holes, indicative of a dedicated (or specialist) herbivore (Fig. 2). It was impossible to study and monitor these leaves without severe stings (similar to a wasp sting), but my curiosity was overwhelming. While this physical defense is toxic to mammals (e.g., dogs have

Fig. 2 Leaves of the giant stinging tree with characteristic lacework defoliation

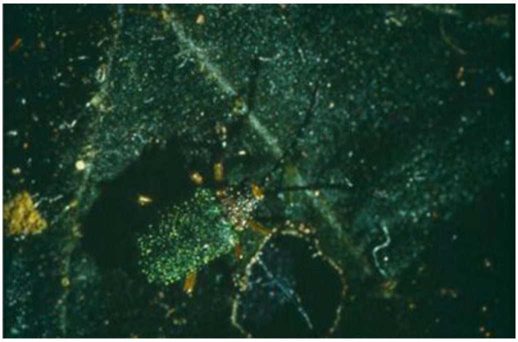

Fig. 3 Giant stinging tree beetle

been killed by wandering into a giant stinging tree thicket, and this species was planted in hedges as part of biological warfare in the Vietnam War) one host-specific beetle (*Hoplostinis viridipenis*) was undeterred by its chemical and physical defenses (Fig. 3). The beetles, gorgeously camouflaged with a brilliant green carapace, ate an average of 32.5% leaf surface area through the stinging tree canopy, losses that were almost doubled energetically for the entire tree since the leaves only lived for an average of 6 months (Lowman 1985, 1992b). Stinging tree hairs may have evolved as a defense against mammalian herbivores in Asia where the family Urticaceae evolved, but they were less effective against specific beetle herbivores that managed to digest and sequester the toxins. Camoflauged perfectly in the stinger foliage, stinging tree beetles gained the added protection of residing on a surface where no insect-eating birds or mammals cared to hunt. Such intricate relations between insects and leaves ultimately became the focus of lifelong research. While leaf longevity was an initial curiosity, it soon became evident that insect herbivores determined the fate – and longevity – of most rain forest leaves.

For analyses of leaf chemistry versus toughness attributes, five leaf age classes were quantified (Lowman 1984):

1. Young leaves: 0–2 weeks old, after budburst but before leaf was full sized
2. Youthful leaf: 2–4 weeks old, full-sized but lacking in heavy cuticle, dark green pigmentation, or other attributes that are characteristic of a mature leaf
3. Mature leaf: from 1 month old to 1 year old, with full size and complete structural attributes
4. Old leaf: second year leaves, distinguished from age class 3 by position on the branch, by dark (shade) or yellow (sun) coloration, and often by early signs of epiphylly or senescence
5. Senescent leaf: distinguished by basal position on branch, loss of chlorophyll, onset of decomposition, or accelerated browning or yellow/red coloration

Giant stinging tree leaves averaged 32.5% (SE 3.6) annual leaf area loss throughout its canopy, almost all of which qualified as sun leaves because these trees grew in gaps. Across different height regimes, leaf area loss averaged 42.6%

Table 1 Differences in herbivory among five different ages and species of Australian ran forest leaves (expressed as the mean proportion of leaf area grazed by insect herbivores, $n = 100$ leaves)

Tree species	% Herbivory by age class					Mean%	Regression (r^2)	
	1	2	3	4	5	Herbivory	Phenols	Toughness
N. moorei	13.0	17.0	1.5	0.1	0.1	30.7	0.30	0.90
T. australis	0.03	1.0	2.9		0.9	4.8	0. 20	0.68
D. excelsa	6.8	10.1	5.7		8.0	32.5	0.19	0.68
C. apetalum	10.5	12.5	3.0	0.6	0.5	26.1	0.40	0.40
D. sassafras	6.2	7.5	0.3	0.3	0.2	14.5	0.55	0.90

Source: (Lawman 1992b)

in the lower canopy (0–2 m), 24.6% in the mid canopy (3–7 m), and 21.2% in the upper canopy (> 7 m) (Table 1). Since more than 95% of herbivory was due to stinging tree beetles, it was obvious that herbivores preferred the understory foliage, although the entire canopy of this tree was consumed significantly more than almost any other Australian subtropical rain forest species. Stinging trees were the only canopy tree species whose young leaves were tougher than older leaves; all other species significantly increased in toughness with age. Stinging trees also underwent the highest level of annual leaf area consumption by herbivores (averaging 32.5%), perhaps in part because mature leaves were relatively soft and herbivores continued to eat mature leaves (which did not happen in neighboring species). Stinging trees also had the lowest levels of phenolics compared with other Australian rain forest species and was the only species measured where phenol contents decreased with age (Lowman and Box 1983). Neither toughness nor phenolic content was effective as a plant defense for stinging trees, and subsequently grazing was high throughout leaf lifespan. Regressions indicated that toughness was a better indicator of herbivory than toughness for stinging trees (Table 1), but that both factors were significantly less important with stingers than compared with a neighboring canopy tree, sassafras (*Doryphora sassafras*). A comparison of herbivory and associated correlations with phenolics versus toughness indicated that toughness was consistently more closely linked to herbivory levels than chemistry (Table 1).

Leaf longevity in Australian rain forests ranged from as short as 3–5 months for stinging trees, up to 20+ years for the sassafras (*D. sassafras*) (Lowman 1992a, 2009). This incredible range was influenced by a complex suite of factors, both temporal and spatial. While insect herbivores significantly affected the fate of young leaves, the location of a new leaf also determined its lifespan – height, light, site, age of canopy, aspect, and forest composition all played an important role in leaf survival among different canopy trees.

While quantifying leaf longevity in Australian rain forest canopies, leaves were continually consumed by herbivores. This led to a more complex research agenda, examining the preferences of herbivores for leaf tissue with respect to the myriad spatial and temporal factors that influenced plant structure, growth, and physiology (Lowman 1985). Even more complicated was the fact that – to accurately assess herbivory throughout forest canopies – all leaves required continual monitoring over their entire lifespan. This was the only method to monitor that otherwise "invisible event": taking account of leaves totally eaten. With short-term or snapshot assessments of hole area in leaves, herbivory was underestimated since leaves that were totally eaten were not included in the calculation. "Long-term leaf monitoring" revealed a significant discrepancy in the conventional literature: forest canopy herbivory was underestimated by between two- and threefold when measured by short-term or discrete methods (Lowman 1984).

Once the most accurate methodology for measuring canopy herbivory was identified, the tedious task of monitoring, measuring, and documenting leaf growth versus insect consumption turned into weeks, months, and even years. With accurate, long-term monitoring, Australian rain forest canopies experienced leaf area losses ranging from as low as 4.8–32.5% annual foliage losses (Table 1). Young leaves were significantly more heavily grazed than mature leaves; and shade leaves were preferred over sun leaves. Given the high levels of variability in herbivory between, within, and among canopy heights, species, light and leaf age, it became obvious that either physical or chemical factors were affecting the susceptibility of foliage to herbivores. The physical attribute of toughness mechanically limited an insect's chewing capacity. (Of note, insects were responsible for more than 95% of Australian rain forest herbivory, with birds or mammals representing insignificant contributions except for tree kangaroos in isolated pockets of North Queensland). Herbivory levels of a suite of Australian rain forest tree species were more positively correlated with toughness than with phenolics, and that softer, younger leaves were more susceptible to herbivory regardless of chemical contents. In those early days of beginning to tease apart the complexity of plant–herbivore interactions throughout an entire forest canopy, obviously a suite of factors – leaf age, physical attributes, chemical contents, and other spatial and temporal attributes – contributed to variability of grazing intensities in tropical foliage.

The production of chemicals by rain forest canopy leaves creates a virtual apothecary of chemicals in the sky. Shamans, from many generations of sharing knowledge, in many tropical rain forests have learned to identify appropriate foliage, bark, or fruits that contain important toxins useful for human medicinal purposes. However, most of these medicines are the byproduct of plant–insect interactions, whereby plants produce toxins in an effort to defend themselves against herbivores. Because plants are not mobile, they cannot run away from enemies – defensive chemicals allow them to develop an arsenal of protection from enemies while standing still. Pharmaceutical companies are wise to collaborate with ecologists to identify potential medicinal plants, seeking out those plants with low herbivory as an indicator that a species has good chemical defenses. By contrast, plants with high herbivory levels are likely to invest relatively little in chemical defense. With the increasing deforestation pressures for tropical rain forests, prospecting for medicinal chemicals in the canopies of tropical rain forest represents a creative conservation solution.

3 Biodiversity Challenges in the Canopy

In addition to temporal and spatial variability of leaves in rain forest canopies, and possible physical and chemical attributes that lend themselves to unique plant–insect interactions, the final (and perhaps most complex) element of the canopy story rests with the herbivores themselves. The diversity and abundance of insects have (to date) eluded even the most thorough, exhaustive and long-term ecological monitoring. In speculating on "how many species exist on Earth", biologists are usually stumped when it comes to insects (May 2010). Ever since C.S. Huldane declared that "God had an unusual fondness for beetles" and since Coleoptera were found to comprise the most important group of herbivores, insect–plant interactions have become the focus of extensive research (Schowalter and Lowman 1998). Not surprisingly, insect herbivores of forest canopies remain relatively cryptic, and so finding specific herbivores in my canopy tree species was akin to locating the proverbial "needle in a haystack".

An embarrassing admission was my discovery that many Australian insect herbivores prefer to feed on canopy leaves at night. While traipsing in the woods in the middle of the night, admittedly to locate the outhouse amidst a dense stand of warm temperate rain forest at a site aptly called Never Never Land in Dorrigo National Park, New South Wales, I was amazed to hear an enormous roaring sound overhead. Thousands of mouthparts were chewing on young coachwood and sassafras leaves, both leafing in profusion. My bladder-incited discovery led to an improved strategy for my canopy research. No longer could I climb during daylight hours and retire at sunset, but instead relied on night climbing, to quantify and observe insects engaged in the act of feeding. Only with direct observations could I create accurate records of who ate what and how much (Lowman 1999).

A few examples of careful, dogged observations have borne out host-specific relations such as the stinging tree beetle and the giant stinging tree. But the majority of insect–canopy foliage relationships involved guilds of similar traits. Rather than feed on only one species, many beetles and weevils fed on different species of canopy tree, but on similar leaf types. For example, the young leaves of both coachwood and sassafras were very similar in toughness and attracted the same species of *Apion* sp., a small black weevil that fed on similar-aged leaves of neighboring tree species (Lowman 1982, 1992).

But the methods to census insect populations in foliage remain fairly primitive – beating trays, insect nets, fogging, light traps, and sticky traps, to name the most common devices (Lowman et al. 1996; Schowalter and Lowman 1998). Each technique attracts a small portion of the wealth of arthropods living in the treetops; taken collectively, these methods do not even closely represent an accurate assessment of either abundance or diversity (butser May 2010). Can an engineer create an insect detector that would count all the six-legged bodies in a cubic meter of foliage? Or can some type of photographic device detect small arthropod bodies within a three-dimensional space? Only time will tell, but unfortunately many of these species are becoming extinct before they are even discovered or named.

The race is on – conservation versus conversation – as policy makers, citizens, scientists, and other stakeholders debate the issues relating to forest economics (Lowman et al. 2006). But one thing is certain – many more insect herbivores lurk in rain forest canopies than meet the eye. And the nature of canopy fieldwork is dogged, tedious, and daunting – yet totally exhilarating for any young and aspiring field biologist. Field work "out on a limb" may reveal increasingly important secrets about how our complex planet operates and how forest ecosystems remain healthy.

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So, You Want to Do Research in the Rainforest?

Arthur S. Edison, Eric Cosio, Stephan Halloy, and Jorge Vivanco

1 Introduction

Tropical rainforests are rapidly disappearing. The earth is warming and its climate is changing. Species are going extinct at an alarming rate. Many people live in poverty, with limited access to the basics of life such as clean water, adequate nutrition, shelter, health care, and education. Much has been written on these topics, and the reader may wonder what they have to do with chemical biology in the tropics. We hope that this chapter serves to introduce the reader to some practical, and sometimes rather complex, aspects of conducting tropical chemical biology research. More importantly, we aim to show how properly conducted research may help improve some of the issues mentioned above.

As you will read in several chapters in this book, there are many wonderful scientific stories to be discovered in tropical chemical biology. Unraveling chemical mysteries in nature is an incredibly satisfying adventure, and most of the chemical biology of the tropics is completely unknown, mainly due to the remote locations of these sites and the lack of appropriate research infrastructure in those

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places. Adventurous children, who in previous centuries would have been explorers discovering new lands and waters, would find tropical chemical biology an exciting and challenging field of study. This natural attraction should be encouraged by governments, schools, and funding agencies as an important component to address some of the major issues that the tropics and the world face.

Tropical rainforests are known for extreme species diversity, and the Tambopata National Reserve (TNR) in Peru's Amazon basin is no exception. The Amazon River basin has one of the highest levels of biological diversity in the world. This region of interconnected lakes, tributaries, flooded forests, and swamplands is roughly the size of Europe and is home to at least 40,000 plant species, 3,000 fish, 1,294 birds, 427 mammals, 427 amphibians, and 378 reptiles (Da Silva et al. 2005; Chavez et al. 2006). There are many national parks and protected areas within the Amazon rainforest, and the TNR in Madre de Dios, Peru, is one of the most pristine sites and the site of several seminal studies of Amazonian biodiversity. One only needs to take a short hike in the TNR to see and hear the incredible insect and bird diversity and abundance. However, closer examination suggests that interactions between species are perhaps the most significant component of the rainforest: insects eat plants; fungi infect insects; ants specifically interact with plants; ants harvest fungi; fungi and bacteria live as endophytes in leaves; nematodes are abundant in the soil and parasitize virtually all plants and animals; microbes are closely associated with roots; nematodes eat bacteria and are killed by other microbes; etc. The unifying feature in most or all of these interactions is chemistry. Chemicals are the language of most communication and the weapons of most of the warfare that is constantly being waged by the organisms in the rich ecosystem of the tropics.

To study the tropics, one needs to take a systems approach: chemistry of interactions, genes, ecology, environment, climate, influence of people, etc. However, one often overlooked component is the geography, politics, and sociology involved in conducting research in the tropics. In this chapter, our aim is to give the reader a snapshot of what is needed to conduct research in one tropical location. We selected the Tambopata National Reserve (TNR) in Peru as the location of choice because of the familiarity we have with this place, with the local people and the governmental regulations, but the overall conclusions can be applied more widely to many other tropical areas.

2 Practical Considerations in Tropical Chemical Biology: Field Work

Obviously, each location will have its own unique challenges. This section focuses specifically on the TNR, but most issues apply to other tropical sites. None of this will be new or surprising to tropical ecologists, but chemical biologists who are accustomed to more controlled environments will need to plan carefully.

Getting to the TNR is an adventure in itself. From Lima, which is on the Pacific coast, one first flies to Cuzco at the top of the Andes. After a short stop in the former Inca capital to refuel and load/unload passengers, the plane flies on to Puerto Maldonado, a sprawling boomtown with anywhere from 30,000 to 40,000 people, and growing by the day. This growth has been fueled by many factors: tropical agriculture, mining, oil, ecotourism, and most recently the construction of a major highway connecting the Atlantic and Pacific oceans linking Brazil, Bolivia, and Peru. How the construction and the implementation of this highway will affect local biodiversity has not been assessed but nevertheless growth in these areas is increasing due to economic opportunities.

Total flying time from Lima to Puerto Maldonado is just over 2 h. Upon arrival, dirt streets, motorized tricycles, and countless motorcycles greet the newcomer's eye. This town is the home base for the majority of ecotourism lodges that work in the southern and central part of the Madre de Dios region. Next, you meet your rainforest guides at the airport. From the Puerto Maldonado airport, there is an hour bus ride along a bumpy dirt road with numerous wooden bridges that sometimes consist of just a few heavy planks of wood. Depending on the season, the road can either be very dusty or be very muddy. A town called Puerto Infierno is at the end of the bus trip. Infierno is the name of a native community of mixed composition. Its members belong to the Ese Eja tribe, one who long suffered slavery and exploitation during the rubber boom years and which led to its near extinction. Puerto Infierno is 2 miles away from the main Ese Eja settlement of the same name and is essentially a boat dock with a small convenience store in which travelers can enjoy a candy bar, sodas, or a quick trip to the toilets (they do exist) before the trip up the Tambopata River. This community is a good role model for ecological tourism entrepreneurship as it is a partner with, or provides services to, major ecotourism lodges in this area. The profits of this partnership are shared between the members of the community and reinvested into ecologically sound initiatives such as the maintenance of a medicinal plant center, training of local people in ecotourism, etc.

The next stage of the trip to the TNR is a motorized river canoe that holds about 12 people and their gear. Most canoes in the area are equipped with 60 HP outboard motors so they are acceptably fast and have enough extra room for a modest amount of personal belongings and equipment. However, extra boats may be required for extensive or bulky scientific equipment and supplies. These canoes are driven by experienced local motorists who work for the lodges and know the river well. The trip up the river can take between 1 and 3 h, depending on the destination lodge/station, and can be done by day or by night.

The location of our research site is within the property of the Explorer's Inn, one of the oldest lodges in this region and the only one located within the TNR. Since the Explorer's Inn is located inside the TNR, all visitors are required to check in at the park ranger post in the La Torre sector before going to the lodge. The post is conveniently located just across the river from the lodge, which has to do with the original TNR having been designed around the Explorer's Inn. At the post, scientists are required to turn in approved research permits and to register in a book before proceeding. This exempts them from payment of the $10 (US) fee paid by tourists who enter the TNR. The boat then crosses the river and, upon docking, you face the steep climb up the tall banks to the Explorer's Inn. Although lodge personnel carry guests' luggage up the hill, the boats, river, and hill all place a physical limitation on the amount, size, and weight of gear that can be brought in for research.

The facilities at Explorer's Inn are perfect for getting away from modern distractions. There is no electricity beyond that provided by solar panels, and a small 3 kW generator used by the scientists. No hot water in sleeping areas, no telephone, and no cell phone reception. Satellite Internet access was recently installed for use by scientists and is surprisingly fast but reception is very dependent on weather conditions. On the other hand, the food and general accommodations are excellent, and there is even a nice bar for socializing at the end of a long day. Interestingly, this bar was used as a brainstorming area in the past by ecologists who were coining the meaning of biodiversity by using the TNR as an example and is now used by chemical biologists to decipher how chemical compounds used in communication among organisms may drive the biodiversity of this area. In short, it is the perfect restful working vacation!

However, conducting research can be a challenge. A few pros and cons can illustrate this fact. For example, the only refrigerator or freezer space is in the kitchen, or bar, and must be shared with the food, or drink, for consumption. However, transport logistics are improving thanks to the highway so that more gas fridges and freezers, from Brazil, are expected shortly. Electricity is very limited and not sufficient to run much scientific instrumentation. However, most portable instrumentation nowadays has significantly lower power requirements and can operate on batteries that can be recharged by a generator. Film teams have been known to drag their generators into the forest in Tambopata, and so have insect collectors using powerful arc lamps above the canopy to bring samples in from far away. There are no formal sample preparation areas, fume hoods, or sterile facilities for microbiology. There is no library or reference material to conduct research beyond the intermittent satellite Internet connection. All this does force the scientist to be creative. In short, nothing even close to the basics of modern research facilities is available, but this is balanced with the fact that the researcher is living within a natural laboratory of organismal interactions and incredible beauty.

Sample collections and fieldwork are quite rewarding and enjoyable in the tropics but not without dangers. The TNR is in a transition of tropical to subtropical rainforest, so regular weather inversions with cold fronts from the southern Andes can send temperatures into the lower 50s (F) for many days at a time. This tends to keep poisonous snakes at bay in the TNR from May to August, but in the remaining months they can be a problem for the unaware. One of us has had one too many close and disagreeable encounters with them. To crawling reptiles you have to add jaguars, ocelots, a fairly large peccari population and several species of caiman. However, the most significant dangers come from insects, with botflies and poisonous ants as good examples, and insect-borne diseases, of which leishmania is a primary danger for those working in the field without protection for long periods of time. Dengue and yellow fever are potential dangers, although presently of low impact, and malaria is not endemic in the TNR but is common in many tropical areas. This may, again, change in the near future. One of the authors recently encountered in Puerto Maldonado a team of U.S. NAMRID scientists (see http://www.nmrc.navy.mil/nmrcd.htm) planning on doing monitoring of vector-borne tropical diseases because of increased traffic between Peru and Brazil with the new highway. Of particular concern are malaria and dengue fever. The Explorer's Inn, as all lodges and stations, provides beds with mosquito netting, and DEET can be considered a definite necessity during the day. It is important to remember that it takes about one full day of travel to get from the rainforest to modern medical facilities in Lima.

As you have read in earlier chapters, some of the most interesting tropical chemical biology research involves the isolation of microbes from soil, rhizosphere, or plants. Many of these bacterial or fungal organisms produce chemicals that might be useful in human health or other economic activities. However, as described in this chapter, many of these microbes have never been identified before and are new to science. Tropical biologists need to recognize the potential dangers of new organisms for plants, domestic and wild animals, and humans outside the tropics. During the PASI, we heard about a bacterium isolated in the Peruvian tropics that was resistant to common antibiotics and a researcher who was infected with a fungus that resisted treatment with known antifungal agents. Isolating novel species of microbes and removing them from their natural environment should be done with great caution, and we recommend that protocols be established for the decontamination of clothes, boots, and equipment before the trip back home. However, this may not be easy with the facilities available in many tropical research areas.

Once samples are obtained, they need to be analyzed. In the case of the TNR, the closest major research facilities are in Lima, about a day's travel. In most cases, dry ice or even regular ice will be very difficult or impossible to obtain, so samples for biochemical analysis will need to be either stable or partially prepped in the field before transport. The scientific infrastructure in many countries with tropical resources is limited. For example, the entire country of Peru has only one 300 MHz NMR spectrometer, so many samples need to be sent out of the country for chemical analysis. This situation is slowly improving in Peru and in other countries so more groups are requesting local analyses rather than go through the work of securing export permits, but it will remain a significant problem in the coming years for any research that requires anything beyond routine chemical analyses. Exporting samples requires a permit and very likely will also require an import permit at the other end. Foreign researchers in Peru are required to work with a local scientific institution and to use local helpers. The best advice is to choose a local partner with experience and contacts in dealing with the park and wildlife authorities who can process the paperwork. This is important due to the political nature of environmental issues in tropical countries. Conditions and authorities change and at times researchers, without appropriate contacts, can spend unnecessary time fulfilling outdated requirements. In the case of Peru, all work to be performed in protected, or any, wildlife area used to involve the Natural Resources Institute, INRENA. If it was a protected area, permit applications had to be sent, following one set of requirements, to the IANP or "Intendencia de Areas Naturales Protegidas". If the work was to be performed in a natural, but not protected area, one had to apply to the Intendencia Forestal, also within INRENA, and follow a slightly different set of requirements. From 2009, all this has changed and now work within protected areas receives permits from the newly created Ministry of the Environment (MINAM), of which SERNANP is the general service in charge of protected areas. If you want to work outside protected areas, then you have to go to the Forestry Directorate of the Ministry of Agriculture. Research applications can be sent directly from abroad, but payment of the fees and follow-up can slow down the process. On the other hand, it must be said that most research permits do get processed within 6 weeks in Peru. All this reinforces the point of getting a reliable and experienced local partner if you plan on doing more than just one observation trip in the jungle. Researchers who chose to ignore permits not only put themselves at risk legally, but are also jeopardizing future studies in this area by violating sometimes already fragile trusts.

Even if a study starts out by simply trying to answer an exciting biological problem, investigators need to be fully aware of the possibility of discoveries having commercial value in areas such as medicine, energy, or agriculture. Although commercial success is extremely rare in any scientific venture, this issue has the potential for great good or great harm, and the responsible scientist needs to consider many facets of the problem.

3 Legal and Ethical Considerations in Tropical Chemical Biology

Every biological study in the tropics should start with serious attention to legal and ethical aspects of the research. Much has been written on this topic (Hyvarinen and McNeill 2003; WRI et al. 1992; King et al. 1999; Barratt et al. 2000; Greene 2004; Rosenthal 2006), and the goal of this section is not to review the extensive literature but rather to introduce some of the key issues to novice tropical biology scientists and naturalists.

The central issue is that many of the most biodiverse regions in the world are in countries with significant economic poverty. Biodiversity is a resource that can be used for short-term profits like logging, gold mining, or oil drilling or for long-term sustainable gain like ecotourism, bioprospecting, or scientific research. The stakes are extremely high, as many of the most biodiverse tropical regions, including the Amazon basin in Peru and the TNR, in particular, are threatened by short-term economic interests, often due to the lack of long-term policies by the local governments. For instance, in 2007 the President of Peru, Alan Garcia, lobbied for the concession of oil exploration rights within a National Park in the Amazon basin to an oil company from China. There was a concerted local and international uproar of protest and fortunately this project did not proceed. The origin of the problem is, however, far from resolved and has to do with nonrenewable natural resources within protected areas, a problem the USA has also faced in Alaska. In many Latin American countries, the zoning for oil and mining exploration is performed by one ministry and protected areas are zoned independently by a different ministry so overlap is common and each case is a separate incident demanding mobilization from the conservationist community.

4 Convention on Biodiversity

The most important international document that addresses the issues of bioprospecting is the Convention on Biological Diversity (CBD), signed in 1992 (CBD 2000) and ratified by 191 countries, with the notable exception of the USA. The CBD entered into force in December 1993 and has three primary goals: "the conservation of biological diversity, the sustainable use of its components, and the fair and equitable sharing of benefits from the use of genetic resources".[1]

Implicit in the equitable sharing of benefits is the issue of prior informed consent (PIC). The people who will be most impacted by the research activities need to understand the project, its goals and expectations, and the benefits, both to the local community and to the researchers, associated with the research. This is often very difficult. As discussed by Rosenthal (2006), PIC is a complicated issue, because it is not always clear what group has authority and the responsibility to negotiate and who are the potential stakeholders who should participate in discussions. The federal government of the given country is an obvious participant, but the participation and support of local communities are also vital, especially when bioprospecting involves the use of indigenous knowledge (Kulip 2003; Uniyal et al. 2003; Carvalho 2004; Gepts 2004). The aims of the CBD have been codified and developed into laws in the signatory countries. In the case of Peru and the countries signatories to the Acuerdo de Cartagena (the tropical Andean countries), the leading rule for prospecting and developing genetic resources is decisión 391, from which local legislation is derived (Halloy et al. 1996).

One of the difficulties in discussions of benefits is the very improbable chance of discovering a blockbuster drug from the bioprospecting or from an indirect chemical ecology study in the tropics, and discussions of instant and great riches can be extremely counterproductive (Mendelsohn and Balick 1997; Lewis 2003). As discussed by Kursar et al. (2006), the most important benefit is the development of research infrastructure in the developing country (Kursar et al. 2006). This involves the training of students, equipping laboratories with instrumentation for modern research, and conducting collaborative research that results in publications.

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[1] http://www.cbd.int/convention/guide.shtml?id=web

5 Socioeconomic Threats in TNR

The TNR is located in a southern corner of Peru, with the much larger Bahuaja-Sonene National Park covering its southern border and Bolivian Madidi forest beyond it. The TNR is organized along current accepted procedures for protected areas. It is surrounded by a buffer zone which, given that its southern flank is covered by a national park, is mainly north of the reserve. The reserve itself is zoned into areas accessible to tourists, those that can be used for sustainable collection activities (Brazil nuts), and strictly conserved areas with no access to the general public. The protected area formed by park and reserve and the Bolivian Madidi is part of a more ambitious project denominated the "Vilcabamba-Amboró Biodiversity Corridor" which follows the north–south axis between the Andes and Amazon forest and covers four national protected areas and one private conservation area in Peru and two public national areas in Bolivia. This large conservation project is, unfortunately, confronted with problems typical of most tropical protected areas. These consist mainly of the influx of displaced peasants looking for new land, legal and illegal logging, and road building. In fact, the "corridor" is now cut in half by the new paved east–west highway connecting Brazil with Peru. The immediate surrounding jungle is undergoing rapid transformation. Slash and burn is a common sight along the roads that lead out of Puerto Maldonado into the official (for now) buffer zone of the TNR. Small farms, ranches, mining operations, and expanded villages are rapidly destroying much of the rainforest outside the protected areas. It is a complicated situation with causes and effects that have been very well described in John Kricher's popular reference book "A Neotropical Companion" (Kritcher 1999). The fast rate of deforestation described by Kricher for Brazil in the 1990s now applies to the southern Peruvian Amazon, an area long isolated from the country's mainstream and now open for oil exploration, gold mining, timber extraction, and tourism. Perhaps, the biggest threat, seen from the scientist's and conservationist's point of view, is the previously mentioned transcontinental highway, which will connect the Atlantic Ocean in Brazil and the Pacific Ocean in Peru. The highway passes through Puerto Maldonado and only a few miles north of the Tambopata River and the TNR and is one of the products of IIRSA, the Initiative for the Integration of Regional Infrastructure in South America, a long-term project involving the development of transcontinental communication networks on land and water.[2] Once completed, it will provide important transportation options to people of South America that people in North America and other more developed regions already enjoy and will certainly bring with it a considerable amount of infrastructure and economic activity to the area beyond just gasoline stations, stores, and hotels. The highway will also provide easy access to many areas in the jungle that were, up until recently, very hard to reach.

The immediate effects of better roads have been seen many times before in other settings. For example, for much of the year, the TNR appears as a pristine

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[2] See http://www.iirsa.org

ecosystem untouched by the influences of humans. However, according to Jan Ygberg, PR manager of the Explorer's Inn, there are now approximately 3 months each year when the smoke from massive forest fires inundates the TNR and Puerto Maldonado areas. Just about 3 years ago, the smoke was only noticeable in the TNR for about 1 month each year. There have been times in recent years that planes could not fly into the Puerto Maldonado airport because of the dense smoke. These fires are started from slash and burn operations that clear the land for ranching and farming. Each burn dries a larger area of uncut forest, making it more flammable in the next burn or lightning strike. The result is that the fires get larger each year (Malhi et al. 2007; Various 2008).

Another effect is that the water in the Tambopata River is unsafe to drink and it is not advisable to eat the fish because of the extensive small-scale gold mining operations that release mercury directly into the river as waste product. Mining activities are increasing dramatically as the price of gold rises on the international market. These operations go from small barges by the river banks to massive, unregulated and unpoliced, efforts that involve bulldozing whole areas of forest. A particularly impressive case of forest and watershed destruction is that going on in Huepetue on the upper reaches of the Madre de Dios basin where hundreds of hectares have been converted into a desert visible from up to 16 km on earth imaging programs such as Google Earth. The impact on river and forest wildlife has, surprisingly enough, not been systematically monitored by either government or NGOs. Isolated reports suggest high levels of methyl mercury in sediments and bottom-dwelling fish, enough to cause concern to long-term conservation efforts such as that being carried out by the Frankfurt Zoological Society on behalf of the giant river otter. Upper trophic level river mammals such as these may very likely be the first casualties of the uncontrolled activities of small miners.

Still, a significant, and growing, part of the economic activity of Puerto Maldonado comes from ecotourism, so there is a developing perception about the need for conservation and sustainable use of the forest in the local population, native or not. If implemented properly, the promotion of scientific research and of bioprospection activities has the potential to contribute to sustainable economic activity (Wilkes 1992; Guarino et al. 2001). However, without careful negotiations and proper legal agreements, bioprospecting can turn into biopiracy, the exploitation of genetic resources without consent or benefit to the local communities (biodiversidadla.org 2005; Halloy 1992).

Increased awareness in developing countries of the potential for misuse in bioprospection activities has resulted in greater scrutiny and more paperwork to be filled out by researchers wanting to carry out their activities in the field. Many countries, like Brazil, do not allow or place extreme restrictions to the export of biological samples. The reality is that research in the tropics demands now significantly more patience and diplomacy than it used to. Restrictions are in place to prevent misuse but also to allow local scientists to partake of the research activities and thereby promote science locally. These efforts vary in effectiveness and have to do with how much money the country devotes to basic research and, in some cases, a sheer luck factor which depends on the degree of local involvement of foreign research groups in the host country. Most tropical Latin American countries can show cases of positive and extensive involvement by foreign academic institutions in the local promotion of research by local scientists. The Smithsonian Institution, the Missouri Botanical Garden, the RAINFOR initiative (Leeds, Oxford and Edinburgh), to select just a few, are all classic examples of foreign researchers working closely with local scientists and institutions on long-term projects. Depending on the country, some interactions are started best at the federal government level, this has worked well in Brazil and Costa Rica, and more recently in Colombia, but is less effective in the central Andean countries (Ecuador, Peru, and Bolivia) with weaker institutional stability. For those aiming for projects on the long run, the best approach in countries with these characteristics is a basic general agreement with authorities coupled to a solid interaction with a local NGO or academic institution with influence in local society and that has proven resilient in time. RAINFOR and the Missouri Botanical Garden in Peru and the German DFG research group 406 on montane tropical forest working in Ecuador are typical examples of this second approach.[3]

An interesting new development in protected area management is a trend toward "outsourcing" of some of the services required in protected areas as suggested by the World Bank and the Interamerican Development Bank in countries where the government has a weaker effective presence in the field.[4] This opens opportunities for research tied to activities linked directly to conservation. It is too early to assess results, but, in Peru, three protected areas have passed to the hands of private contractors either for total management (Mangroves of Tumbes, Salinas Aguada-Blanca for vicuña protection) or for specific activities (TNR and Bahuaja Sonene for research and biological monitoring activities). At the time of this writing, the promotion and administration of research and monitoring activities in the TNR are being transferred to an NGO, AIDER, which will work in close cooperation with a consortium of two private universities for the next 7 years in the promotion of scientific research in the area.

6 PASI Discussions on Legal and Ethical Issues

During the PASI, we had several interesting and lively discussions about legal and ethical issues in bioprospecting. The participants in the PASI came from many countries and had backgrounds ranging from chemistry to ecology and included graduate students, postdoctoral associates, professors, and nonprofit conservation groups. Some investigators were well informed about legal issues related to bioprospecting and others had not had much or any exposure to the topic. This section summarizes some of the discussions.

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[3] http://www.tropenregenwald.de

[4] http://www.profonanpe.org.pe/peru01_eng.html

One of the major obstacles in conducting research in tropical chemical biology is funding. It was noted by several investigators from Latin American countries that funding from local governments is often very limited or nonexistent. This creates a major hurdle for local researchers to train students and to conduct basic or applied research utilizing the rich biological resources in their own country. Funding from other sources is often needed, and one of the most innovative is the International Cooperative Biodiversity Group (ICBG) program funded by the NIH, NSF, USDA, and DoE (Rosenthal 1999). ICBGs fund collaborative research projects between scientists in the USA and countries with rich biological resources. Components of the ICBG include the search for compounds with the potential for new drugs for human health, education and infrastructure building in the host country, promotion of conservation by providing new models and benefits for the use of biological resources, and cataloging local biodiversity.

Other funding opportunities are less specifically focused on international collaborations in tropical biology than ICBG but include the Human Frontier Science program (HFSP), other USA federally funded projects, and private foundations such as the Bill and Melinda Gates Foundation. In general, there are relatively few funding opportunities that, like the ICBG, provide the resources necessary for all components of a successful bioprospecting research program.

The PASI group recognized the need to approach research in tropical chemical biology with a big picture and with several different techniques, because the most interesting problems involve investigations on every level, from a global view that incorporates understanding of economics and law to the smallest scale of chemical analysis. Some people thought that the legal issues of bioprospecting should be left to the lawyers and not the scientists, but many others argued strongly that scientists themselves understood the details and goals of the project and had a responsibility to be fully involved in the legal and ethical issues associated with the research.

Traditional knowledge is a major issue in bioprospecting, and the PASI group recognized that traditional knowledge is distributed and often spans many generations and communities. There are significant hurdles associated with prior informed consent and benefit sharing, because it is difficult or impossible to identify a group of people who can represent these interests. These complications led many PASI investigators to favor either ecologically based discovery or even random screens of biological activity over the use of traditional local knowledge. This sentiment was expressed despite the fact that traditional knowledge has been used to help narrow the search and hence reduce overall costs and increase probability of success.

Nearly everyone agreed with the importance of benefit sharing to the local community or country, but there were several different ideas about how to best do this. Discovering a new drug is extremely rare and unlikely to happen from most chemical biology investigations. Therefore, many PASI participants felt that discussions of royalties from the unlikely riches of a new blockbuster drug were counterproductive and distracted attention from much more important issues (Kursar et al. 2006). However, export permits of samples need to specify that nothing will be used commercially until all groups are in full agreement with benefit sharing, essentially an agreement to agree.

In contrast to discussions of big, abstract, and unlikely royalty checks, the group favored the training of local scientists and building the scientific and technical infrastructure of local institutions as the way to have the greatest positive impact. In addition to formal training of graduate students, postdocs, and professors in technical areas of science, efforts should be made to obtain appropriate equipment for chemical analyses to be done on a local level. A good example of this was the purchase of a 300 MHz NMR spectrometer in Panama by the ICBG in that country (Kursar et al. 2006). Another line of thought points out that international projects like these provide incentive for the return of young qualified PhD scientists to their countries of origin and local universities. It was through this return path of researchers that the new 300 MHz NMR in Peru was purchased, using exclusively local funds, but foreign contacts. The same incentive motivated the recent purchase of an LC-MS, again paid with local funds, but supported through international projects. International projects of this type, rather than being only a welcome source of funding for these returning researchers, function as a network and also as an incentive for matching equipment funds in the present international context in which most Latin American and African countries have been showing significant rates of economic growth. This serves a virtuous circle where more returning scientists bring more contacts and more visibility to research which in turn brings in more local money and improves the scientific infrastructure and local capacities.

At a local service level, investigators involved in chemical biology research could teach short classes to local schools and ecotourism guides. Investigators can also offer field trips and science clubs to children from local communities to increase awareness of the importance of the rainforest environment. In the course of conducting these local activities, exceptionally talented students could be identified and encouraged to pursue more advanced education and training. However, making the right personal connections in the local communities can often be difficult. Importantly, care must be taken to prevent over-harvesting when local people are trained in sample collections.

Other ideas for benefit sharing were reduced prices for drugs discovered from the resources of that country and advanced payments for the right to conduct research. Some countries require a percentage of the total costs of the research to be paid for access, but this often is difficult or impossible for small academic programs. Finally, companies involved in major projects such as roads, logging, and oil drilling could be potential sources of funds for groups to help develop conservation plans that are required by most governments. The present IIRSA projects, one of which is the already mentioned transcontinental highway, are good examples, with large amounts of funding to mitigate environmental and social impacts which could be tapped into for this kind of research, training, and capacity building (Killeen 2007).

7 Conclusion

The coming of age of “portable chemistry” holds the promise of many important discoveries to be made in tropical chemical ecology. Researchers more acquainted with labcoats may exchange them for rubber boots and head for the forest with the newest in small high-resolution equipment. However as easy as travel may have become, the tropics remain what they have been all along, an unforgiving place with, at times, complicated politics to handle. Although many of the issues discussed in this chapter have taken Peru and the TNR as an example, they are generally valid for all tropical research locations. Therefore, we can attempt to draw a tentative checklist for the potential chemical researcher aiming to leave the seclusion of his laboratory and heading for the tropics.

Unless your project is site specific, do some comparison shopping for existing research facilities in the tropics, which may alleviate some of the logistical requirements, including lodging, transport, and equipment. Better yet, look for those offering support in securing research and, if required, export permits. In the Americas, Barro Colorado Island (BCI) in Panama and La Selva in Costa Rica are among those prized destinations for tropical researchers. Those lucky enough to have their object of study in those locations and to find free space at any time (high demand) can fully concentrate on their research and the nightmare of field trip logistics where reagents are not just one call away.

Stays of a month or longer on tropical locations place a heavy toll on scientists from more temperate climates. Air conditioning in the laboratory is not the main concern but humidity, mud, high temperatures, physical stress, and discomfort outside that realm. The authors have witnessed more than one case where perfectly fit individuals from wildlife programs in the USA and Europe have collapsed after a few days of flat 2-mile hikes simply because they had flu a week before coming to the tropics. Apart from the general fitness issues, all countries have lists of recommended vaccinations and behavioral practices to reduce the chance of falling prey to disease or parasites.

Should you be among those not heading toward fairly complete research facilities in the tropics, a reliable local partner, not just one for legal requirements, is highly recommended. Both NGOs and local academic institutions tend to be the most likely candidates and the choice of any one of them will depend on your needs for equipment, transport, or experience in dealing with the authorities. In many cases, it is easy to find one partner that covers all these aspects, whereas in other cases you may need to work on several fronts. Regardless of your need, the trend goes toward tropical countries requiring that you necessarily involve a local partner to grant a research permit. Larger institutions may have wider logistical advantages for the foreign researcher. As an example, the Pontifical Catholic University of Peru (PUCP) has had agreements offering one of the two options, either scientific cooperation plus logistical support, or only logistical support with permits, for groups going into areas of the Amazon where they work. In the Madre de Dios area in Peru, NGO research operators such as the NGO ACA (Amazon Conservation Association) operating two research stations in the area offer this type of support to researchers also. Private tourist operators, such as Rainforest Expeditions, also offer help with permits for researchers and film teams, although one can expect fees to be higher.

If your project is medium or long term, you can provide an educational component for the local community, which will contribute to create awareness in the locals of the importance of scientific research for their own interest. Most large research programs are including educational and environmental awareness themes for local communities and many European granting agencies are making this a requirement. Eventually and due to these outreach activities, the response of local authorities to scientists is beginning to change from seeing them as adventurers and potential plunderers to considering them as a valuable resource for the community. We have seen this happen in many places now and we can say with total conviction that this approach greatly enhances the pleasure of scientific discovery.

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