Cellular Origin, Life in Extreme Habitats and Astrobiology 16

Joseph Seckbach
Zvy Dubinsky
Editors

All Flesh Is Grass

Plant-Animal Interrelationships

Springer

ALL FLESH IS GRASS

Cellular Origin, Life in Extreme Habitats and Astrobiology

Volume 16

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Joseph Seckbach
The Hebrew University of Jerusalem, Israel

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INTRODUCTION TO ALL FLESH IS GRASS: PLANT–ANIMAL INTERACTIONS

> "What shall I proclaim? **All flesh is grass,** All its goodness like flowers of the field"
>
> Isaiah 40: 6

Plants are the base of life; without the green world of the photosynthesis performers there is almost no life on earth. The rise of angiosperms (flower plants) in the Early Cretaceous (~140 million years ago) was accompanied by coevolution of a variety of insects, including flies, bees, and wasps required for pollination (**Ollerton and Coulthard**, 2009). Plants and animals interact; the animals release CO_2 to the atmosphere and produce organic fertilizers in their nutrient-laden excretions. While the plants absorb CO_2 (during photosynthesis) and uptake the organic animal excretion and use these materials to build the new tissues of their botanical bodies. The animal manure may also serve for making clean fuel and natural gas.

There is age-old respect for the edible trees, even the Bible prohibits cutting down fruit trees, not even during war time or when waging siege against an enemy city (Deuteronomy **20**:19–20). This command aims at keeping ecology in balance and expresses appreciation for the useful vegetation. Actually, the Bible contains numerous statements about man-plant relationships, starting from Adam and Eve in the Garden of Eden to the later books of the Holy Scriptures; in Psalms are many mentions of the interrelations between the green world and zoological life.

In this book, the authors discuss the evolution of plant-animal relations and phenomena of symbiosis between algae, plants, and other living organism. This volume contains twenty-two chapters contributed by thirty-nine authors from the following countries: Argentina, Australia, Czech Republic, Germany, India, Israel, Italy, United States, and Venezuela.

Marine environments where these relations occur are the focus of a number of chapters. The important bioactivities of the transfer of pollen grains for the production of fruit are described in the chapters on pollination. Another section of this volume highlights the interesting and exceptional carnivorous plants that trap and consume insects and other small organisms so as to extract vital nutrition from their prey. Other chapters deal with the Aphids, Galls, and chemical plant protection against grazing and animal attacks.

This volume is number 16 of COLE series edited by J. Seckbach and published by Springer. We hope that the readers will draw updated information from the rich chapters contributed by experts in their field.

Hebrew University of Jerusalem Joseph Seckbach
Jerusalem, Israel
November 2009

Biodata of **Joseph Seckbach,** editor, with Zvy Dubinsky, of this volume and author of opening and *Xanthium* chapter in this volume.

Professor Joseph Seckbach is the founder and chief editor of *Cellular Origins, Life in Extreme Habitats and Astrobiology* ("COLE") book series. He has co-edited other volumes, such as the *Proceeding of Endocytobiology VII Conference* (Freiburg, Germany) and the *Proceedings of Algae and Extreme Environments meeting* (Trebon, Czech Republic). See http://www.schweizerbart.de/pubs/books/bo/novahedwig-051012300-desc.ht). His co-edited the recent volume (with Richard Gordon) entitled *Divine Action and Natural Selection: Science, Faith, and Evolution* published by World Scientific Publishing Company

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PART 1:
EVOLUTION OF PLANT ANIMAL RELATIONSHIP

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Professor Claudio Tuniz earned his doctorate in Physics in 1974 at the University of Trieste, Italy, where he carried out research in nuclear physics and its applications from 1974 to 1990. He was a post-doctoral fellow at Rutgers University in New Jersey, USA, from 1981 to 1983, becoming involved in pioneering applications of cosmogenic radionuclides to understand the cosmic record in meteorites and lunar rocks. From 1984 to 1990, he promoted accelerator mass spectrometry (AMS) and cosmogenic radionuclide dating at the Italian Institute of Nuclear Physics and at the University of Trieste. During this period, he carried out experimental activities at several laboratories in Europe, USA, and Australia.

He moved to Australia in 1991, following an invitation by the Australian government to lead the AMS group at the Lucas Heights Research Laboratories in Sydney. The world-class AMS center developed under his leadership carried out research programs in global climate change, Antarctic research, nuclear safeguards, biomedicine, and archeology. Later, he became the director of the Physics Division at Lucas Heights Research Laboratories (1996–1999) and coordinated a broad spectrum of interdisciplinary research based on the use of ions, neutrons, and synchrotron radiation. Tuniz was a Counselor for science policy matters at the Australian Permanent Mission to the United Nations Organisation in Vienna between 1999 and 2004. Tuniz served as a Chairman of the Executive Committee for the Australian Program on Synchrotron Radiation Research, Chairman of the Neutron Scattering Committee for the Australian Replacement Reactor, Co-Chairman of the Australasian Archaeometry Conference, and Co-Chairman of the International Conference on Accelerator Mass Spectrometry. He is a fellow of the Australian Institute of Physics, member of the Italian Physical Society, and of the Italian Association of Archaeometry. He is the author and co-author of over 100 international journal and conference publications, including one book and several book chapters, mainly in the interdisciplinary studies based on the use of ion accelerators in environmental studies, biomedicine, materials science, and archeology. Tuniz started his work at ICTP in 2004 as a Special Advisor to the Director. He is presently Assistant Director and Head of the ICTP Multidisciplinary Laboratory. Currently, his main field of interest is the use of advanced physics methods in paleoanthropology and human evolution.

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EVOLUTION OF PLANT–ANIMAL INTERACTIONS

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1. Introduction

The evolution of plant–animal interactions goes back to the Early Archean, when the first signals of photosynthesis may have been detected in the Isua Peninsula in Greenland, a phenomenon that is related to the isotopic anomalies of carbon. The first evidence of reliable fossils of photosynthetic microorganisms has been identified by micropaleontologists in the Late Archean and Early Proterozoic. A closely related topic in this geologic time interval is the evolution of trophic relations and metabolic diversification in the microbial world. In the context of the three domains of life, Archea, Bacteria, and Eucarya, the bifurcation of multicellular organisms into plants and animals becomes evident only during the Paleozoic. Cell evolution also leads up to the unicellular dichotomy of autotrophs and heterotrophs. Symbiosis has a strong role to play in the transition to plants and animals in the Phanerozoic. It is timely to focus on the details of evolution in the Cretaceous and Tertiary, where detailed pathways of evolution have been gathered in many geographical regions, including the Karst region of northern Italy. Various experimental techniques have contributed to elucidate the coevolution of plants and animals. A special case of plant–animal interaction is the evolution and dispersal of hominins, including their impact on the ecosystems. A significant development in understanding the evolution of plant–animal interactions is based on the possibility of identifying reliable biomarkers that can characterize its different stages, from the earliest microbes to the extant plants and animals. Such identification of biomarkers labeling different stages of evolution may orient the search for life in the exploration of the Solar System.

2. Evolution of the Microbial World in the Proterozoic–Early Phanerozoic

2.1. THE TREE OF LIFE AND LAST UNIVERSAL COMMON ANCESTOR

The dichotomy between plants and animals evolved from the microbial world that lasted for the major part of Earth's history (Archean and Proterozoic). The microbial cellular plan consists of prokaryotes, lacking a central nucleus, and eukaryotes that evolved later containing a central nucleus enveloping the genetic material. Eukaryotes were earlier considered similar to eubacteria and archeobacteria and diversified from a universal last common ancestor (Fig. 1).

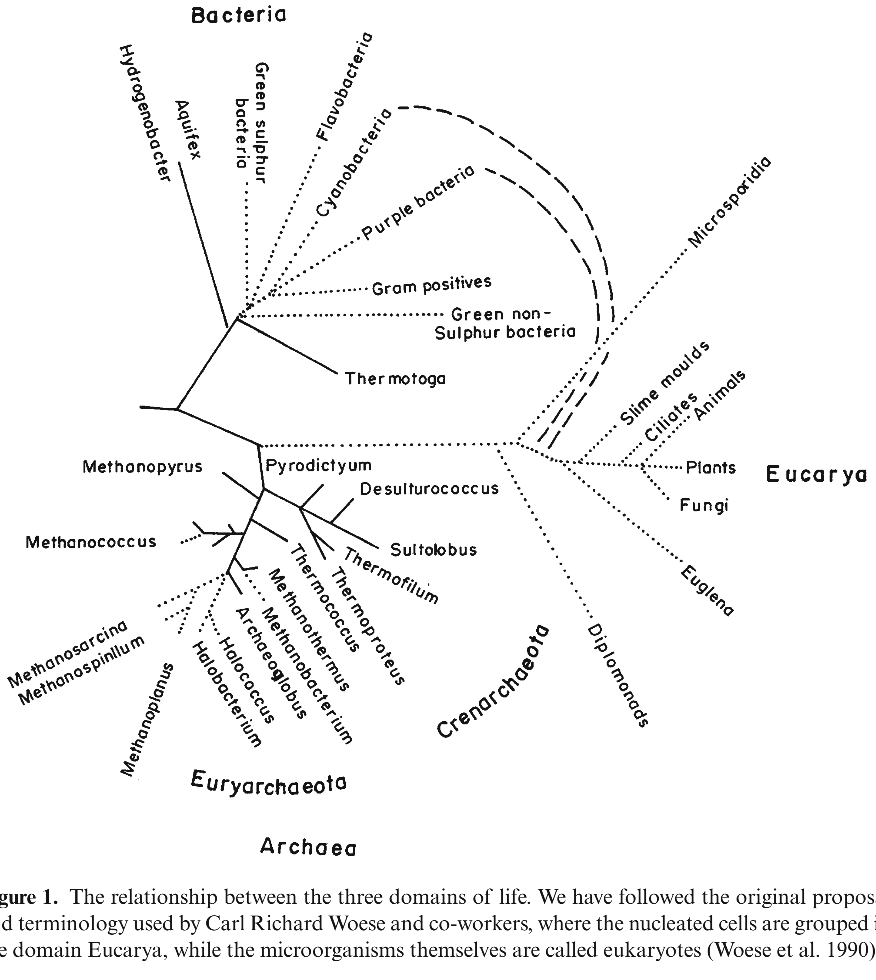

Figure 1. The relationship between the three domains of life. We have followed the original proposal and terminology used by Carl Richard Woese and co-workers, where the nucleated cells are grouped in the domain Eucarya, while the microorganisms themselves are called eukaryotes (Woese et al. 1990).

In the Archean biosphere, the microbial communities were dominantly marine; however, unicellular eukaryotes must have been present. The unicellular organisms that can survive in extreme conditions are very close to the eukaryotes evolved in Paleoproterozoic around 2.1 billion years (Ga) before the present (BP). Cyanobacteria were the main prokaryotic microbial fossils reported from rocks dating from 3.5 Ga BP. Life on Earth has been classified into three domains: Archea, Bacteria, and Eucarya (eukaryotes) (Woese et al., 1990). The evolution of eukaryotes is a debatable subject. The Archean oceans may have been colonized by prokaryotes and proto-eukaryotes. The prokaryotes are single-celled microorganisms and can be easily distinguished by their DNA structure that is simple and not bounded by membrane and nucleus. The mitochondria, chromosomes, and chloroplasts are also absent in the prokaryotic cells.

The symbiosis between prokaryotes and eukaryotes resulting in modern mitochondria and chloroplasts are well established. This remark lies at the basis of the eventual separation of multicellular organisms between plants and animals (cf., Section 3.1). It is believed that the modern eukaryotic cell resulted from the symbiosis of eubacterial organelles into an Archea-like rootstock (Margulis and Cohen, 1994). The Archea domain includes, among others, hot-spring bacteria and methanogens. The Bacteria domain includes cyanobacteria, anoxygenic photosynthetic bacteria, and the mitochondria and chloroplasts of the eukaryotes (Wheelis et al., 1992). Archea and Eucarya shared a common ancestor that was not shared by Bacteria. Hyperthermophiles diverged from the Archea–Eucarya domains (Woese et al., 1990; Wheelis et al., 1992).

Paleobiological evidence indicates that eukaryotes evolved more than 2 Ga BP. Modern unicellular organisms surviving in the extremely hot conditions may be their modern analogs (Tewari et al., 2008). This view differs from the earlier view that the eukaryotes share a common ancestry with Archea. This evidence was based on genes that code for RNA of ribosomes in Eucarya, Archea, and Bacteria. The nuclear genome contains genes that are of specific eubacterial origin, and some genes are specific to eukaryotes (Woese, 1987). The first appearance of endosymbiont eukaryotes is not clear; however, they must have originated in the late Archean. Multicellular eukaryotes evolved later, around 1.5–1.0 Ga BP. These metazoans appeared as a major eukaryotic radiation in the Mesoproterozoic period around 1.0–0.8 Ga BP (Knoll, 1984). Terminal Neoproterozoic (0.65–0.57 Ga BP) was the time of Ediacaran explosion of diploblastic animals (Conway Morris, 1989; Narbonne and Hofmann, 1987). Their possible modern analogs are coelenterates, such as jellyfish, corals, and sea anemones. The Cambrian explosion of triploblastic animals with three germ layers is the major event of multicellular metazoans. In the present paper, we discuss the physical and paleobiological evidences of prokaryote to eukaryote evolution on Earth. The presence of microorganisms in Antarctic lakes including eukaryotic diatoms and cyanobacteria not only supports the idea that life can survive in extreme environments, but also that life may thrive on Mars and Europa (Chela-Flores, 1998; Tewari, 1998, 2001a; Chela-Flores et al., 2008).

2.2. EVIDENCE FOR THE PRECURSORS OF PLANTS AND ANIMALS

Life is generally accepted to have evolved on Earth between 3.8 and 3.77 Ga BP, but an earlier onset of life cannot be excluded. This would have important implications for the current debate on the possibility of temporal overlap of earliest life with a bolide impact scenario terminating at ~3.8 Ga BP (Whitehouse et al., 1999; Moorbath, 2005). The early organisms probably formed around submarine hydrothermal vents. Prokaryotes are the oldest known organisms on Earth and constitute about 70% history of life till eukaryotes appeared around 2.1 Ga BP. The fossil bacteria are found well preserved in black cherts associated with stromatolitic carbonates in most of the Archean and Proterozoic period (Knoll and Walter, 1992; Schopf, 1993, 1994; Tewari, 2004; Shukla et al., 2006). Westall (1999) has shown experimentally that earlier eukaryotes were identified under thin sections, mainly by internal spots as the supposed remains of organelles, tetrahedral tetrads of cells, scars indicative of meiotic cell division, large cell size, and mitotic cell division; however, these features are reported from silicified bacteria. Tewari (1989, 2004) has also reported these features from the Deoban cherts of the Lesser Himalaya. Thus, it is difficult to recognize the exact time of the first appearance of eukaryotes in fossil records. Cyanobacteria from the early Archean rocks of Australia and South Africa have been reported from the hydrothermal environment (Schopf, 1993; Westall et al., 2001).

The microbial assemblage was photoautotrophic, but the oxygen level was rather low in the Achean period. The Proterozoic microbiota is highly diversified and well preserved in subtidal to intertidal cherts associated with microbially mediated stromatolites (Tewari, 2004, 2007, 2009; Schopf et al., 2008). Meso- to Neoproterozoic Deoban and Buxa stromatolitic carbonates of the NW and NE Lesser Himalaya are classical examples of preservation of these microfossils (Tewari, 1989, 2004; Shukla et al., 2006; Schopf et al., 2008; Tewari et al., 2008). The atmospheric oxygen increased during Paleoproterozoic to Mesoproterozoic around 1.9–1.6 Ga BP, and perhaps, eukaryotes started appearing as leiosphaerid acritarchs in China (Zhang, 1984). Eukaryotic algae are known from 2.0 Ga BP Duck-Creek Dolomite of Western Australia. The diversity in fossil microorganisms is also environmentally controlled in some classical sections like Draken Formation of Spitzbergen (Knoll and Swett, 1987) and Deoban-Blaini-Krol-Tal Formations of the Lesser Himalaya (Tewari, 1989, 2001b, 2004, 2007). Subtidal to intertidal stromatolites and microbiota of Deoban Group include: *Kussiella kussiensis*, *Colonnella columnaris*, *Baicalia nova,* and *Tungussia* (*stromatolites*), and *eukaryotic algae Kildinosphaera,* along with *Eomycetopsis robusta*, *Siphonophycus kestron*, *Oscillatoriopsis media*, and others (Tewari, 1989, 2004, and references therein). Eukaryotic algae diversified in open marine tidal flat environment.

The discovery of *Vendotaenia* (and a new genus, *Krolotaenia gniloskayi*) from the Lesser Himalaya shows that eukaryotic alga evolved during Ediacaran or Vendian/Terminal Proterozoic period, and that the Vendotaenid assemblage coincides with Ediacaran assemblage in the Krol Formation of the Lesser Himalaya, India, Canada, and elsewhere (Tewari, 1988, 1989, 1993, 1999, 2004, 2007; Hofmann, 1994). The Krol Formation also contains Neoproterozoic diverse organic walled microfossils, stromatolites, and Ediacaran metazoans (Tewari, 2004 and references therein). *Vendotaenia* is also found in Dengying Formation of China in association with eukaryotic spheromorphic acritarchs. This is a coevolution of plants and animals during Ediacaran/Vendian times (650–540 million years before the present, Ma BP, after the Neoproterozoic glaciation, best known as "Snow Ball Earth," Tewari (2001b)). Recently, we have discussed the $\delta^{13}C$ and $\delta^{18}O$ during Meso- to Neoproterozoic period in the Lesser Himalaya with reference to global paleoclimatic events, paleobiological evolutionary changes, and their global correlation (Tewari, 2007; Tewari and Sial, 2007).

Ediacaran metazoans were diploblastic animals and related to the increase in the atmospheric oxygen of about 4–8% of the present atmospheric level (PAL). Triploblastic organization of these metazoans is further increased by atmospheric oxygen level up to 10% PAL. Cambrian radiation event of multicellular life is linked to 40% PAL around 0.57–0.5 Ga BP. Prokaryotic to eukaryotic cellular evolutionary change has taken place during one billion years of Earth's early history. Additional micropaleontological discoveries combined with the use of recent instrumental techniques, such as Laser Raman Spectroscopy and Confocal Laser Scanning Microscopy (three-dimensional imaging) of microfossils (Schopf et al., 2002, 2008) are important to establish the biogenicity of early life and the presence of organic matter (biomolecules) in them. Further detailed Carbon isotope chemostratigraphy of the key stratigraphic boundaries, such as the Proterozoic–Cambrian (Tewari and Sial, 2007; Tewari, 2007) and Cretaceous–Tertiary boundary in the Karst region of northern Italy (Tewari et al., 2007), will eventually shed more light on the evolution, diversification, and catastrophic extinction of life on Earth.

2.3. THE EVOLUTION OF TROPHIC RELATIONS SINCE THE ARCHEAN

A closer and more detailed understanding of the origin of life on Earth has forced upon us a more significant view on the Late Archean and Early Proterozoic evolution of trophic relations in the microworld.

Presently, we are more aware that hydrothermal vents in the Earth oceans may have played a role in the origin and evolution of the three domains of life (cf., Fig. 1). Indeed, it is possible that throughout evolution, entire ecosystems depend on geothermal, rather than solar, energy. This is not only evident on the Earth, but is also likely to be the case on the other oceans of the solar system, for example, on the moons of Jupiter: Europa and Ganymede. On these satellites, this particular hypothesis for the origin of life may be tested in the foreseeable future. The Europa-Jupiter System Mission is now being seriously considered by the main space agencies of Europe, the United States, Japan, and Russia, after the initial proposal of the LAPLACE mission (Blanc et al., 2009).

The primary sources of organic matter for the microbial autotrophs are photosynthesis, methanogenesis, and sulfate reduction. In the limited space available, it is most important to single out and highlight that the group of sulfate reducers may contain some of the earliest forms of life on Earth. Thus, this special form of metabolism may hold the key to understanding the primordial state of life because sulfate-reducers are in deeply rooted branches of the phylogenetic tree of life (Shen and Buick, 2004).

The morphological simplicity of the primitive sulfate reducers is one drawback in probing the fossilized remains of these microbes. Instead, we must rely on the science of biogeochemistry when our objective is to enquire on the antiquity of life and its trophic relations. From the early papers of Manfred Schildowski and co-workers, the stable isotope geochemistry of sulfur and other biogenic elements (H, C, O, N) has been reviewed extensively (Schidlowski et al., 1983; Strauss and Beukes, 1996).

For a proper understanding of the Archean S-isotopic record, we should first realize that the abiotic fractionation of carbon isotopes can produce effects comparable with the geomicrobiological effects, as described in detail elsewhere (Horita, 2005). However, the situation is more favorable for sulfur. Indeed, microorganisms mediate the reduction of sulfate to sulfide. The resulting fractionations can be reliably taken as good markers for the geological record, especially for the Archean S-isotopic record, where we have hinted that the oldest signatures for life are to be retrieved. The biology that underlies this significant aspect of our quest for the evolution of trophic relations is as follows: the preferential use of ^{32}S over ^{34}S by microorganisms depletes the sulfide in the environment of ^{34}S with respect to the original sulfate. Several species of bacteria and Archea can make this happen via the metabolic pathway known as dissimilatory sulfate reduction. Sulfur itself is not incorporated into the cell, but it ends up in the oxidation of organic matter. In normal marine sediments of sulfate, fractionation can range from 10‰ to 49‰, but this effect can be as large as 70‰. On the other hand, unlike the case of carbon described earlier, abiotic isotope fractionations can yield fractionations in the range 15–20‰, for example, in the magmatic reduction of gaseous sulfate to hydrogen sulfide (Rollinson, 2007). This leaves an ample margin for distinguishing the microbial activity in rocks at a hydrothermal vent and abiotic fractionations.

The S-isotopic record of sulfide and sulfate in Archean sedimentary rocks ranges between Isua of ~3.8 Ga BP (pyrite in banded-iron formations) and ~3.47 Ga BP (barite deposits). In these early times, sulfate reducers were beginning to leave measurable traces, but there are still some difficulties in fully understanding their sources and the role of atmospheric contributions. In the more recent pyrites in black shales of ~2.7 Ga BP (Shen and Buick, 2004, Fig. 6), these traces are better understood. In conclusion, the stable isotope geochemistry of the ~3.47 Ga barite deposit suggests that the reactions mediated by microorganisms were already fractionating sulfur much in the same way as the present-day sulfate-reducing microorganisms.

2.4. METABOLIC DIVERSIFICATION

In the previous section, we have sketched some of the most ancient microbes lying deepest at the root of the tree of life. We proceed to discuss briefly how the evolution of the Earth atmosphere repressed these Archea into distinct niches. The main driving force was the gradual oxygenation of the atmosphere after the discovery of photosynthesis. Ecosystems adopted a layered mat-morphology and sediments. Such ecosystems are well known in present-day environments, such as the dry valley lakes of Antarctica.

The microbial mats found today in these environments are composed primarily of cyanobacteria, heterotrophic bacteria, protozoan cysts, eukaryotic algal cells, and minerals associated with microbial activity occurring throughout much of the benthic regions of the dry valley lakes (Wharton et al., 1983; Mikell et al., 1984; Vincent, 2007). In the Archean, the photosynthesizers were distributed in the upper layers, while the anaerobic microorganisms, such as the sulfate-reducing Archea, were relegated to the lower layers. The eventual consequence of this atmospheric factor was not only to segregate the sulfate reducers (and others such as the methanogens) to lower layers of the mat formations, but these microorganisms were further relegated to restricted niches. Consequently, it was inevitable that evolutionary diversification would follow. Indeed, today, we have a large number of obligate anaerobes, not only Archea but also mesophilc bacteria. The microfossil record testifies that as oxygenation was gradually driven towards PAL by ~2.1 Ga BP, sulfate reducers and methanogens left their imprint supporting the general outline of Darwinian evolution of the three main domains of the tree of life (Fig. 1). Beyond the consequences of natural selection and adaptation, a new force in evolution, symbiosis, emerged and it was to play a crucial role in the eventual dichotomy of multicelullar life: plants and animals, as we shall discuss in the following sections.

3. Cellular Evolution and the Dichotomy Plants–Animals

3.1. THE ROLE OF SYMBIOSIS IN EVOLUTION

We may separate living organisms into two large groups: prokaryotes (Bacteria and Achea) and eukaryotes (Encarta, cf., Fig. 1 and Section 2.2). The first group consists exclusively of unicellular organisms, considered vegetal organisms since immemorial times (Gerola, 1988) and the second, on the contrary, consists of all multicellular plants and animals inhabiting the Earth. On the basis of this division, we assume that bacteria and unicellular algae, in their quality of autotrophic organisms are vegetal organisms, and we focus our attention on the importance of the interactions of these organisms among themselves, with eukaryotes, and with the multicultural organisms. Frequently, these interactions have developed a sort of dependence along the history of life, which very often comes into a real coevolution for both the organisms. The interactions between unicellular autotrophic organisms among themselves, and between prokaryotes and eukaryotes (unicellular and multicultural), play a major role in speciation and evolution.

In general, the close ecological relationship between the individuals of two (or more) different species is defined as symbiosis. Ecologists use a different term for each type of symbiotic relationship: mutualism is defined as the symbiotic relationship between the individuals of two (or more) different species, where both species benefit; commensalism implies only one of the interacting species being benefited, and the other being unaffected; finally, parasitism implies one species being benefited and the other being harmed. Today, microbial symbiosis is known to be a ubiquitous aspect of life.

According to contemporary conceptual consensus, the mitochondria of all eukaryotic cells and the chloroplasts of plants and protists were once free-living bacteria (alpha-proteobacteria and cyanobacteria, respectively) that became incorporated in a primitive host cell (Gray, 1992; Margulis, 1992). It was first suggested that chloroplasts originated as symbionts (Schimper, 1883). The concept was further developed by Merezhkowsky (Sapp, 2005), who coined the word "symbiogenesis" for such a synthesis of new organisms. He maintained that nucleus and cytoplasm had originated by symbiogenesis. Microbial evolutionists are also considering whether the cell nucleus may have arisen from some sort of fusion of symbiosis between two different kinds of bacteria.

Neodarwinism considers the Cambrian explosion as the "big bang" of biology. During this period, between 560 and 495 Ma BP, many now-extinct plants and animals burst onto the scene. Why the Cambrian explosion occurred is not fully understood, but at the cellular scale, the real "big bang" for plants and animals occurred some 1,800 Ma earlier, with the first appearance of eukaryotic cells (Sapp, 2005). With its membrane-bound nucleus and all the associated features, such as mitosis, meiosis, and multiple chromosomes to package tens of thousands of genes per cell, it provided the material and the conditions for the differentiation of tissues, organs, and organ systems of plants and animals. Symbiosis is at the foundation of our being. Multicellular organisms, including humans, probably evolved and were maintained by bacteria.

Symbiosis has not only played a principal role in the emergence of eukaryotes, it has been vital throughout eukaryote evolution. Eukaryotes are the most morphologically complex microbes, with the largest biomass on earth, and they have the greatest biochemical complexity (Whitman et al., 1998). Microbial symbionts perform many chemical reactions that cannot be carried out by their hosts. Collectively, they can photosynthesize, fix nitrogen, metabolize sulfur, digest cellulose, synthesize amino acids, provide vitamins and growth factors, and ward off pathogens.

The fact that microbial symbiosis is a fundamental aspect of life was first suggested by botanists of the late nineteenth century. The dual nature of lichens, nitrogen-fixing bacteria in the root nodules of legumes, fungi in the roots of forest trees and orchids, and photosynthetic algae living inside the bodies of protists, hydra, and the flat worm *Convoluta roscoffensis*, suggested a temporal continuum of dependency of microbe and host from transient to permanent interdependence. When these phenomena were considered together with cytological evidence for reproducing organelles within the cells of plants and animals, they led several biologists of the late nineteenth century to a conception of the cell itself as a symbiotic community (Sapp, 1994). Speciation induced by parasitic or mutualistic symbionts has been suggested for taxa ranging from plants to insects to monkeys (Thompson, 1987). Models for symbiont-induced speciation have been proposed based on hybrid inferiority and selection for reinforcement genes. However, taken on their own, such models have severe theoretical limitations and little empirical support. Thompson highlighted the importance of the environment on the symbiont-induced speciation. He established two conditions for symbiont-induced speciation: first, interaction norms in which the outcomes of host/symbiont interactions differ between environments; and second, differential co-adaptations of host and symbiont populations between environments or along an environmental gradient. Thompson defined symbiont-induced speciation as a form of "mixed-process coevolution," namely, reciprocal evolution in which adaptation of a population of one species to a population of a second species (or co-adaptation of the populations) causes the population of the second species to become reproductively isolated from other populations.

The term "symbiosis" was introduced from the Greek language ("living together") into biology by Anton de Bary in 1879 (Darlington, 1951). He was discussing the presence of algae in the leaves of the aquatic fern *Azolla* and the stunning discovery that lichens were double organisms, a combination of algae and fungi. Today, we know that all of the 15,000 species of lichens are made of fungi and either algae or cyanobacteria. A whole new structure, the thallus, emerged out of their association. For de Bary, lichens and other examples of symbiosis offered proof of evolution, and they indicated a means of macroevolutionary change in addition to Darwinian gradualism, based today on gene mutations and recombination.

Frank (1885), who had used the term *symbiotismus* a year before de Bary, reported another important symbiosis between fungi and the roots of forest trees, an association that he named "mykorrhizen" (fungus root). Although the discovery of mycorrhiza and its ubiquity is generally attributed to Frank, the claim that root fungi may be beneficial to plants had been made a few years earlier for the Indian pipe, *Monotropa hypopitys* (Kamienski, 1881; Trappe and Berch, 1985).

Mycorrhizas are known to occur in practically all terrestrial plants: the plants benefit from receiving essential nutrients, and the fungi benefit by receiving organic compounds from the plants (Allen, 1991; van der Heijden and Sanders, 2002). This symbiosis is thought to have been instrumental in the colonization of land by ancient plants some 450 Ma BP (Malloch et al., 1980; Redecker et al., 2000).

Mycorrhizas are also at the very foundation of the world's most complex and biodiverse ecological systems. Trees of tropical rain forests, in nutrient-poor soils, rely on these fungi to bring them precious nitrogen and minerals (Went and Stark, 1968).

Nitrogen-fixing bacteria in the root nodules of legumes have also been central to discussions of symbiosis since the nineteenth century. Plants cannot metabolize molecular nitrogen abundant in the atmosphere, and require nitrogen in the form of nitrate converted to $NH4^+$. Legumes overcome this problem by their symbiotic union with a group of N-fixing bacteria, rhizobia, which live in specialized root nodules, where excess $NH4^+$ is exchanged for sugars exuded by the plant. Symbiotic N fixation underpins the whole global N cycle. During the production of the nodule, there is gene transfer between the host and bacteria: some of the plant DNA is transmitted to the bacteria (Pirozynski, 1991). It is not known whether the reverse is also true (Quispel, 1998).

For plants, the associations with fungi and bacteria were the innovative key in the colonization of land and specific habitats. The eukaryote-associated microbes act as metabolic partners for accessing limiting nutrients and also as protectors, producing toxins that ward off herbivores or pathogens. Similar associations have arisen with animals, allowing colonization of diverse niches, such as the specialized feeding on plant or animal tissues, and the use of deep-ocean hydrothermal vent habitats. Often, the associations are persistent for the hosts, frequently being transmitted vertically across generations, from mother to progeny. The symbionts may be sufficiently fused such that they cannot live apart or be easily recognized as distinct entities (Moran, 2006).

3.2. EFFECTS OF SYMBIOSIS BETWEEN AUTOTROPHS AND HETEROTROPHS

The physiological and morphological effects of symbiosis between photosynthetic autotrophic organisms (plants) and heterotrophic organisms (animal) are varied. Sea anemones, hydra, giant clams, sponges, and tropical corals capture algae from the ocean, harbor them in their cells, and are nourished by their symbionts' photosynthetically produced carbon compounds. Corals acquire up to 60% of their nutrition from *Symbiodinium*, which in return obtain nitrogenous compounds from the coral polyp, which are scarce in the crystal-clear tropical waters. A worldwide catastrophe for tropical corals has resulted from disrupting this delicate balance (Hughes et al., 2003). Prolonged high sea-surface temperatures, sedimentation, and inorganic pollutants cause coral "bleaching": corals lose their algae, leaving their tissues so transparent that only the white calcium carbonate skeleton is visible. Without the algae, corals starve to death.

Lichens are intimate and long-term symbioses of photosynthetic algae or cyanobacteria and heterotrophic fungi. As intimate symbioses where the photosynthetic partner is inhabiting its heterotrophic partner (Law and Lewis, 1983; Ahmadjian, 1993), lichen symbionts are often hypothesized to have undergone long-term coevolution, especially when one or both symbionts appear obligate and specialized (Ahmadjian, 1987). However, coevolution has not been rigorously tested for lichen associations.

To demonstrate coevolution directly requires an assessment of increased fitness resulting from reciprocal genetic change (Thompson, 1994), although coevolution could be demonstrated indirectly by showing parallel cladogenesis or cospeciation between symbiont lineages (Page and Hafner, 1996). A hypothesis of parallel cladogenesis would be accepted with highly specific associations between algal and fungal partners, especially if there is strict vertical transfer of inhabiting algal partners throughout a fungal lineage. In contrast, this hypothesis would be rejected in the case of horizontal transfer of algal partners among fungal lineages. Here, this phylogenetic process is called algal switching.

When reciprocal evolution leads to cospeciation, coordinated speciation events, equal numbers of species among symbiont partners should evolve – a situation not predicted from morphological studies of these algae (Tschermak-Woess, 1988) and their fungal partners (Hawksworth et al., 1995).

3.3. THE ROLE OF THE ENVIRONMENT IN COEVOLUTION

Evidence is accumulating that interactions of animals with environmental microbes have resulted in the coordinate evolution of complex symbioses, both benign and pathogenic (McFall-Ngai, 1998; Henderson et al., 1999; Hooper et al., 2001), and that coevolved animal–bacterial partnerships represent a common, fundamental theme in the biology of animals. Most environmentally transmitted symbioses in animals, such as the alimentary canal and squid–vibrio associations, are extracellular and often remain open to the environment throughout the life history of the host. Thus, the host/symbiont community must not only develop mechanisms by which to achieve a balanced, functioning population ratio, but it must also ensure specificity of the interaction from the inception of the relationship throughout its persistence. Available evidence suggests that such controls are mediated by: the direct interaction of the bacterial cells with the host cells that are colonized, and by the immune system, both innate as well as adaptive (when present), which samples the population and keeps the host informed of the state of the interaction (McFall-Ngai, 2001). For example, interactions with the gut microbiota induce the mammalian intestinal mucosa to produce mucins and alpha-defensins, which inhibit the symbionts from invading host tissues (Hooper et al., 2001).

We remark, then, that humans are also engaged in several mutualistic relations with other species (cf., Section 5). These relations range from our gut flora (without which we would not be able to digest food efficiently) and our immune system, to domesticated animals like dogs or horses. These interactions provide protection, or other services in return for food and shelter. Furthermore, man takes benefits from mutualism of other species (second degree mutualism): in traditional agriculture, men exploit the mutualistic beneficial functions of many plants as companion plants, providing each other with shelter, soil fertility, and the repelling of pests; for example, the cultivation of beans, which may grow up cornstalks as a trellis while fixing nitrogen in the soil for the corn.

Therefore, symbiosis is recognized to be a ubiquitous aspect of life. The mechanisms at the origin of these relationships, and how and why species might cooperate is a question that has been raised by a number of writers who philosophically question the conception of evolutionism and the notion of linear historical progress.

4. Evolution in the Cretaceous and Tertiary of the Trieste Karst

4.1. EXAMPLES OF PLANT–ANIMAL INTERACTIONS

The geological history of the Karst area encompasses a time spanning from the early Cretaceous to the mid-Eocene (Cucchi et al., 1987; Pugliese and Tunis, 2006). This stratigraphic sequence includes carbonate deposits (from the early-Cretaceous to the mid-Eocene partim) overlaid by mid-Eocenic torbiditic beds (marls and sandstones of the flysch). Most carbonate beds record shallow water marine-to-lacustrine settings, where the development of the relationships between plants and animals took place. Considering the carbonate sequence, paleontologists may propose examples of these relationships and reconstruct some hypothetical environmental scenarios through the analysis of well-defined fossil taxa. The most representative taxon was the calcareous microalgae (especially Cyanophyta, Dasycladaceae, Charophyta, Corallinaceae), organic builders (including rudists and corals), and microfaunae (benthic foraminifers).

Calcareous algae populated several shallow substrates of the depositional environments recorded in the Karst stratigraphic sequence. The main taxa correspond to Cyanophyta, Charophyta, Dasycladaceae, and Corallinaceae. The calcareous algae may represent a favorable basis for a food chain and a good house for the animals.

Rudists and, subordinately, corals were the organic builders (especially requienids, radiolitids, and hippuritids). Organic builders are usually symbiont-bearing taxa. Symbiosis consists of a host–symbiont interdependence between them and the microalgae. This interdependence is advantageous for both: the symbiont gets nutrients and place to live, and the host gets food and oxygen. Moreover, photosynthesis induces calcification in settings that are poor in carbon dioxide. However, there is no data about the symbiosis between the Cretaceous rudists and microalgae. Corals were sporadically present in both Cretaceous and Tertiary beds, and produced small build-ups, especially during the Tertiary. Similar to the modern corals, they can be considered symbiont-bearing taxa (cf., for example, Pearse and Muscatine, 1971).

Foraminifers are usually representatives of the most abundant taxon in the fossil record. They show several kinds of relationships. Like corals, the larger foraminifera are a symbiont-bearing taxon (cf., for example, Hohenegger, 1999). Moreover, following Langer (1986, 1993), who studied the recent foraminifers from some Mediterranean sectors, we focus on the adaptation of the epiphytic foraminifers to a well-defined microenvironment:

A. Permanently immobile (flat, trochospiral, conical) taxa that live attached to plants; some arborescent taxa live on shaded parts (rhizomes) of plants
B. Temporarily mobile conical, trochospiral taxa that glide over flat substrates (algae, sea-grasses) extending their pseudopodia towards moving direction
C. Mobile taxa living within the algae that build three-dimensional meshworks; some plane-spiral or annular species suspend their tests and, like the spiders, produce a pseudopodial network to capture their food
D. Mobile ovate, elongate, or planispiral taxa living over the plants

4.2. HYPOTHETICAL ENVIRONMENTAL SCENARIOS

Considering the previously described examples of plants–animals relationships, some environmental scenarios can be interpreted as follows.

4.2.1. Low-hydrodynamism Lagoons (Inner Lagoons)

More or less restricted lagoons mostly occurred in the Cretaceous and Paleocene beds, evidencing anoxic or oxygen-deficient conditions. In particular:

Early Cretaceous dark limestones usually present an oligotypical biota consisting of opportunistic taxa and limestones with calcareous algae (Cyanophyceae, *Thaumatoporella parvosiculifera*, rare Dasycladaceae). Also, stromatolitic layers suggest shallow vegetated bottoms, where animals grazed. Thin-shelled both vegetarian and predator gastropods might have lived in these environments. Vegetated bottoms may be suggested by foraminifers that were mostly represented by morphotypes D (mobile small Miliolidae) and A (arborescent Nubeculariidae and rare small cone-shaped species). The organic builders activity is sporadic (and is due to the requienids) may be in relation to an increase in the hydrodynamism and related temporary improvement of oxygenation. Caffau et al. (2000) described the corresponding biota that was characterized by the requienids giving rise to thin banks. Moreover, a relatively rich biodiversity emerged: foraminiferal morphotypes A (Nubeculariidae) and D (small Miliolidae) suggest possible vegetated bottoms, in the presence of grazing gastropods.

Just above the early Cretaceous limestones, a Cenomanian dolomitic unit testifies very restricted conditions, in relation to a sea regression. It represents extreme life conditions (innermost lagoon, tidal pool), where only gastropods and the agglutinated foraminifer *Glomospira* have been found. It is presumable that these very restricted settings might have been occasionally vegetated, as also demonstrated by sporadic stromatolitic layers that may have been colonized by grazing taxa.

The boundary between this unit and the overlying late Cenomanian dark limestones is marked by an interval characterized by paleosoils. The dark limestones correspond to a renewal of the benthic life that was linked to a transgressive phase. The community showed opportunistic taxa very similar to those already observed in the early Cretaceous unit.

Algae (Cyanophyceae and *T. parvosiculifera*) might have provided a favorable substrate for prey/predators life, also in the presence of the most opportunistic radiolitids, which gave rise to small patch-reefs. Within a better-diversified biota, the foraminifers showed possible morphotypes A (Nubeculariidae), B (Nezzazatidae), C (*Biplanata peneropliformis*, *Biconcava bentori*, *Broeckina balcanica*), and D (*Spiroloculina*, small Miliolidae), suggesting a vegetated nature of the substrate.

During the Senonian, some levels were characterized by poor-oxygen conditions showing opportunistic biota. They consisted of taxa that were similar to those found in the early Cretaceous. Brackish water wetlands occurred during the Danian and Selandian. Most beds yield small foraminifers represented by the morphotypes B (discorbids), C (*Bangiana hanseni*), and D (small Miliolidae). In relation to the fresh water influence, the scenario also included Charophyta, mostly thin-shelled gastropods and ostracods, together with very rare foraminifera (morphotype B, discorbids). Charophyta were represented by their girogonites, encrusted stems, and *Lagynophora* that probably produced meadows, where animals grazed.

4.2.2. High-hydrodynamism Lagoons (Open Lagoons)

These settings are represented mainly by Senonian (partim) and Tertiary limestones with a high fossiliferous content. They present a very rich life including algae, foraminifera, and molluscs.

The late Cretaceous beds include the *alga T. parvovesiculifera* and a rich rudist fau na (radiolitids and, subordinately, hippuritids and requienids). Foraminifers were dominated by mobile morphotypes C and D (*Moncharmontia apenninica* and *Spiroloculina*, and Miliolidae, respectively) with rare morphotypes B (*Stensioina surrentina*). Among the molluscs, the gastropods were sometimes abundant, may be grazing on the vegetated substrates, as also suggested by the presence of epiphytic foraminifers. In addition, radiolitids and requienids provided a builder activity scenario.

The limestones from the Danian to Thanetian contain abundant algae that were mostly represented by Dasycladaceae. Foraminifers were constituted by abundant miliolids (morphotype D) and agglutinated and rotaliid species. Moreover, isolated and colonial corals were present during the Danian. They were rare in some thin beds of the Padriciano section (Pugliese et al., 2000) and common in Slovenia beds (Drobne et al., 2000). Thus, it is presumable that a zooxanthellae/corals symbiosis occurred.

4.2.3. Peritidal Settings

Their most common findings are signaled just below and above the K/T boundary. Their sequences were repeated several times, denoting changes in intertidal/

subtidal/supratidal biota. Concerning the aqueous assemblages, the remnants obviously testify unstable environmental conditions, where an opportunistic biota of lagoon occurred. In particular, lagoon layers contain foraminifers represented by mobile morphotypes (*B. hanseni* and small Miliolidae in Tertiary beds) and grazing thin-shelled gastropods and ostracods. Wherever present, the stromatolites testified a substrate favorable for grazing gastropods.

4.2.4. Open Marine Settings

Ramps and, in general, coastal settings are common during the Senonian and after the Thanetian. The limestones often present a high bioclastic content, where only rare corallinacean algae were found.

Senonian biota was well diversified. In agreement with Caffau et al. (2004), the Senonian ramps included organic builders consisting of hippuritids, sometimes together with radiolitids and requienids. Moreover, rare zooxanthellate thamnanasteroid corals occurred (A. Russo, 2004, personal communication). Among the foraminifers, the mobile lenticular porcelaineous macroforaminifer *Keramosphaerina tergestina* was present. This scenario corresponds to an oligotrophic scarcely vegetated bottom. Moreover, corallinacean algae may have highlighted an epibiosis: they might have encrusted shells of other taxa.

Tertiary ramps and coastal marine settings were characterized by limestones with a more or less high muddy content. Corallinacean algae and larger foraminifera were very common. Foraminifers were probably mobile morphotypes (large Miliolidae, Alveolinidae, Nummulitidae, and *Orbitolites complanatus*). Thamnasteroid zooxanthellate corals (Turnšek and Drobne, 1998) and rare sea-urchins were present. This scenario suggests oligotrophic conditions.

4.3. SYMBIOSIS SCENARIOS

Karst limestones yield a host–symbiont system in several horizons of its stratigraphic sequence. The existence of these systems may be confirmed, in agreement with the taxonomic uniformitarian (*sensu* Dodd and Stanton, 1990). Actually, modern corals and larger foraminifera are symbiont-bearing taxa (cf., for example, Pearse and Muscatine, 1971; Hohenegger, 1999). Obviously, skeletal parts of the symbiont-bearing taxa can be easily fossilized.

This manner of plant–animal interaction took place during episodes of environmental stability. This stability corresponds to environments populated by a well-diversified biota, consisting of several species in equilibrium. Following Travé et al. (1996), Hottinger (1998), Hottinger and Drobne (1988), and Drobne et al. (2000), this biota was k-strategist (*sensu* Dodd and Stanton, 1990). This biota is well recorded in the deposits of ramps and, in general, in the coastal settings during the Senonian, the Thanetian, and after the Thanetian. Senonian ramps mainly included hippuritids, together with radiolitids, thamnanasteroid corals, some Corallinaceae, and foraminifers, such as the lenticular porcelaineous larger foraminifer *K. tergestina*. Thus, corals and larger foraminifera might testify a symbiosis between zooxanthellae/corals and zooxanthellae (or other algae)/ larger foraminifera. Tertiary ramps-coastal marine settings confirm plant–animal interactions that are described for the Senonian. Corals (Turnšek and Drobne, 1998; Hohenegger, 1999) and larger foraminifera (Alveolinidae, Nummulitidae, Orbitolites) were in symbiosis with algae.

If the open lagoons, ramps, and shallow marine settings represented stable environments with a well-diversified biota, the more or less restricted lagoon testified unstable settings. The former settings showed interactions based on a K-strategist biota, together with the possible and subordinate coexistence with r-strategists. The latter settings evidenced r-strategist opportunistic taxa only. In this regard, data concerning possible r-strategists as symbiont-bearing taxa are not confirmed.

Larger foraminifera/microalgae systems may also provide detailed paleo-depth data. Hohenegger (1999) proposed a precious synthesis of the extensive literature on larger symbiont-bearing foraminifera. In general, foraminifera should protect the symbionts from the intense illumination in a shallow-water environment.

Moreover, the presence of zooxanthellate hermatipic corals constitutes a geological thermometer. In fact, the system zooxanthellae corals is active if the seawater temperature is at least 18°C in relation to the presence of an anhydrase enzyme that inhibits the system at a temperature less than 18°C. The existence of the host–symbiont system was surely inhibited during oxygen-poor conditions (inner lagoon, wetlands, peritidal settings). Moreover, within the paleo-environmental evolution recorded by the Karst rocks, the impact of the meteorite in correspondence with the K/T transition produced remarkable effects. Among the evidence of this event, δC^{13} showed a negative shift, denoting a decrease in the photosynthesis activity (Pugliese et al., 1995) and the possible occurrence of host–symbiont systems.

5. Interaction of Plants and Animals Since the Appearance of Hominins

5.1. THE FIRST HOMININS

Hominins emerged in the animal kingdom around 6–7 Ma BP. The hominin clad includes the humans and all their ancestors back to the human–ape separation. They are identified by two main traits from the great apes: bipedalism (with the associated differences in pelvis, spine, knee, and foot) and skull shape (with the associated change in brain size and shape), both probably induced by evolutionary effects that can be classified as animal–plant interaction.

Australopithecins are the hominins preceding the *homo* species and showing their presence in the fossil record approximately between 4 and 1 Ma BP. They have characters common to apes, such as the small 300–400 g brain, and to humans, such as bipedalism and small teeth. During times when australopithecines were in their hay days, around 3.5–3 Ma BP, the Sahara desert did not exist and these hominins could move freely in a vast land of forests and savannahs extending from the Cape of Good Hope to Java. In fact, around 3.5 Ma BP, australopithecines were present not only in southern and eastern Africa, but also 2,500 km west, in Chad. One of the oldest *Australopithecus,* Little Foot, is the 4 million-year-old hominin found in Sterkfontain (Clarke, 1999). The anatomy of the ankle joint shows that Little Foot was bipedal but able to climb in trees by virtue of a divergent big toe. He felt comfortable walking upright, but he might have escaped back into the trees for safety or foraging. Australopithecines were well adapted to bipedalism, but were not consistent bipeds. A nearly juvenile complete *A. afarensis* (nicknamed Selem) was found in 2005 at Dikika, 4 km from where Lucy, the most famous 3.5 million–year-old Australopithecus was found. Its body confirms the mosaic evolution patterns found in other australopithecines (Alemseged et al., 2006). The passage from quadrupedalism to bipedalism started in the lower limbs and pelvis, keeping the primitive characters in arms and shoulders.

One of the differences between *Homo* and *Australopithecus* is the smaller size of *Homo*'s jaw muscles. Australopithecines needed powerful muscles for processing nuts and other hard foods that became necessary after the environmental change in Africa during the Pliocene. The recently discovered human gene MYH16 is responsible for encoding the protein, myosin heavy-chain 16 that has a key role in human muscles (Stedman, 2004). They are much weaker when this protein is absent or altered. For example, in both humans and macaques, MYH16 is encoded by a particular gene that is expressed in the temporalis muscle. At some point, the human gene had a mutation that strongly reduced the function of the protein. After that mutation, the muscle mass became 8 times smaller than that of the macaque. But *when did this mutation occur?* By comparing the number of changes in the human gene with that in other species, one can infer that the mutation happened 2.1–2.7 Ma BP, exactly at the time when the genus *Homo* originated. Also, living primates such as chimps have strong jaw muscles and in fact have the MYH16 gene that fully activates the function of the corresponding protein.

On the other hand, muscle size can influence bone growth. Reduction in the jaw muscle attenuates the stress on skull bones. The larger and thinner skull of humans could be the effect of smaller jaw muscles. In conclusion, there could be a strong connection between the size of jaw muscles and that of the brain. In addition, a less massive jaw muscle could have allowed a better coordination of the mandible function, improving speech capabilities. The first *Homo* species is *H. habilis* that emerged around 2–2.5 Ma BP. At that time, the climate was becoming drier and cooler, inducing dramatic changes in vegetation and fauna. This is a challenging moment for hominins in Africa, who needed to change their diet and way of life in order to survive.

5.2. THE INFLUENCE OF CLIMATE

At that time, not only Africa but the whole planet was going through dramatic environmental changes. A progressive 50 million years of slow cooling during the Tertiary had brought the planet into the Pleistocene, when the Arctic ice cap also developed. Despite being referred to as "Ice Age," the Pleistocene was characterized by glacial advance as well as recess. Long periods of glaciations were punctuated by briefer warm periods (interglacial – lasting less than 20 thousand years (ka)). Between 2.6 and 1.1 Ma BP, a full cycle of glacial advance and retreat took about 41 ka. During the past 1.1 million years, the cycle lasted 100 ka. The paleotemperature record is being obtained with increasing detail, measuring the oxygen isotopes in ice cores from Antarctica and Greenland. The recent EPICA project has provided a detailed temperature record for the last 700 ka (EPICA, 2004).

Homo with a larger brain and stone tools suddenly appeared in the African record following an evolutionary selection induced by environmental change. He was selected for survival, while all the other small-brained were not, except for *Paranthropus Robustus*, who stayed around for a while and then became extinct at about 1 Ma BP.

Between 2.5 and 1.5 Ma BP, *H. habilis* and *H. ergaster* were sharing the African savannas with robust australopitecines. This long coexistence was made possible due to the different strategies of adaptation for coping with increasing seasonality. Some Australopitecines, such as the *Paranthropus robustus*, with their powerful chewing muscles anchored to the characteristic sagittal crest and their large molar teeth with thick enamel, were well adapted to eat tough fibrous plant foods. Carbon and oxygen isotope analysis using laser ablation mass spectrometry show that the australopithecines adapted their diet to seasonal and interannual variations (Sponheimer et al., 2006). *Homo ergaster*, with his smaller jaw muscle and smaller molar teeth, had a diet of softer food, including fruits and meat. It is generally believed that the carnivorous diet provided the energy needed for the increase of brain in *Homo*. The species *Australopithecus* survived until about 1.5–1.3 Ma BP, when the African savannahs were fully inhabited by *H. ergaster*, capable of controlling fire. We know little on the details of the extinction of australopithecines and on the role that *H. ergaster* and environmental change had on their demise. *Who was ergaster? Was he really the product of evolutionary events in Africa as maintained by most paleoanthropologists?*

The details of the true ancestor remain uncertain. Some say *habilis* was the ancestor of all species once called *erectus* and now called *ergaster* in Africa, *erectus* in Asia, and *heidelbergensis* in Europe. Other claim *rudolfensis* and not *habilis* was the ancestor of *erectus*. According to Meave Leakey, keniantropus was its ancestor. In any case, *erectus* suddenly appeared in Africa at about 1.8 Ma BP. The best representative of this species is the Nariokotone skeleton, 1.75-cm tall (twice the size of australopithecines) with modern body proportions and only 20% size difference between males and females. Being a carnivorous creature with a brain of 900 cc and armed with advanced stone tools, he was the first species to migrate out of Africa, conquering India, China, Sudan, and eventually reaching Europe. It is generally believed that meat was the key source of energy for the growth of *Homo*'s brain that led to the bifurcation of this species from other hominids at the end of the Pliocene.

Less orthodox viewpoints have been gaining ground recently (Dennell and Roerbroeks, 2005). It has been suggested that the speciation event that produced

Homo could have occurred in Asia. This is based on a number of assumptions. First, *Australopithecus* and/or *habilis*-like hominins were living in the vast savannahs going from western Africa to China during 3.5–2.5 Ma BP. There are several sites with stone tools that have chronologies older than the date assumed for the "out of Africa 1" event. "Taphonomic circumstances and lack of field work" are possible reasons explaining the absence of australopithecine fossils in Asia. Second, Asia was the center of global climate change around 2.5 Ma BP with the rising of monsoon seasons that caused the expansion of grassland in a more dramatic way than in Africa. Climate change was also due to the collision between Indian and Eurasian Plaques that formed the Tibetan plateau. If climate change was the cause of speciation that originated *H. ergaster/erectus*, one should remember that according to recent paleoclimatic studies, Asia and not Africa was at the center of the cooling event at the Pliocene/Pleistocene boundary. Third, the more advanced *Homo* species that developed in Asia as a response to the cooling event disperses back to Africa. This would explain the sudden appearance of *H. ergaster*, a species apparently without immediate ancestors in Africa, at about 1.8 Ma BP.

The main morphological effects of the evolution from australopithecines to *H. ergaster* had been an increase in body size and brain volume (from 300–400 cc to 1100–1200 cc). The size of the brain is also linked to the linguistic aptitude and paleoanthropologists believe *H. ergaster* had already developed some capacity to communicate in a rudimentary manner through spoken language. According to recent hypotheses, brain size is related to jaw structure that was smaller in *H. ergaster* than in earlier hominids. This was probably the effect of a diet rich in cooked meat, more available due to the sophisticated stone tools with sharp blades, very efficient for butchering large animals.

Climate variability must have been the key catalyst in the evolution of the *H. sapiens genome* in Africa, possibly from *H. ergaster* and *H. heidelbergensis*, and its dispersal into Eurasia. The climate change witnessed by modern humans in the last 100–200 ka has been recorded, as stated earlier, in polar ice cores, deep-sea sediments, corals, and varves. These terrestrial archives show with great detail the dramatic variability of climate during this period, a feature that is common to the whole Pleistocene epoch. Isotopic analyses provide high-resolution information on the average temperature of the planet, dropping nearly 10°C below the present mean values during glacial times and reaching higher values than the present during interglacials. The movement of modern humans must have been seriously conditioned by the corresponding changes in landscape, vegetation, and sea level. The Sahara was a critical area influencing the diffusion of North–South of modern humans. Its occupation was favored during the warm and wet interglacial times, while aridity produced by cold and dry glacial periods would have made life more difficult for humans. Climate change increased the flux of people in the direction of North Africa and the Levant during glacial times, facilitating Middle Paleolithic/Middle Stone Age cultural interactions between these areas.

Cultural and social progress during the Middle Paleolithic/Middle Stone Age was very slow. Rock shelters and caves became the home base for relatively large groups, which would occasionally break into small bands for hunting and foraging.

The first use of seafood and the grinding of plant seeds were introduced, probably during the Last Interglacial. This diversified the diet for modern humans, introducing new elements of flexibility, useful during their geographic spreading.

5.3. ISOTOPIC SIGNATURES OF PLANT–ANIMAL INTERACTIONS

DNA analysis in extant human populations suggests that anatomically modern humans (AMHs) left Africa around 70 ka BP, reaching Australia around 50 ka BP and Europe 40 ka BP (Wells, 2006). Archeological sites with human presence need to be dated in the relevant areas to confirm these predictions. A precise chronology is also required to evaluate the impact of AMH on the ecosystem they were invading, including their possible involvement in the extinction of the ice-age megafauna and "less evolved" human species they were encroaching (Barnosky et al., 2004).

For example, Australia lost 85% of its terrestrial animals with body mass exceeding 44 kg during a short period of time, around 50,000 ka BP, in coincidence with the arrival of humans. One of these animals was the large flightless bird *Genyornis newtoni*. Its eggshells can be found across Australia, together with those of an extant large bird, *Dromaius*, the emu. The chronology related to the presence of these birds during the late Late Pleistocene have been determined using radiocarbon dating by accelerator mass spectrometry, amino acid racemization, and thermal ionization mass spectrometry U-series analysis on eggshells and luminescence dating on associated sediments (Miller et al., 1999). More than 700 dates have been obtained, showing the continuous presence of Genyornis between 100 and 50 ka BP, when it suddenly disappeared from the archeological records. On the other hand, the eggshells of emu show their continuous presence until the present. The diet of the two birds was reconstructed using the $\delta^{13}C$ ratio of the eggshells, which reflects the plants that were a part of their diet. The results show that Genyornis ate mainly C3 plants, whereas emu had a broader diet including both C3 and C4 plants. Hence, the emu could adapt its feeding strategy to different ecological conditions and this might have been critical for surviving through environmental changes that took place 50 ka BP ago, probably caused by the arrival of the first humans in Australia (Miller et al., 2005).

6. Discussion and Perspectives

6.1. WHAT HAVE WE LEARNT SO FAR?

The bicentenary of Charles Darwin's birth in 2009 as well as the 150th anniversary of the publication of *The Origin of Species* seems an appropriate time to raise questions that Darwin was unable to do because he was constrained to the world-view that was typical of the nineteenth century. For this reason, it seems timely to review, as exhaustively as possible, the main outline of what we now have learnt so far from the evolution of life, especially the evolution of plant–animal interactions. Within the limitations of a short review, we have attempted to sketch a comprehensive view of evolution of life on Earth spanning from bacteria to humans. We have pointed out some insights that have already been gained from the earliest traces of life in the Lower Archean, due to the significant combined progress of stable isotope geochemistry and micropaleontology (Section 2). The ascent of multicellular life can by now be documented convincingly (Section 3). There is robust evidence for the evolution of multicellular life over a billion years just before the Ediacaran fauna (Section 2.1) till the origin of the primates (Section 5.1). This is especially convincing in the coevolution of plants and animals, known in a given geographical region. In such cases, evolution can be followed up in exhaustive detail, as we have done in our region for the sake of illustration (Section 4). With the fast progress of technology, especially the instrumentation of physical sciences, we have been able to present some microfossil evidence for both the precursors of plant and animals (Section 2.2). Furthermore, how the trophic relations evolved from solar energy to geothermal energy (Section 2.3) has also been discussed. The transition from these two forms of energy, which are available for living processes led to an inevitable layered segregation that even led to new niches for anaerobes. These events left a geochemical record that convincingly testifies the general lines of Darwinism (Section 2.4). New instrumentation has also enabled us to demonstrate how plant–animal interactions have influenced the origin of humans (Section 5). This is relevant because stable isotope variations have shed insights into the climate and the diet of our ancestors. The relevant parameters are $\delta^{13}C$ and $\delta\ ^{18}O$ (Ambrose, 2006).

6.2. NEW PERSPECTIVES ON EVOLUTION

Chemical evolution has been able to successfully sketch the pathway from molecules to the first cell. However, research in astrochemistry has also outlined the pathway from nucleosynthesis in stellar interiors to the spread of the biogenic elements (C, H, O, N, S) through supernova explosions into interstellar gas, leading to the inevitable synthesis of organic molecules that will eventually be the building blocks for the origin of life on Earth and eventually elsewhere in the solar system and around other stars. This is the way that evolution from the Archean, the world of Archea and Bacteria, to the Holocene, the world of the humans, should be viewed. Some questions that could not be formulated during Darwin's lifetime now begin to be forced upon us, and answers might be within our grasp in the foreseeable future. This short review only allows us to touch one of them, namely, *What can be learnt from the evolution of life on Earth that might contribute to the search for life elsewhere?*

This question is especially relevant for understanding the intelligibility of nature (Chela-Flores, 2009). Many space missions are currently testing the laws of physics, the geophysical conditions of planets and satellites of the Solar System, and searching for evidence showing that evolution may have taken place elsewhere. Careful probing of the different stages of evolution that are available to us in great detail, as we have described in the present review, will contribute to identifying useful biosignatures.

Other topics of current interest for the evolution of life are beyond the scope of our work, and the reader is referred to the excellent reviews elsewhere. Among such questions, we should mention: *Can the Modern Synthesis of Darwin's evolution and Mendelian genetics be enlarged to take into account present-day insights of developmental biology?* (Pennisi, 2008).

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PART 2:
INSECTS INTERACTIONS

Abril
König
Dröge
Iluz
Raman

Bio data of **Adriana Abril**, author of ***"The Leaf Cutting Ant–Plant Interaction from a Microbial Ecology Perspective"***

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THE LEAF-CUTTING ANT–PLANT INTERACTION FROM A MICROBIAL ECOLOGY PERSPECTIVE

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1. Introduction

Herbivory is a key ecological process in both terrestrial and aquatic ecosystems. The interaction between plants and herbivores is responsible for energy and nutrient flow, population regulation, and the shaping of vegetation physiognomy and species composition (Barbour et al., 1999). Digestion of plant material is a complex chemical process that in some cases goes beyond the metabolic capacity of multicellular organisms. A key metabolic bottleneck for multicellular organisms is their lack of the enzyme set required for degradation of those cell wall components (cellulose, lignin) that provide structural strength to terrestrial plants (Robbins, 1993). This limitation has been solved by some herbivores through evolutionary processes that led to interactions between herbivores and microorganisms, because microorganisms are capable of performing metabolic processes, such as decomposition of structural biopolymers, nitrogen fixation, and oxidation of inorganic compounds (Paul and Clark, 1996).

Classical examples include vertebrate ruminants and termites that host cellulolytic bacteria in their digestive tract, which are capable of releasing glucose and other low-weight monomers that are utilized by the host (Maynard et al., 1979). Moreover, microbial activity may involve not only polymer decomposition, but also production of vitamins and other growth factors, such as in the case of bacterial community found in the cecum intestine of some birds and mammals (Robbins, 1993; Klasing, 1998).

A special case of herbivore–microbe association results from insects consuming fungi that grow on plant material they collect and store, including xylophage beetles, some termite groups, and leaf-cutting ants (LCA). Because of their diversity, abundance, and volume of plant material consumed, LCA represent a singular, highly interesting case of microorganism-mediated herbivory (Cherrett et al., 1989; Wirth et al., 2003).

LCA are members of the Attini tribe and make up a distinctive group restricted to the New World. Most of its 12 genera inhabit the tropical regions of the American continent and a few species live in subtropical arid and temperate-cold regions. The true LCA belong to the so called "higher attine" group because they feed on the fungus growing in the harvested material, whereas the remaining ones feeds on other substrates (insect frass, seeds, etc.). Higher Attines include the genera *Atta* and *Acromyrmex*, with 15 and 24 species, respectively (Wirth et al., 2003).

Traditionally, it has been assumed that the fungus cultivated by LCA is capable of transforming structural compounds of the plant cell wall into fungus biomass, an easily digestible food for the ants (Cherrett et al., 1989; Hölldobler and Wilson, 1990). Moreover, it has also been assumed that the fungus cultivated by ants is a single species (Lepiotaceaea family) resulting from a very long ant–fungus coevolutionary process (Mueller et al., 2001; Wirth et al., 2003). However, in recent years it has been demonstrated that the ant–fungus interaction does not fit entirely with the accepted paradigm, at least regarding the metabolic capability of the fungus and the fungal species composition of the ant garden.

In the first place, it was shown that the fungus cultivated by LCA is not capable of degrading large polymers, such as cellulose. Instead, LCA fungus behaves as a typical saccharolytic fungus, metabolizing soluble carbons and leaving recalcitrant compounds untouched, which are later disposed of by ants in the internal or external refuse places (Abril and Bucher, 2002, 2004). This finding has profound implications with regard to the ecological niche of LCA as herbivores.

The specificity of the fungus–ant relationship is also under scrutiny because recent research results demonstrated that instead of a single, highly specialized fungus culture, the LCA garden is composed of a multispecies fungus community, without any conclusive indication of the presence of a single, putative mutualistic species (Abril and Bucher, 2007; Mikheyev et al., 2007). These findings not only have scientific, but also significant economic implications, taking into consideration that LCA are important agricultural pests throughout the Neotropical region (Wirth et al., 2003).

Based on the new evidences described previously, I feel that it is timely to re-assess our understanding of the complex ant–fungus functional interaction, as well as its implications in terms of the ecological niche occupied by LCA as herbivores.

In this chapter, I provide a revision of the current knowledge on the topic and explore some new aspects and implications of the ant–fungus interaction. Accordingly, my analysis addresses the three basic herbivory processes: (a) foraging behavior of the LCA, (b) the metabolic and ecological characteristics of the microorganisms involved in the ant–fungus interaction, and (c) the chemical and biological characteristics of the waste material (refuse).

Finally, based on these analyses, I present a novel interpretation of the ant–fungus relationship from a soil microbial ecology perspective. This perspective allows us a deeper understanding of the metabolic relationship of its components and to include aspects of the habitat where such successful interaction takes place.

2. Foraging Behavior of LCA

Several works have attempted to determine foraging behavior of LCA with respect to: (a) dynamics of annual, seasonal, and daily activity, (b) amount, type, and size of harvested material, (c) factors determining LCA's activity (rainfall, temperature, etc.), (d) trail systems and frequency of trail use, and (e) preference for and selectivity of harvested plants. The latter aspect is of great significance for the understanding of plant-ant–fungus functional interaction (Rockwood, 1976; Pilati and Quirán, 1996; García et al., 2003; Lopes et al., 2003; Wirth et al., 2003).

The analysis of all the information available neither reveals a clear general behavior pattern nor allows us to determine constant and conspicuous actions of foraging behavior. For example, dynamics of annual, seasonal, and daily activities is very variable, depending on whether the species are of humid tropical (rain forest) or dry subtropical environments. Wirth et al. (1997) indicated that some *Atta* species in rain forests have greater activity between 11 and 17 h, whereas Claver (2000) reported that in dry shrubland, activity of *Acromyrmex lobicornis* is more intense between 9–11 and 18–20 h. The latter author also observed foraging activity in summer nights (between 20 and 2 h) because of the high diurnal air and soil temperatures. It has also been indicated that in highly seasonal climates, peaks of foraging activity are concentrated in spring–summer, in agreement with ants' reproductive season (Pilati and Quirán, 1996). In desert climates, intense foraging activity has also been observed after the classical rain pulses throughout the year (Claver, 2000).

The amount, type, and size of the material harvested is also very variable, depending on the ant species, size of workers, weight of fragments, distance to nest, and type of plant (Howard, 1988). Wirth et al. (2003) found that succulent, sclerenchymatous, and pubescent leaves are cut into smaller pieces than glabrous leaves, whereas other authors reported reduced fragment size with leaves that are thick (Breda and Stradling, 1994), hard, pubescent (Roces and Hölldobler, 1994), and with greater water content (Bowers and Porter, 1981).

The material harvested by LCA mainly includes green leaves, but also has a variable amount of non-green material (flowers, fruits, litter, etc.), depending on the availability, material size, and ant species (Wetterer et al., 1998). It is well known that *Acromyrmex striatus* is the LCA that carries the greatest amount of litter (Bucher and Montenegro, 1974). However, it should also be noticed that much of the litter harvested by some *Acromyrmex* species is not incorporated into the fungus garden, but is used to construct protective coverings over the nest (Fig. 1) (Claver, 2000). Likewise, it has also been observed that much of the material harvested by ants is soon discarded as refuse, as for example, fruit teguments and other dry or hard material (Camargo et al., 2003a). Therefore, not all the materials harvested by LCA are metabolized by the ant–fungus herbivory entity.

A very interesting aspect of ants' foraging activity is seed dispersal. LCA are known to consume fleshy parts of fruits and discard seeds, which are deposited in the refuse dump, where they are dispersed by the wind or other animals (Dalling and Wirth, 1998; Varela and Perera, 2003; Wirth et al., 2003).

Figure 1. Litter cover and refuse on an *Acromyrmex lobicornis* nest from Western Chaco of Argentina.

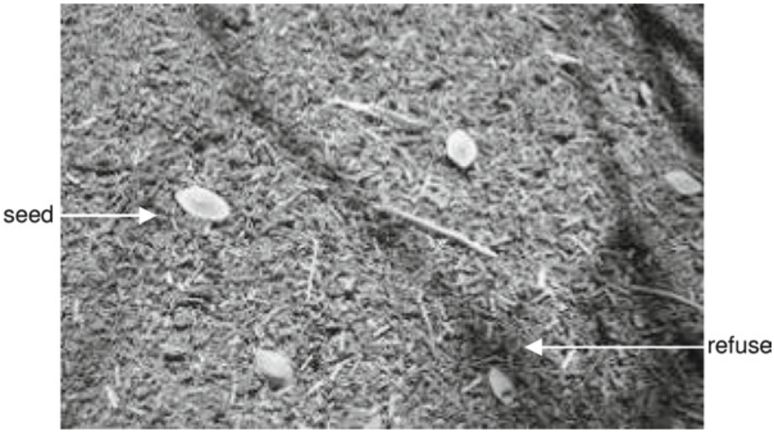


Figure 2. *Prosopis* spp. seeds on *Acromyrmex lobicornis* refuse.

In arid zones, refuse piles usually have a great amount of seeds (*Prosopis* spp. or *Celtis* spp.), completely free of glucose-amylaceous substances that usually cover them inside the fruit (Fig. 2). These observations indicate that seminal teguments are not metabolized in the fungus garden and that seeds remain viable.

2.1. PREFERENCES AND PLANT SELECTION

There is general consensus that LCA are polyphagous herbivores and that they preferably select young leaves; however, observations on selectivity of LCA are at times confusing. For example, it is known that ants can forage exhaustively on a single species and on a single individual for a short period and that they do not attack identical specimens that are nearby (Rockwood, 1976; Howard, 1988).

The patterns determining the selectivity of material harvested by ants have been a subject of heated debate. Most of the several works studying LCA have focused on the search of morphological and chemical characteristics of plants that might determine selectivity, using criteria usually applied to other higher herbivores (insects and vertebrates).

2.1.1. Morphological Characteristics of Selectivity

The morphological characters that have been most frequently analyzed are leaf hardness, thickness, and hairiness. Howard (1988) stated that LCA prefer thin and glabrous leaves and discard densely hairy ones because hairs hinder ant movement and leaf cutting. This finding is consistent with other observations indicating that LCA feeding on sunflower seedlings stop consuming leaves when they become very hairy (D. Irastorza, 2000, personal communication). However, it has also been mentioned that hairy leaves are not always discarded by ants, which would be related to hair structure, position, and density (Howard, 1988).

Moreover, Farji-Brener (1996) found that LCA from Argentine Patagonia prefer exotic species (*Rosa eglanteria* and *Sarothamnus scoparius*) to native species (*Nothofagus dombeyi*, *Austrocedrus chilensis*, *Maytenus boaria*, *Mulinum spinosum*, *Schinus patagonicus*, *Lomatia hirsute,* and *Carduus nutans*), because the former lack xerophytic traits (thick cuticles). Nichols-Orians and Schultz (1990) also mentioned that ants prefer young, tender leaves to mature leaves, despite young leaves having high load of chemical compounds and low nutrient content. They attribute this preference by ants to the fact that mature leaves are thicker and therefore harder to cut.

2.1.2. Chemical Characteristics of Selectivity

The influence of plant chemical characteristics on ant selectivity has been analyzed focusing on the presence of: (a) nutrients, (b) amount and type of antiherbivore substances (phenols, tannins, saponins, etc.), and (c) presence of latex and essential oils.

Nutrient content: most of the works available in the literature have focused on determining the concentration of biogenic elements, such as C and N, in terms of total content. On a few occasions, the chemical composition of the material harvested has been determined from the perspective of foraging quality; for example, analyzing the amount of polymerized (cellulose, lignin, fibers, proteins, tannins, etc.) and soluble organic compounds of the material (Bucher et al., 2004). The oversimplification of the chemical analysis may have hindered a deeper understanding of the selectivity in material harvested by LCA.

For example, Wirth et al. (2003) did not find a selectivity pattern relative to N or C content, or C/N ratio. These authors observed great variability in N content and overlapping ranges of N between plant species selected and not selected by LCA. Therefore, the total N content appears to be a poor indicator of plant selection by LCA (Howard, 1988). However, the deeper analysis conducted by Bucher et al. (2004) determined that all the material harvested by LCA has a high proportion of soluble compounds and that the remaining components (proteins, phenols, and fibers) are very variable.

Antiherbivore substances: in general terms, no direct relationship has been found between antiherbivore level and selectivity of plant material by ants. Nichols-Orians (1991) observed that despite higher concentrations of condensed tannins in foliage, LCA found these leaves more acceptable. Folgarait et al. (1994) analyzed a great amount of antiherbivore substances in the material selected by LCA and found no relationship between the percentage of each plant species in the diet and type of chemical defense in the mature or young leaves.

Studies on the relationship between the presence of antiherbivore substances and selectivity by LCA have always considered these substances as digestive enzymes agglutinant in herbivores' stomach (Reese, 1979). However, this concept is not applicable to LCA because plant material is "digested" by enzymes of microorganisms, which are usually different from digestive enzymes of higher organisms. For example, it has been observed that fungi cultured by LCA can produce phenoloxidase, which allows them to degrade tannins (Nichols-Orians, 1991; Powell and Stradling, 1991).

Latex and essential oils: it has been often reported that leaves containing latex and some essential oils (species of *Canavalia*, *Sesamum*, *Ipomea*, *Ricinum*, etc.) are not selected by ants. This is explained by the fact that LCA ingest sap when they cut the leaves (Hubbell et al., 1984; Howard, 1988; Hebling et al., 1996, 2000a, b; Pagnocca et al., 1996); hence, latex would act as a taste-repellent factor. Furthermore, latex has been mentioned as a mechanical barrier due to sap adhesion to the ants' mouth parts and appendage. Likewise, Hubbell et al. (1984) observed that LCA do not select leaves with non-polar chemical compounds (e.g., terpenoids); however, orange peel is usually used to impregnate toxic baits because of the ants' high preference for these fruits (Camargo et al., 2003b).

Nichols-Orians and Schultz (1990) also stated that comparative bioassays focusing on a small set of variants often fail to capture the complex multivariate nature of harvest selection. In a study of the multiple factors determining selectivity, Howard (1988) found that antiherbivore substances act as attractants only when interacting with nutrients.

Another important aspect is that most of the works on selectivity are conducted with nests maintained in laboratory conditions and the ants fed on materials that are barely common in nature (rice flaks, impregnated and dried pieces of paper, etc.) (Hubbell et al., 1984; Hebling et al., 2000a; Camargo et al., 2003b). Hence, many characteristics of LCA's foraging behavior are probably very different under field conditions.

2.2. FORAGING BEHAVIOR SYNTHESIS

In summary, the information available does not allow us to define a clear and definite pattern of plant material selection. In addition, reports on selectivity of LCA are confusing and contradictory to the point that Howard (1987) stated that foraging pattern observed in tropical LCA for several years did not fit with any of the three herbivory hypotheses known: optimum foraging, conservational, and nutrient-allelochemical balancing.

However, I can extract the most common and relevant aspects. LCA: (a) select buds and young leaves, flowers, and fleshy fruits (materials that can be easily cut); (b) discard hairy leaves and latex-containing plants; (c) do not select material of high cellulose and/or N content; and d) do not show a clear behavior pattern relative to antiherbivore substances. These aspects agree with the idea that LCA select plant tissues that maximize the proportion of live cytoplasm and minimize the proportion of plant cell wall (Abril and Bucher, 2004).

3. Characteristics of the Fungus Garden

The so-called "fungus garden" is a mass of spongy material composed of the plant material carried by the workers, closely mixed with fungal mycelia that is visible to the naked eye. Similar to the agriculture performed by humans, the ant "cultivates" the fungus on which it feeds, which involves preparing the plant material and managing conditions suitable for fungal growth (Cherrett et al., 1989; Mueller et al., 1998, 2001; Wirth et al., 2003).

Ants process plant material before depositing it in the fungus garden. The intensity of this activity, which includes licking, shredding, chewing and crimping of vegetal fragments, and incorporation of fecal fluids, depends on the size, type, and thickness of plant fragments, and is generally carried out by special ant castes (de Andrade et al., 2002). Moreover, ants control humidity in the fungus garden by: (a) collecting young plant material with high water content, (b) incorporating saliva and fecal fluids into the material before depositing it in the fungus garden, (c) locating fungus gardens in deep chambers (with greater edaphic humidity) (Roces and Kleineidam, 2000), and (d) managing nest cover. In environments with low seasonal rainfall, ants usually deposit great amounts of litter on the nest, which captures and retains water from rain and dew (Claver, 2000). Much of the water that ants incorporate in the fungus garden through saliva and fecal fluids comes from the sap they ingest when they cut the leaves. Interestingly, in experiments using artificial nests, in which ants are fed on previously cut leaves, ants have been frequently observed collecting water from the lab pipelines (D. Igarzabal, 2000, personal communication).

Another factor that ants manage to optimize fungus growth is gas balance in the nest atmosphere. The high fungus growth rate involves high consumption of O_2 and great amounts of CO_2 emissions. This change in gas concentration inside the fungus garden may negatively affect not only the fungi, but also the ants' life, especially in actively growing larvae. Kleineidam and Roces (2000) reported that ants ventilate the fungus garden with openings parallel to wind direction. Furthermore, I observed that during the period of greatest growth of the fungus garden (and greatest nest activity), ants perform a high number of openings on top of the nest for ventilation purposes.

Moreover, Farji-Brener (2000) found that ants regulate the nest's internal temperature by rearranging the shape and internal structure. The low surface/volume ratio of the semi-spherical mound minimizes thermal exchange with the external environment as well as the change in the chamber depth.

3.1. TAXONOMY OF THE FUNGUS

To start a new fungus garden, ants take a fraction of mycelium of previous "crops" to the new harvested plant material. Therefore, it has been assumed that the fungus cultured by ants belongs to the same species that is spread vegetatively (hence, it would be a clone). Thus, the fungus would have coevolved with the ant for approximately 50 million years (Chapela et al., 1994; North et al., 1997; Mueller et al., 1998, 2001; Bot et al., 2001; Currie, 2001a, b; Green et al., 2002; Mueller, 2002; Poulsen and Boomsma, 2005).

One of the major scientific concerns in this field has been to elucidate how LCA maintain the fungus clone pure (axenic culture), free of contaminating or pathogenic organisms, in a medium with naturally high microbial load like soil and plant material. This concern has encouraged a wide range of works that study the mechanisms through which ants control, such that the culture conditions remain axenic.

Among the control mechanisms that have been proposed are physical processes, such as ants' licking of the leaf surface to remove natural populations in leaves, and sophisticated biological control processes through the symbiotic relationship with actinomycetes for the production of antibiotics (Cherrett et al., 1989; Currie, 2001a; Poulsen et al., 2002, 2003; Zhang et al., 2007). However, some historical records (Papa and Papa, 1982; Möller, 1893) indicate that the culture is not axenic (because several types of fungi have been found inside the fungus garden), which is consistent with the recent biomolecular genetic studies.

The first works on molecular taxonomy were conducted on isolated fungi of lower Attine nests, which clearly showed the existence of several OTUs of fungi genetically related to species naturally occurring in the soil. The authors interpreted that lower ants are still in process of coevolution with their symbionts, allowing the entrance of other populations. However, they pointed out that this process would not take place in higher Attine (genera *Atta* and *Acromyrmex*), which are considered more advanced from the evolutionary standpoint (Adams et al., 2000; Bot et al., 2001; Green et al., 2002; Doherty et al., 2003; Solomon et al., 2004; Villesen et al., 2004).

Later, evidence of a certain degree of genetic variability in the mycelium of fungi cultured by higher LCA was provided, which introduced some flexibility to the strict paradigm of an "axenic culture of a single clone" that prevailed until the end of the twentieth century. Thus, the idea of clone variability within a single fungus species has gradually gained support (Doherty et al., 2003; Mueller et al., 2004).

As it is assumed that ants cultivate a single fungus species, its isolation is a matter of concern due to the high amount of contaminants usually present in the fungus garden. Accordingly, most of the works recommend conducting multiple seriated cultures and adding antibiotics to obtain the pure culture (Bot et al., 2001; Currie, 2001a; Michels et al., 2001, Mueller, 2002). Because of this difficulty in isolating the fungus, most of the works on biomolecular genetics did not use new isolates and, instead, employed cultures maintained in laboratory strain collections (Mohali, 1998; Doherty et al., 2003). Hence, the detection of relatively low genetic variability in the fungus cultured by higher Attine might be due to the strong pre-selection of the fungus for its isolation and because works are conducted with few collection clones.

Moreover, it is important to carefully examine the current criteria considered to isolate the symbiont fungus. There is wide consensus on the use of the presence of gongylidia, structures that consist of a tip swelling of the hyphae, as the sole distinctive feature. Therefore, hyphae lacking gongylidia are considered contaminant fungi in the fungus garden (Michels et al., 2001; Mikheyev et al., 2007).

However, this selection criterion is arguable because morphological studies of Lepiotaceae fungi do not mention gongylidia as morphological structures in any fungus group (Johnson, 1999), which has led to strong support of the coevolution hypothesis of a very particular species that has developed gongylidia to feed ants. Alternatively, the presence of gongylidia can be interpreted not as a permanent feature, but as a healing structure when the hypha is cut by the ant (Spegazzini, 1922). This would explain the absence of gongylidia when fungus gardens are abandoned by ants (Bass and Cherrett, 1996; Bass, 1997; Mohali, 1998).

These remarks supported the study conducted by Abril and Bucher (2007), who did not follow the criteria based on the presence of gongylidia, and assumed that all the fungi present in the fungus garden might be those cultured by ants. The authors worked under extreme aseptic conditions, both in the field and in the laboratory, to ensure that the isolated fungi were those belonging to the fungus garden and not external contaminants.

This genetic study showed that all nests analyzed had more than one fungus OTU and that the genetic differences detected indicate great variability between large groups of fungi (not only species or clones) (Fig. 3). The study also showed that the number of isolated strains increased linearly with the number of nest samples and that the variability pattern is similar between nests of different species and different localities. These findings suggest that the pool of species available to integrate the nest community is very high and probably comes from fungi usually present in the soil (Abril and Bucher, 2007).

These authors state that their results are in agreement with the following: (a) Papa and Papa (1982), who found that Attini ants cultivate associations of a wide variety of fungal symbionts, including members of the subdivision Ascomycotina and Deuteromycotina; (b) Mohali (1998), who found that several fungus genera characteristic of the early stages of plant material decomposition (*Penicillum, Aspergillus*, and *Trichoderma*), were associated with the mutualistic fungus;

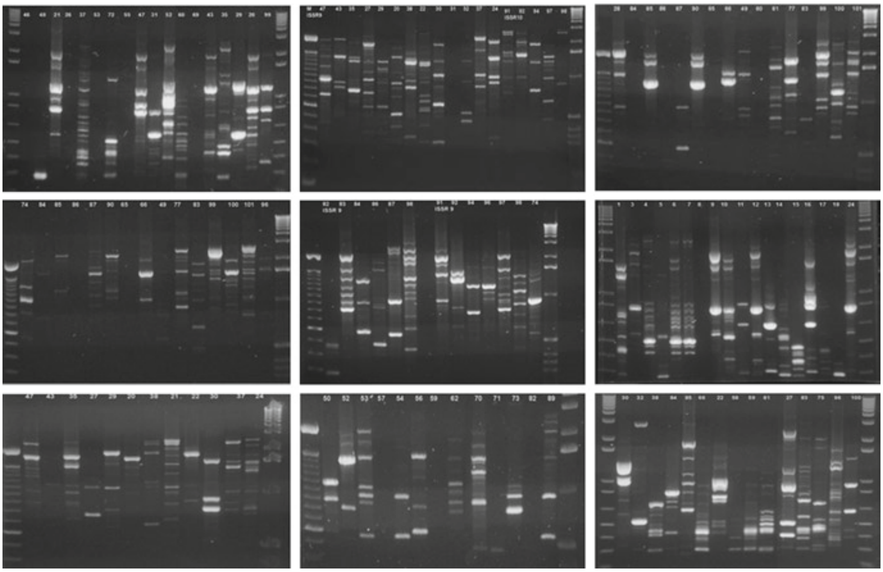


Figure 3. Genetic diversity of fungus occurring in leaf-cutting ant nest (Abril and Bucher, 2007).

(c) Van Borm et al. (2002), who found great genetic variability of fungi in the ant gut; and (d) several personal observations of fungus gardens and fungus isolates showing great morphological differences in the size and shape of the hyphae (with or without gongylidia, with or without septa) and in the characteristics of the colonies formed in the culture media (color, texture, etc.) (Fig. 4).

3.2. OTHER MICROORGANISMS OCCURRING IN THE FUNGUS GARDEN

Laboratory procedures usually applied in the study of the fungus cultured by LCA (presence of gongylidia for the selection and use of antibiotics in the culture media) hindered the analysis of other microorganisms that might be usual components of the fungus garden. I conducted some isolation assays without adding antibiotics to the culture media and found the proliferation of a great variety of bacteria. This observation is consistent with the numerous works mentioning bacterial contamination in cultures, and with some studies that found yeast (Carreiro et al., 1997) and the microfungus *Scovopsis* spp. (which is considered a pathogenic fungus), in practically all the fungus gardens (Seifert et al., 1995; Currie, 2001b; Taerum et al., 2007). These evidences led me to think that control mechanisms used by ants are not so strict and efficient, and to formulate the question as to whether bacteria play a functional role in the fungus garden.

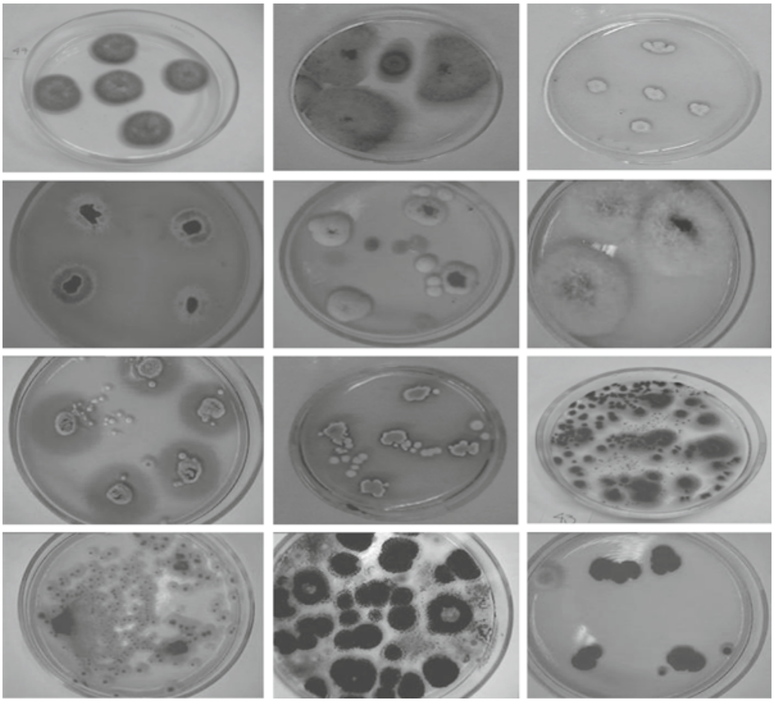


Figure 4. Morphological diversity of fungus occurring in leaf-cutting ant nest.

3.3. MICROORGANISM METABOLIC ROLES IN THE FUNGUS GARDEN

Until the beginning of the twenty-first century, the role of the fungus cultured by LCA was unquestionably assumed to be the degradation of the major structural polymers of the plant cell wall. However, several reports have failed to demonstrate a significant cellulose-degrading activity (Bacci et al., 1995; Gomes de Siqueira et al., 1998; D' Ettorre et al., 2002). In 2002, Abril and Bucher clearly demonstrated that the fungus cultured in the fungus garden does not degrade cellulose.

Based on this evidence, Abril and Bucher (2004) conducted another work analyzing in detail the compounds metabolized by the fungus as C and N sources. This study showed that the fungus cultured by LCA does not degrade polymerized compounds (does not excrete extracellular enzymes), except for the peptide compounds of plant cell medium lamellae, and that their nutrition is based on cytoplasm-soluble compounds of the plant cells (amino acids, glucose, etc.).

Following the same method of metabolic analyses used by Abril and Bucher (2004), I quantified the abundance of functional groups of microorganisms in the fungus garden, including all the microorganisms present. Surprisingly,

Table 1. Abundance of microbial functional group and nitrogenase activity (mean ± SD) in the fungus garden of eight nests of *Acromyrmex* spp. in the western Chaco of Argentina.

Microbial functional group (log g^{-1})	
Ammonifiers	22.1 ± 1.1
Nitrifiers	0
Cellulolytic fungi	0
Cellulolytic bacteria	0
N_2-fixing bacteria	2.4 ± 0.5
Nitrogenase activity (nM g^{-1})	0.4 ± 0.3

I observed that none of the microorganisms present in the fungus garden can degrade cellulose (cellulolytics) or oxidize ammonium (nitrifiers), but noticed the presence of N_2-fixing bacteria (with nitrogenase activity) and a great amount of ammonium-producing bacteria (ammonifiers) (Table 1).

The high abundance of ammonium-producing bacteria detected in the fungus garden would be related to the presence of a great amount of organic N from the ants' fecal fluids deposited in the fungus garden (Cherrett et al., 1989). Likewise, the lack of nitrifying organisms might be associated with the high amount of ammonium consumed by the fungus. It is widely known that ammonium-oxidizing organisms have an inefficient metabolism and very low growth; therefore, they cannot compete for ammonium with other more abundant microbial groups (Paul and Clark, 1996; Abril et al., 2005).

3.4. FUNGUS GARDEN SYNTHESIS

In summary, the ant–fungus herbivory entity is not a specific symbiotic relationship, but a system that includes a microbial community regulated by the availability of resources and environmental conditions. From this point of view, the system may not be such a complex and special association as it has been regarded until now.

4. Refuse Characteristics

The exhausted fungus garden is removed by ants as refuse material. Depending on the ant species, refuse can be dumped outside the nest or deposited in isolated internal chambers in the fungus garden. Morphologically, refuse material is a dry granulose mass composed of plant detritus that is not metabolized in the fungus garden, along with ant feces and dead ants (Cherrett et al., 1989; Hölldobler and Wilson, 1990; Farji-Brener and Medina, 2000; Verchot et al., 2003). Depending on the diet of LCA, harvested plant fragments can be relatively identified in the refuse. If ants harvest gramineous plants, leaf fragments can be clearly identified. However,

Figure 5. Plant seedling on LCA refuse.

if the diet is based on dicotyledonous species, then refuse is a very homogeneous mass where only dead ants can be observed (AA, 2003, personal observation).

Refuse has been extensively studied because it is a fairly atypical process in nature. The most commonly known studies include aspects of the chemical composition, but refuse has been also analyzed focusing on its usefulness. For example, Zeh et al. (1999) and Farji-Brener and Sasal (2003) reported that refuse has a deterrent effect on LCA.

Seed germination and establishment (Fig. 5) and presence of living fine roots have been found to be higher in refuse than in the soil near nests (Farji-Brener and Medina, 2000). Hence, some studies that analyzed the chemical characteristics of fertility reported high mineral nutrient content (Farji-Brener and Silva, 1995; Guerra et al., 2007; Sternberg et al., 2007; Tadey and Farji-Brener, 2007). Likewise, other authors evaluated the chemical elements and caloric potential of refuse with the aim of determining energy and nutrient balance in soils with and without the presence of ant nets, showing that nutrients are cycled more rapidly in ant nests than in soils not disturbed by ants (Lugo et al., 1973; Haines, 1978; Wirth et al., 2003).

4.1. BALANCE OF NUTRIENT COMPOUNDS

Another approach to the study of refuse has focused on its importance in understanding the mechanism underlying LCA–fungus interaction. As refuse comprises all the waste from the herbivory entity, and can be used to estimate the digestibility and degree of use of nutrient compounds from the harvested plant material. These estimations determine the difference in the amount of each organic compound between the material consumed and feces, and are widely used in studies on herbivory in higher vertebrates (Maynard et al., 1979). Martin and Weber (1969) and Jonkman (1977) are among the first authors to compare some chemical compounds of the fungus garden and refuse material to estimate the degradation of organic compounds inside the LCA fungus garden.

However, balance of compounds in LCA is difficult to estimate because the time that the harvested material is maintained in the fungus garden is uncertain, and therefore, the source of the refuse analyzed cannot be determined. Most of the studies that evaluated the amount of time the material remains in the fungus garden have been conducted in artificial nests; therefore, in natural conditions, the time is probably very different (Guerra et al., 2007; Sousa-Soto et al., 2007). It is known that in strongly seasonal climates (like those in central Argentina), the greatest amount of refuse is deposited at the start of ants' annual activity, and hence, refuse would correspond to material deposited in the previous season (S. Claver, 2002, personal communication).

To avoid distortion in estimations, Bucher et al. (2004) performed a nutritional balance of LCA–fungus interaction using the digestibility index. This value determines the percent relationship between components of a non-metabolizable substance. Cellulose was used as an indicator because it is well known that this substance is not metabolized in the fungus garden (Abril and Bucher, 2002). These authors determined that only soluble C compounds are consumed in the fungus garden and that there is a greater amount of soluble N compounds in the refuse material than in the leaves (Fig. 6), and suggested that this increase in the amount of N in the refuse material might be due to the contribution of ant feces (Martin, 1970; de Andrade et al., 2002) and N biological fixation taking place inside the fungus garden and/or in the refuse, as it occurs in termite nests (Waller, 2000). Wirth et al. (2003) also observed a decrease in the C/N relationship in refuse with respect to plant material harvested by ants, which they attributed to a greater amount of microbial biomass in the refuse.

4.2. BIOLOGICAL ACTIVITY IN REFUSE MATERIAL

Based on the concepts mentioned by Bucher et al. (2004) and Wirth et al. (2003), and considering the great amount of organic compounds that persist in the refuse, I analyzed the abundance of functional microbial groups, respiration rate, and N biological fixation at different depths of the refuse piles, and compared them with those obtained in the soil not disturbed by nests (Table 2). Thus, I intended to define the extent and type of biological activity developed in the material discarded by LCA.

The results of this study show that: (a) on the surface, refuse humidity is low, organic matter content is high, and total heterotrophic activity (respiration) is also high; (b) at 10-cm depth, refuse shows higher humidity content and lower organic matter content than in the surface layer, great amount of cellulolytic

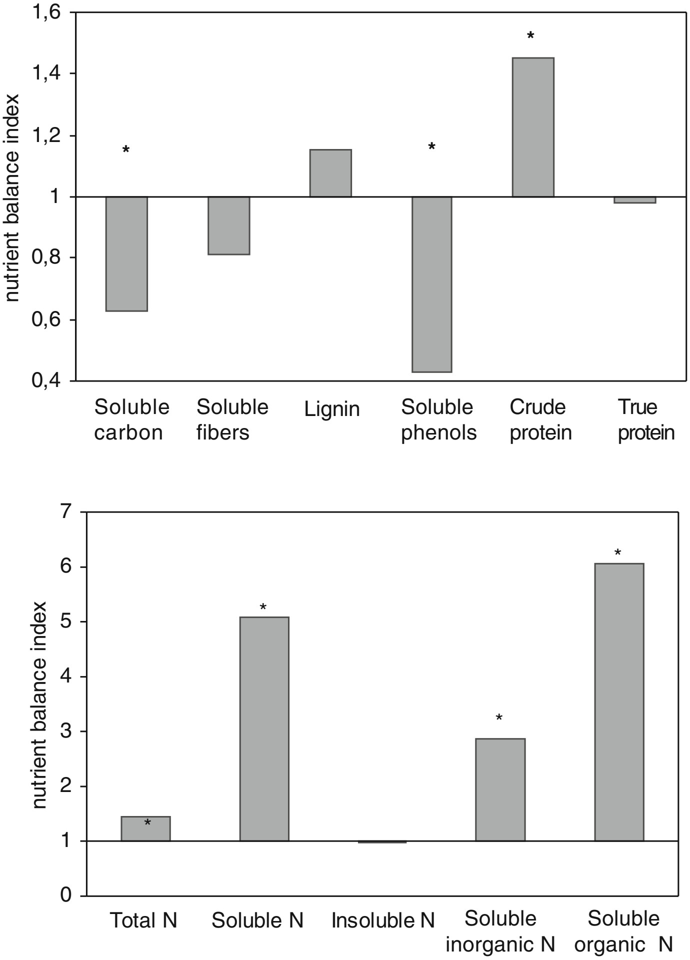


Figure 6. Nutrient balance between refuse and harvested material for six *Acromyrmex lundi* nests. * indicates significant differences (t–test; $P < 0.05$) (Bucher et al., 2004).

organisms and high N biological fixation; and (c) at 20-cm depth, all the values of abundance of microorganisms and biological activity decrease, although these values are always higher than in the soil that is not disturbed by ants.

I attribute the greater abundance of cellulolytic organisms at 10-cm depth to the beneficial effect of high humidity on hydrolysis of the high amount of cellulose present in the refuse. Likewise, the high N biological fixation rate would be consistent with active cellulose degradation and low O_2 tension conditions (due to water saturation) that promote nitrogenase activity (Vitousek et al., 2002; Kavadia et al., 2007; Abril et al., 2008).

Table 2. Variation in humidity content, organic matter content, and microbial abundance and activity at three depths of the refuse pile of *Acromyrmex* spp. nests and on the soil that is not disturbed by nests (n = 8) in the western Chaco of Argentina. Letters indicate significant differences (Tukey's test, $P >$ 0.05). nd: not detectable.

	Surface refuse	**10 cm in depth**	**20 cm in depth**	**Surrounding soil**
Humidity (%)	0.98 d	26.01 a	13.43 b	2.80 c
Organic matter (%)	53.3 a	25.6 b	10.14 bc	5.36 c
N-fixing organisms (log g^{-1})	4. 87	4.65	4.70	4.68
Ammonifiers (log g^{-1})	5.95	6.30	6.14	6.20
Cellulolytics (log g^{-1})	2.20 b	4.11 a	3.95 a	1.4 c
CO_2 (mg g^{-1} 7 day^{-1})	52.81 a	14.96 b	2.20 c	0.32 d
Nitrifiers (log g^{-1})	0.2 c	3.14 a	2.45 b	1.2 bc
Nitrogenase (nM ethylene g^{-1})	0.051 c	4.398 a	0.776 b	nd

4.3. SYNTHESIS OF REFUSE CHARACTERISTICS

In summary, the refuse material removed by ants is characterized by high fiber (cellulose and lignin), soluble and total nitrogen content, and high microbial activity, including N biological fixation. Therefore, the question arises as to why ants do not use such nutritious material. The answer that has been proposed so far is that debris harbor microorganisms harmful to both the ants and their symbiotic fungus (Bot et al., 2001; Currie, 2001a), and it has been even suggested that the pathogens in refuse piles might be nest-specific (Farji-Brener and Sasal, 2003).

5. New Insight into the Functioning of the Plant–Fungus–Ant Interaction

Several interesting works interpret the functioning of LCA–plant interaction and provide an extensive analysis of the role of the fungus in the herbivory association. Most of these works are based on the paradigm that the fungus cultured by the LCA degrades the complex plant polymers and that the symbiont fungus is a single one that resulted from a long coevolutionary history; the fungus is protected from invading alien species by complex mechanisms (Cherrett et al., 1989; Currie, 2001a; Mueller et al., 2001; Mueller, 2002).

The analysis conducted in the previous sections indicates that this paradigm, although based on concepts widely accepted until recently, is not supported by the evidences observed in the twenty-first century (Abril and Bucher, 2007; Mikheyev et al., 2007). Therefore, it is necessary to provide a new synthesis integrating and explaining the recently acquired knowledge on the ant–fungus interaction. The approach I propose has been based on the soil ecology perspective, because the soil is the habitat of this herbivory association. This perspective provides a broader vision because it is not focused specifically on any of the interaction components.

The review and update of the three factors analyzed in this work (foraging behavior,, characteristics of the fungus garden, and the refuse) provide the following evidences: (a) ants select young and soft plant material with high water and soluble compound content; (b) the mutualistic fungus species occurring in the fungus garden, on which the ant feeds, is not a single species; (c) the fungus garden is a microbial community, not a fungal axenic culture; and (d) the refuse is a highly nutritious material (high cellulose and N content), which has low humidity when the ant dumps it outside the fungus garden.

I understand that LCA's preference for fresh material (with high soluble contents) and refuse with high fiber content indicates that the use of plant soluble compounds is the main process in the ant–fungus interaction. Fungi that use soluble compounds are known as saccharolytic, which distinguishes them from polymer-degrading fungi (Paul and Clark, 1996). In soil microbiology, it is well known that polymer-degrading fungi need to synthesize and excrete extracellular enzymes to hydrolyze polymers and subsequently metabolize monomers. This physiological characteristic is responsible for the slow growth in polymer-degrading fungi, and therefore, for their poor efficiency when compared with saccharolytic fungi. For this reason, while there are soluble compounds in the media, polymer-degrading fungi behave saccharolytic.

In general, saccharolytic fungi belong to the group considered as lower fungi (those having coenocytic hyphae), whereas polymer-degrading fungi belong to the higher fungi group (those having septate hyphae). This classification is consistent with the presence of a great variety of fungi, in the fungus garden, behaving as saccharolytic that have both morphological traits (coenocytic and septate hyphae) and that are genetically very diverse (Abril and Bucher, 2004, 2007).

Moreover, the presence of an abundant bacterial community in the fungus garden indicates that bacteria play a significant role in the ant–fungus interaction and is consistent with the repeatedly reported culture contamination problems involved in the isolation of a single symbiotic fungus following the criteria that the fungus should have gongylidia and be septate.

5.1. FUNGUS CULTURING

What nutritional benefits does the ant find in transforming plant-soluble compounds into fungal biomass? Why does not the ant itself consume soluble compounds? What contribution does fungal cytoplasm make to ants?

To answer these questions, some interesting aspects should be considered: (a) some lower Attini ants use plant-soluble compounds directly, but these are colonies of few individuals when compared with the huge LCA colonies (Wirth et al., 2003); (b) fungi degrade antiherbivore soluble compounds (phenols) that can be toxic to the insects (Bucher et al., 2004); (c) N content of plant-soluble compounds is very scarce to cover actively growing larvae demands; (d) ants' fecal fluids contribute with additional N to the growth of fungi whose biomass has low

C/N ratio; and (e) there is a great amount of ammonium-producing bacteria in the fungus garden.

Therefore, I consider that by favoring growth of saccharolytic fungi, ants are benefited in the following ways: (a) they can forage on a great variability of plants (polyphagy) because they are not affected by poor nutritional quality (N content or antiherbivore substances); and (b) they consume easily degradable food and with low C/N ratio (the fungus does not have cellulose or lignin walls). Regarding the latter aspect, it should be noted that chitinase present in the ant labial glands allows them to degrade chitin from the fungal cell wall (Erthal et al., 2004) and that ants prefer to consume actively growing gongylidia (with weak or no walls).

In summary, I understand that the fungus garden functions in the following ways: (a) ants contribute with plant fragments (high-soluble compound content) and manage the fungus-garden conditions (processes the material, humidity, temperature, and gas exchange in the nest and provides organic N from their fecal fluids); (b) bacteria mineralize organic N from feces by releasing NH_4 available to fungi; (c) saccharolytic fungi find optimal growth conditions and rapidly cover all the plant material with the hyphae, thus inhibiting development of other, less efficient microorganisms of slower metabolism; and (d) ants obtain a great amount of biomass of fast-growing fungi for larval feeding (and feeding of the entire population when sap is scarce) in a short period.

From the microbial point of view, the fungus garden functions as a continuous culture with incorporation of substrate and extraction of fungal biomass. Hence, when ants take inoculum from the fungus garden to start a new colony, they are replicating a combined culture of saccharolytic fungi (highly adapted to nest conditions) that is at an exponential growth phase.

5.2. END OF THE FUNGUS GARDEN

Why and when does the ant abandon the fungus garden? I understand that when in the continuous culture of the fungus garden, the amount of material incorporated is lower than the demand for fungal biomass, the soluble compounds for culturing saccharolytic fungi become exhausted. At this point, the microbial community in the fungus garden changes and triggers the growth of organisms capable of degrading polymerized compounds (especially cellulose) that have not been used by the sacchrolytic fungi.

As cellulolysis is a very slow process, microorganisms have low growth rate and do not meet ants' demands. Thus, once the soluble compounds are exhausted and symptoms of degraded polymerized compounds appear, ants reduce humidity in the fungus garden and, once dried, the half-degraded material is dumped outside the fungus garden and deposited as refuse.

This change in the fungus-garden appearance has been observed in numerous nests and has been attributed to the invasion of fungal parasites of the mutualistic fungus (Currie, 2001a). However, because of the conspicuous presence of these fungi in the middle and bottom portions of the garden (Currie, 2001b;

Mikheyev, 2008) and the low host–pathogen specificity (Gerardo and Caldera, 2007; Taerum et al., 2007), they are probably not pathogens, but microorganisms that occur when soluble compounds in the fungus garden become exhausted and polymers of the plant cell walls start to degrade.

My understanding of fungus-garden functioning and the metabolic reasons of culture abandonment fully support the physical, chemical, and biological characteristics of the refuse. When the refuse is dumped outside the fungus garden, it looks like a pile of sawdust (high amount of fibers and low humidity) that, when humid, exhibits high activity of cellulolytic microorganisms (that degrade remaining material) and fixing organisms that produce N for the microbial biomass.

5.3. ECOLOGICAL IMPLICATIONS

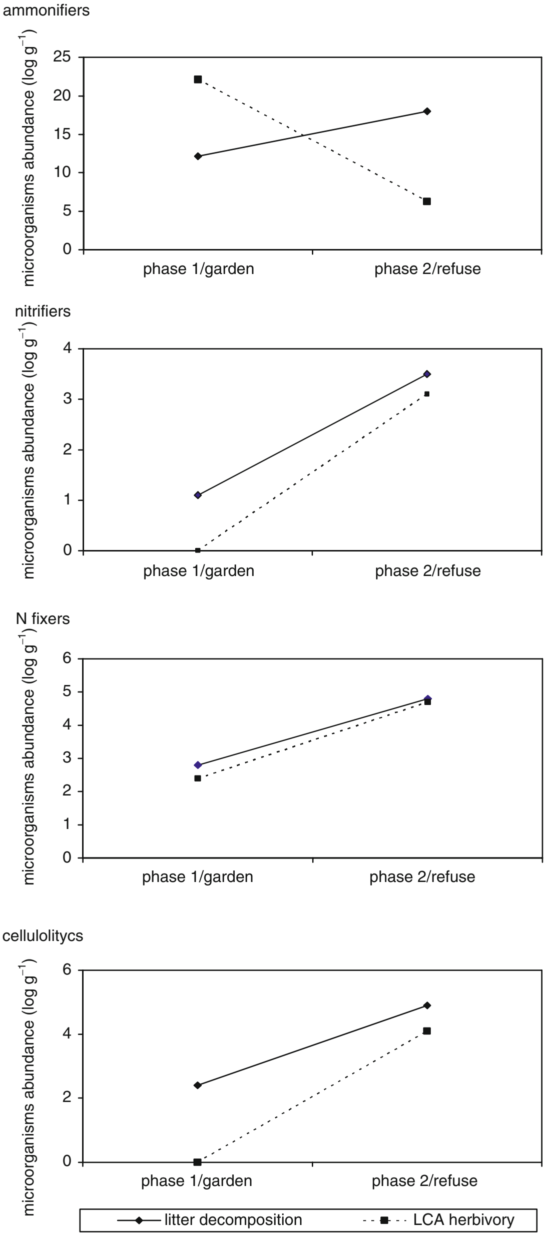
I found great similarity between the succession of metabolic processes of the fungus garden and refuse and litter degradation in the ecosystems. For example, the degradation processes of plant material occurring inside the fungus garden correspond to the so-called "first phase" of litter decomposition because it is carried out by microorganisms of rapid growth that use the soluble compounds present in the plant material. Biological processes taking place in the refuse correspond to the "second phase," which involves slow degradation of the recalcitrant compounds (Paul and Clark, 1996).

This comparison is not only supported by the chemical aspects of both phases, but also by the differential presence of those microorganisms responsible for decomposition. In a microbial analysis of fungus gardens and refuses conducted in the same study area, Torres et al. (2005) found similar microbial succession in litter degradation (Fig. 7): (a) high amount of saccharolytic fungi and ammonifiers at the first phase, and (b) high amount of cellulolytic, N fixers and nitrifiers at the second phase. The only difference was the much higher amount of ammonifiers in the fungus garden than in the litter, in agreement with the contribution of organic N present in the ants' fecal fluids (Fig. 7). Likewise, N biological fixation found in the refuse pile is consistent with that observed in litter decomposition of high fiber content (Vitousek and Hobbie, 2000; Abril et al., 2008).

My approach shows that the LCA–fungus herbivory association modifies the natural decomposition process through: (a) an overweighted first phase (by the accumulation of live tissues and management of fungus-garden conditions); and (b) a second phase concentrated in a reduced area, favoring the formation of patches of increased soil fertility (Sternberg et al., 2007).

In summary, from the perspective of microbial ecology, the LCA–plant interaction may not be a complex and special association as it has been regarded until now, but a modification of natural microbial decomposition processes mediated by ants. I expect that this new insight contributes to the integration of the available information on the plant–fungus–LCA interaction, and helps to the advance and deepening of the knowledge from an ecologically more realistic perspective.

Figure 7. Comparison of microbial functional group dynamics between litter decomposition phases and LCA herbivory process.



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INTESTINAL SPIROCHETES OF TERMITES

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1. Introduction

Spirochetes differ from all other bacteria by their unique morphology and mechanism of motility. The cells possess a helical shape, and the flagella (axial filaments) are located in the periplasmic space. The flagella are attached to the cell poles and wrapped around the protoplasmic cylinder. The flagella and the protoplasmic cylinder are surrounded by a multilayered outer sheath or outer cell envelope (Canale-Parola, 1984). The 16S rRNA sequences demonstrated that the spirochetes represent a monophyletic phylum within the bacteria (Paster and Dewhirst, 2001).

Spirochetes are widespread in different environments. They occur either as free-living cells mainly in marine and limnic sediments, or host-associated as commensals or parasites of animals including humans. One of the spirochetal habitats is the digestive tract of termites and wood-eating cockroaches. Termites have developed a unique hindgut flora consisting of bacteria, archaea, and yeasts. Lower termites also possess flagellates. This symbiotic microbial community is involved in the decomposition of complex organic compounds and thus enables the termites to feed on wood or soil material (Eutick et al., 1978; Breznak and Brune, 1994; Varma et al., 1994; König et al., 2002; König and Varma, 2006). Whereas most spirochetes usually exist free-living in the gut fluid, they have also been found as ectosymbionts attached to the surface of protists (Kirby, 1936, 1941a, b, c, d, e; Radek et al., 1996; Rösel et al., 1996; Noda et al., 2003; Brune and Stingl, 2006; Jeon 2007). *Mixotricha paradoxa,* which inhabits the gut of the lower wood-eating termite *Mastotermes darwiniensis,* is a well-known example (Cleveland and Grimstone, 1964; Wenzel et al. 2003; König et al., 2007).

The first pure cultures of hindgut spirochetes were obtained from the lower termite *Zootermopsis angusticollis* (*Treponema primitia* strains ZAS1 and ZAS2 and *Treponema azotonutricium* strain ZAS-9; Leadbetter et al., 1999; Graber et al., 2004). These isolates exhibit physiological pathways, which were previously unknown within the spirochetal group, including acetogenesis from H_2 plus CO_2 (Leadbetter et al., 1999) and nitrogen fixation (Lilburn et al., 2001). Other isolates such as *Spirochaeta coccoides* and *Treponema isoptericolens* (Dröge et al., 2006, 2008) produce acetate from carbohydrates. These processes are beneficial for termites, because acetate is their major carbon and energy source (Odelson and Breznak, 1983) and N_2 fixation by symbiotic hindgut bacteria can supply up to 60% of the nitrogen in termite biomass (Lilburn et al., 2001). Spirochetes are one of the most abundant bacterial groups in this habitat representing about 50% of the prokaryotic cells (Paster et al., 1996). These observations suggest that this bacterial group plays an important role within the complex symbiotic system in the termite hindgut.

2. Detection of an Ancient Association

At the end of the nineteenth century Leidy published the first description of spirochetes in the termite gut (Leidy, 1874–1881, 1877). He detected a symbiotic association of microorganisms and termites. It exists at least since 15 million years, because Wier et al. (2002) found spirochetes in termites of Miocene amber. Most probably, the termite microbiota date back to the emergence of the termites (Isoptera) and cockroaches (Blattodea) (Nalepa and Bandi, 2000) about 140 million years ago. The recent developments in the investigation of this unique symbiotic microbial community have been summarized by Abe et al. (2001) and König and Varma (2006).

3. Morphology

Spirochetes of different sizes (3–100 μm in length, 0.2–1.0 μm in width) are consistently present in the hindgut of all termites (Margulis et al., 1979; Breznak 1984, 2006; Breznak and Leadbetter 2002, 2006). They possess between 2 and more than 100 periplasmic flagella. Some spirochete species such as *Pillotina calotermitidis*, *Diplocalyx calotermitidis*, *Hollandina pterotermitidis*, *Clevelandina reticulitermitidis, Cristispira pectinis,* and *Canaleparolina darwiniensis* have been described solely on the basis of their morphometric criteria such as number of the flagella, ultrastructural features of the outer membrane, and the cell wavelengths and amplitude (Hollande et al., 1967; To et al., 1978, 1980; Margulis et al. 1981; Breznak, 1984; Bermudes et al., 1988; Margulis and Hinkle, 1991, 1999).

The spirochetes in pure culture such as *Treponema primitia* and *Treponema azotonutricium* (Graber et al., 2004) or *Treponema isoptericolens* (Dröge et al., 2008) exhibit the typical spirochete morphology, while the cell shape of *Spirochaeta coccoides* (Dröge et al., 2006) is quite atypical.

In deep agar, *Treponema isoptericolens* strain SPIT5^T forms white colonies with diffuse spherical edges and a higher density toward the center (cotton ball-like) (Dröge et al., 2008). The cells are highly motile with wavy or rotating movement. The cell-size ranged from 0·4 to 0·5 μm in diameter and mainly 12–20 μm in length. The poorly coiled cells have a wavelength of 6–7 μm and an amplitude of 1·5–1·8 μm (Fig. 1). Individual cells possess six flagella, which were inserted near the end of the protoplasmic cylinder. Spherical bodies appeared in the late exponential growth phase and became dominant in the stationary phase. The cells stained Gram-negative.

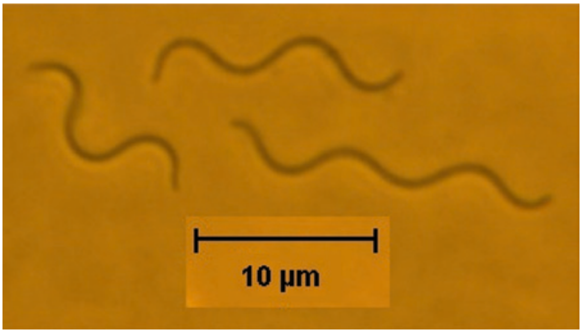

Figure 1. Phase-contrast micrograph of *Treponema isoptericolens* strain SPIT5^T.

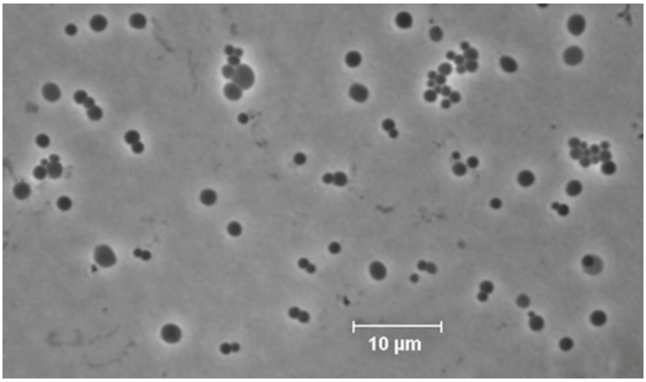

Figure 2. Phase-contrast micrograph of *Spirochaeta coccoides strain* SPN1^T.

Spirochaeta coccoides Strain SPN1 was isolated by repeated deep-agar dilution series from the hindgut of the termite *Neotermes castaneus* (Dröge et al., 2006). Strain SPN1 showed an atypical cell form, because the cells were coccoid. It was immotile (Fig. 2). Axial filaments were not observed. The cells had an average cell diameter of about 1.5 µm and were surrounded by an outer envelope. In the early growth phase, aggregates are formed. The cell shape of strain SPN1 was unexpected, because spirochetes are usually characterized by their typical helical cell structure. Furthermore, all known spirochetes are motile by their axial filaments, which are attached to the cell poles. However, the formation of cocci by morphological regular spirochetes in the stationary growth phase is known (Canale-Parola, 1984). Probably, this coccoid stage is the origin of the unusual morphology of strain SPN1. Recently, novel spirochetal isolates have been obtained from freshwater sediments, which also reproduce only in round forms (Ritalahti and Löffler, 2004). This may indicate that “nonspiral spirochetes” are more widespread in nature and not restricted to specialized ecosystems like the termite hindgut.

The application of cryotomography revealed unique novel cell structures in the case of *Treponema primitia* such as bowls, arcades of hooks, cones at the cell ends, and two layers of wall material (Murphy et al., 2008; Wolgemuth et al., 2008).

4. Phylogeny

The evolutionary distance between spirochetes from termite guts and other members of this group have been well demonstrated by several studies, which were based on culture-independent investigations of 16S rRNA gene sequences (Berchtold et al., 1994; Berchtold and König, 1996; Paster et al., 1996; Ohkuma and Kudo, 1996, 1998; Kudo et al., 1998; Lilburn et al., 1999; Ohkuma et al., 1999; Breznak, 2002; Wenzel et al., 2003; Schmitt-Wagner et al., 2003; Berlanga et al., 2007; Mackenzie et al., 2007). All spirochetal clones obtained from termite gut contents are only distantly related to known spirochetes from other habitats. However, phylogenetic analyses have shown that most symbiotic spirochetes form separate clusters within the *Treponema*- or *Spirochaeta*-group of the spirochetes (Table 1). The phylogenetic position and features of *Treponema primitia* and *T. azotonutricium* were recently summarized by Breznak (2006).

The morphology of *Spirochaeta coccoides* strain SPN1 (Dröge et al., 2006) differs from all known validly described spirochetes, but according to its 16S rRNA gene sequence the isolate could be assigned to the order spirochetales, based on phylogenetic analysis of strain SPN1 forming a monophyletic cluster with different free-living spirochetes and a clone obtained from swine intestine (Table 1) (Ritalahti and Löffler, 2004). Consequently, strain SPN1 did not cluster in one of the so-called "termite *Treponema* clusters I–III" of the treponemes.

Table 1. Phylogentic grouping of spirochetes from the termite gut.

Cluster	Representative species/clones
Treponema cluster I	*Treponema primitia* strain ZAS-1 (AF093252) *Treponema azotonutrcium* strain ZAS-9 (AF320287) *Treponema isoptericolens* strain SPIT5 (AM182455) clone mpsp 15 (Ectosymbiont of *Mixotricha paradoxa*) (X89051)
Treponema cluster II	Termite gut clone HsPySp20 (AB032004) Termite gut clone RFS2 (AF068429) Termite gut clone RsDiSp3 (AB032001)
Treponema Cluster III	Termite gut clone NkS34 (AB084961) Termite gut clone LK057 (AY739140)
Spirochaeta cluster I	*Spirochaeta coccoides* strain SPN1 (AJ698092)
Spirochaeta cluster II	Termite gut clone Za29 (AJ419823) Termite gut clone Rs-B68 (AB088897) Termite gut clone Rs-H09 (AB088910)

Nucleotide sequence accession numbers in brackets.

However, the isolate seems to be permanently in the hindgut, because the 16S rRNA gene of strain SPN1 was detected with semi-specific PCR in hindgut contents of *Neotermes castaneus*.

The rDNA sequence of *Treponema isoptericolens* strain SPIT5^T was similar to sequences from the spirochaetal genus *Treponema*. The highest similarities were observed with molecular clones obtained from hindgut contents of the termite *Neotermes koshunensis* (about 95% sequence identity with NkS53, NkS97, and NkS-Oxy3; Noda et al., 2003). The closest validly described relative of strain SPIT5^T was *Treponema primitia* strain ZAS-2^T (DSM 12427^T) with 92.3% sequence similarity. Earlier investigations with culture-independent molecular approaches had shown that the majority of termite spirochetes belonged to one of the so-called termite-clusters I–III of the treponemes (Table 1), whereas few could be assigned to the genus *Spirochaeta* (Dröge et al., 2006). Based on the phylogenetic analysis, strain SPIT5^T belonged to the termite-cluster I of the treponemes.

Because of the difficulties in the isolation of spirochetes from the termite hindgut, several investigations concentrated on culture-independent approaches. These investigations clearly verified that the termite hindguts are an enormous reservoir of novel spirochete species. Recently, more than 40 different spirochetal phylotypes were recognized in one termite species (Noda et al., 2003). Altogether, less than 30 well-characterized and named spirochetes of the genera *Spirochaeta* and *Treponema* have been described from different habitats (Olsen et al., 2000). Corresponding to the phylogenetic positions of spirochetal clones obtained from termite gut contents, it was previously speculated that all termite spirochetes represented a separate phylogenetic branch of the treponemes (Berchtold et al., 1994; Berchtold and König, 1996; Paster et al., 1996). Further investigations showed a second cluster of *Treponema*-related spirochetes in the termite hindgut (Ohkuma et al., 1999; Lilburn et al., 1999; Iida et al., 2000; Noda et al., 2003). This cluster, designated as "termite *Treponema* cluster II," is closely related to rumen spirochetes such as *Treponema bryantii* and *Treponema pectinovorum*. However, the phylogenetic variety within these clusters is large and free-living spirochetes species like *Spirochaeta stenostrepta* and *Spirochaeta caldaria* were included in Cluster I. According to Ochman and Wilson (1987), 1% of sequence divergence in 16S rRNA gene corresponds to roughly 50 Ma of evolution. Concerning the establishment of the spirochete/termite symbiosis, the high genetic distances (>0.10) of spirochetes within individual termite species as well as between different termite species can lead to the speculation that the last common ancestor of termite spirochetes must have existed earlier than 500 Ma and the diversification of the gut spirochetes started long before the origin of termites (about 300 million years ago) (Emerson, 1965). Spirochetes were found in the gut of termites preserved in the Miocene amber, which demonstrates that spirochetes thrive in the termite gut for at least 20 million years (Wier et al., 2002). This may indicate that different spirochete species colonized the ancestral termites and evolved within the hindgut to their recent diversity. Recently, spirochetal clones from gut contents were obtained, which could not be assigned to the *Treponema* cluster of termite spirochetes (Hongoh et al., 2003; Noda et al., 2003). The first clone of this group (Za29) from *Zootermopsis angusticolis* was sequenced by our group. The same is true for the isolate *S. coccoides,* which is relatively closely related to the genus *Spirochaeta*. This indicates that the phylogenetic diversity of the gut spirochetes is greater than that previously expected. It seems that, in addition to the *Treponema* cluster I–III, the spirochetes from the termite hindgut also form two clusters related to the genus *Spirochaeta* (Table 1).

On the surface of certain flagellates, about six clones of ectobiotic treponemes can be found, which are attached to restricted regions (Fig. 3). All identified clones belong to the *Treponema* clusters (Iida et al., 2000; Noda et al., 2003; Wenzel et al., 2003; Radek and Nitsch, 2007; Inoue et al., 2008). Spirochetal ectosymbionts have been found on the surface of many devescovinid flagellates (Kirby, 1941a, b, c, d, e) and, e.g., the flagellates *Mixotricha paradoxa*,

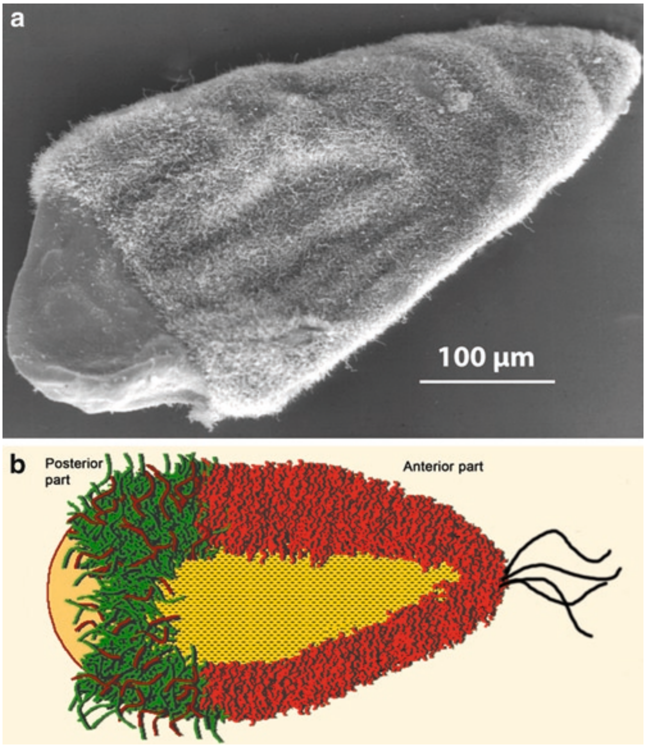

Figure 3. Electron micrograph of the larger cristamonad *Mixotricha paradoxa*. (**a**) Scanning electron micrograph of a whole cell. Bar = 100 µm. Some larger spirochetes are also visible. (A. Breuning and H. König, unpublished). (**b**) Schematic drawing showing the proposed distribution of the bacterial ectosymbionts on the cell surface of *Mixotricha paradoxa* (Wenzel et al., 2003)
Spirochete clone mpsp 15 ∿
Spirochete clone mp1 ∿
Spirochete clone mp3 ∿
Rod-shaped clone B6. |

Deltotrichonympha sp., and *Holomastigotoides mirabile*. In the case of *Mixotricha paradoxa,* they propel the flagellate cells (movement symbiosis) (Cleveland and Grimstone, 1964; Wenzel et al., 2003).

Representative termite species from all termite families except Serritermitidae have been investigated (Table 2), which can be assigned to four clusters (Table 1). Treponemal 16S rDNA clones from a termite species could be closer related to each other than to clones of other termites, but in some cases clones of a termite species are also closer related to clones from other termites. Berlanga et al. (2007) investigated the spirochete diversity from the termite species *Cryptotermes cavifrons*, *Heterotermes tenuis*, *Kalotermes flavicollis*, *Neotermes mona*, *Reticulitermes grassei*. They grouped 37 phylotypes to spirochete cluster I, three phylotypes to spirochete cluster II, and three phylotypes to spirochete cluster III. These authors concluded

Table 2. Systematically investigated spirochetes from termites or wood-eating cockroaches.

Host family	Host species	Spirochete genus/ species/16S RNA clones	References
Cryptocercidae	*Cryptocercus punctulatus*	*Hollandina*	Hollande and Gharagozlou, 1967
Kalotermitidae	Cryptotermes cavifrons	*Treponema*	Berlanga et al., 2007
	Incisitermes tabogae	*Treponema isoptericolens*	Dröge et al., 2008
	Kalotermes flavicollis	*Diplocalyx*	Gharagozlou, 1968
		Diplocalyx calotermitidis	Bermudes et al., 1988
		Treponema	Berlanga et al., 2007
	Kalotermes minor (*Incisitermes minor*)	*Hollandina*	Margulis et al., 1981
	Kalotermes praecox	*Pillotina calotermitidis*	Bermudes et al., 1988
	Kalotermes schwarzi (*Incisitermes schwarzi*)	*Hollandina,* *Pillotina*	Margulis et al., 1981; To et al., 1978
	Kalotermes synderi	*Hollandina*	Bermudes et al., 1988
	Marginitermes hubbardi (*Kalotermes hubbardi*)	*Diplocalyx*	To and Margulis, 1978
	Neotermes castaneus	*Spirochaeta coccoides*	Dröge et al., 2006
	Neotermes mona	*Treponema*	Berlanga et al., 2007
	Neotermes koshunensis	*Treponema*	Noda et al., 2003; Nakajima et al., 2006
	Postelectrotermes praecox (*Kalotermes praecox*)	*Pillotina*	Hollande and Gharagozlou, 1967
	Pterotermes occidentis	*Hollandina*	To et al., 1978
		Hollandina pterotermitidis	Bermudes et al., 1988

(continued)

Table 2. (continued)

Mastotermitidae	*Mastotermes darwiniensis*	*Hollandina*	Cleveland and Grimstone, 1964
		Treponema	Berchtold et al., 1994; Berchtold and König, 1996; Wenzel et al., 2003
		Canaleparolina darwiniensis	Wier et al., 2000
Rhinotermitidae	*Coptotermes formosanus*	*Hollandina*	To and Margulis, 1978
		Treponema	Shinzato et al., 2005; Lilburn et al., 1999
	Heterotermes tenuis	*Treponema*	Berlanga et al., 2007
	Reticulitermes flaviceps	*Clevelandina, Pillotina*	Breznak, 1984
		Treponema	Lilburn et al., 1999
	Reticulitermes grassei	*Treponema*	Berlanga et al., 2007
	Reticulitermes hesperus	*Clevelandina*	Bermudes et al., 1988
		Hollandina	Margulis et al., 1981
		Pillotina	
	Reticulitermes speratus	*Treponema*	Hongoh et al., 2003
	Reticulitermes tibialis	*Clevelandina, Hollandina*	Margulis et al., 1981
		Clevelandina reticulitermitidis	Bermudes et al., 1988
Termopsidae	*Hodotermopsis sjoestedti*	*Treponema*	Iida et al., 2000; Nakajima et al., 2006
	Zootermopsis angusticolis	*Treponema primitia, HT.azotonutricium*	Graber et al., 2004
Termitidae	*Macrotermes michaelseni*	*Treponema*	Berlanga et al., 2007
	Nasutitermes lujae	*Treponema*	Paster et al., 1996
	Nasutitermes takasagoensis	*Treponema*	Hongoh et al., 2006

Modified from Bermudes et al., 1988, 2007.

that certain spirochete species are closely related to other members of the same termite family and have coevolved with their species of the host termites.

5. Properties of Isolated *Spirochete* Species

Leadbetter et al. (1999) obtained the first spirochete species from the termite *Zootermopsis angusticollis* in pure culture. They were described as the new species *Treponema primitia* and *Treponema azotonutricium* (Graber et al., 2004). Two further isolates *Spirochaeta coccoides* and *Treponema isoptericolens* were obtained by Dröge et al. (2006, 2008). The isolates are characterized by the features described below.

Treponema primitia strain ZAS-2: cells 0.2 µm in diameter and 3–7 µm long, with a wavelength of 2.3 µm. Motile by two periplasmic flagella inserted at opposite ends of the cells. Anaerobe. Possesses NADH and NADPH peroxidases. Optimum temperature for growth is 30°C. Optimum pH for growth is 7.2. Homoacetogen. Energy source used for growth include glucose, maltose, mannitol, xylose, and 80% H_2 plus 20% CO_2. Strain ZAS-1 also uses arabinose and cellobiose, whereas strain ZAS-2 can grow slowly by acetogenic demethylation of methoxylated benzenoids (e.g., syringate). Growth by mixotrophy (H_2 and organic substrates). Yeast autolysate is required for growth. Strain ZAS-1 requires folinate, whereas strain ZAS-2 requires folic acid or folinate. Cells exhibit nitrogenase activity. It produces acetate from the fermentation of carbohydrates as well as from H_2 and CO_2 (acetogenesis) and from the methyl group of methoxylated aromatic compounds. It can also use both substrates mixotrophically (Leadbetter et al., 1999; Graber and Breznak, 2004). Yeast autolysate and a folate derivative are required for growth (Graber and Breznak, 2004, 2005). The biomass yields are between 0.1 and 0.2 g dry cell mass/mole substrate for H_2 and CO_2 and between 6.3 and 7.9 g dry cell mass/mole substrate for glucose. A NADH and NADPH peroxidase is present. Genome sizes are 3,461 kb (strain ZAS-1) and 3,835 kb (strain ZAS-2). The G + C contents of DNA are 51 mol% (strain ZAS-1) and 50.9 mol% (strain ZAS-2). Each strain possesses 2 *rrs* gene copies. Source: hindgut of the termite *Zootermopsis angusticollis* (Hagen). Type strain is ZAS-2^T (DSM 12427^T, ATCC BAA-887^T)

Treponema azotonutricium strain ZAS-9: cells 0.2–0.3 µm in diameter by 10–12 µm long, with a wavelength of 1.2 µm. Motile by two periplasmic flagella inserted at opposite cell ends. Anaerobe. Catalase negative. Yeast autolysate required for growth. Optimal growth temperature is 30°C. Energy sources include glucose, fructose, ribose, xylose, maltose, and cellobiose. It produces acetate, ethanol, CO_2, and H_2 (5.2 mol/mol maltose). It can fix dinitrogen (Lilburn et al., 2001). It possesses a conventional glycolytic pathway and a pyruvate ferredoxin oxidoreductase-mediated cleavage of pyruvate yielding 5.2 mol H_2, 3.6 mol CO_2, 2.8 mol acetate, and 0.8 mol ethanol per mol maltose (Graber et al., 2004). It exhibits nitrogenase activity. The genome size is 3,901 kb. It contains 50.0 mol% G + C and 2 *rrs* gene copies. The isolate belongs to the *Treponema* cluster. Source: hindgut of the termite *Zootermopsis angusticollis* (Hagen). Type strain ZAS-9^T (DSM 13862^T, ATCC BAA-888^T).

Spirochaeta coccoides strain SPN 1: cells are cocci, 0.5–2 µm in diameter. Cell aggregates are formed. Axial filaments were not observed. Immotile. Anaerobic. Catalase negative. Yeast extract is required for growth and could serve as sole energy and carbon source. Optimal temperature for growth is 30°C. The pH range for growth was between 5.5 and 9.5 with an optimum at 7.4. Pentoses (arabinose, xylose) and oligosaccharides (maltose, cellobiose, maltotriose, and maltotetraose) stimulated growth in a medium containing 0.4% yeast extract and 0.2% peptone. Maltose is fermented to ethanol, formate, and acetate as the main fermentation products. Glucose, galactose, lactate, pyruvate, amino acids, and polysaccharides are not utilized. Activity of β-D-glucosidase, α-D-glucosidase, α-D-galactosidase α-L-arabinosidase, β-D-fucosidase, and β-D-xylosidase is exhibited. G +C content is 56.6–57.4 mol%. Based on nucleotide sequence of the 16S rRNA gene, this spirochete belongs to the genus *Spirochaeta* (accession number AJ698092; EMBL). Source: strain SPN1 was isolated from the hindgut contents of the lower drywood termite *Neotermes castaneus* (Burmeister) (Isoptera: Kalotermitidae). Type strain is strain SPN1^{T} deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany (DSM 17374^{T}), and with the American Type Culture Collection, Manassas, USA (ATTC BAA-1237^{T}).

Treponema isoptericolens strain SPIT5^{T}: cells are helical-shaped with 0·4–0·5 µm in diameter and mainly 12–20 µm in length. Motile by six periplasmic flagella, inserted near the opposite ends of the protoplasmic cylinder. Gram-negative. Spherical bodies are formed at the end of the exponential growth phase. Strictly anaerobic and catalase negative. Yeast extract is required for growth and could serve as sole energy and carbon source. Optimal temperature for growth is 30°C. The pH range for growth was between 6.5 and 8.0 with an optimum between 7·2 and 7·4. Chemoheterotroph with fermentative metabolism. Grows on arabinose, xylose, fructose, maltose, cellobiose, trehalose maltotriose, and yeast extract. Maltose is fermented to ethanol and CO_2 as main fermentation products. Glucose, galactose, lactate, pyruvate, fatty acids, amino acids, and polysaccharides are not utilized. Activity of α-L-arabinosidase, β-D-cellobiosidase, β-D-fucosidase, α-D-galactosidase, α-D-glucosidase, β-D-glucosidase, and β-D-xylosidase is exhibited. The G + C content of the DNA is 47.7 mol%. The type strain, SPIT5^{T} (= DSM 18056^{T} = JCM 13955^{T}) was isolated from hindgut contents of the lower drywood termite *Incisitermes tabogae* (Snyder) (Isoptera: Kalotermitidae).

6. Microhabitats

The bacteria of the termite gut are not randomly distributed. Many occur in certain microhabitats (Berchtold et al., 1999). Spirochetes can account for 50% of the prokaryotic cells in the termite gut (Paster et al., 1996). They occur in three microhabitats: (1) free living in the paunch contents, (2) as ectosymbionts of flagellates, and (3) in flagellate vesicles.

Ectosymbiotic spirochetes are characteristic for many devescovinid flagellates (Kirby, 1941a, b, c, d, e) One of the first detected symbioses between flagellates (*Pseudodevescovina uniflagellata*) and spirochetes was described by Kirby (1936). *Pseudodevescovina uniflagellata* lives in the gut of the Australian dry wood termite *Neotermes insularis*. Only 3 years earlier, Sutherland (1933) published an article about *Mixotricha paradoxa* where the attached spirochetes were misconceived as cilia. A detailed description of the fine structure of *Mixotricha paradoxa* and the role of the ectosymbiotic bacteria in cell locomotion was provided by Cleveland and Grimstone (1964).

Mixotricha paradoxa is a rare example of movement symbiosis between prokaryotic spirochetes and a eukaryotic flagellate (Fig. 3; Cleveland and Grimstone, 1964; Tamm, 1982; Breznak and Leadbetter, 2002; Brugerolle, 2004; Breznak, 2006; König et al., 2007; Radek and Nitsch, 2007), which were identified by Wenzel et al. (2003). One large surface spirochete was described as *Canaleparolina darwiniensis* (Wier et al., 2000). Over the years, more and more examples of surface symbiosis between protists and prokaryotes from the termite gut appeared, but examples of movement symbiosis in the termite gut could be rarely detected (Smith et al., 1975; Bloodgood and Fitzharris, 1976; To et al., 1980; Tamm, 1982).

Since the detection of the ectosymbiotic spirochetes (Kirby, 1936, 1941a, b, c, d, e), more details about the attachment sites were investigated by Smith et al. (1975) and Radek and Nitsch (2007). Radek and Nitsch (2007) found that in the case of *Mixotricha paradoxa* the spirochetes could be removed from the flagellate surface by Triton X-100 and 8 M urea. Application of antibiotics reduces the number of spirochetes and leads to a disintegration of the cortical attachment system. *Mixotricha paradoxa* becomes immotile. The spirochetes were transformed in spherical cells, which represent probably cysts.

Recently, large pillotinaceous spirochetes of the genus *Canaleparolina* and smaller spirochetes were found in spherical vesicles of parabasalid *Staurojoenina assimilis* living in the gut of the kalotermitid *Neotermes mona* (Wier et al., 2007).

7. Maintenance

Treponema primitia strains ZAS-1 and ZAS-2, *Treponema azotonutricium* strain ZAS-9, and *Treponema isoptericolens* strain SPIT5 can be grown in *Treponema* ZAS medium (DSMZ medium 843; http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium843.pdf), while *Spirochaeta coccoides* strain SPN1 grows in *Spirochaeta zuelzerae* medium (DSMZ medium 169: http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium169.pdf).
DSMZ medium 843

NaCl	1.00 g
KCl	0.50 g
$MgCl_2 \times 6\ H_2O$	0.40 g
$CaCl_2 \times 2\ H_2O$	0.10 g
NH_4Cl	0.30 g
KH_2PO_4	0.20 g
Na_2SO_4	0.15 g
Resazurin	0.50 mg
Distilled water	1,000.00 ml

Prepare the basal medium anaerobically under 80% H_2 + 20% CO_2 gas mixture. Dispense 5 ml amounts in anaerobic Hungate or Balch tubes under the same gas and autoclave. Prior to use, add 5 ml via injection of the following anaerobic, sterile solutions:

Trace element solution SL-10 (tenfold diluted)	0.05 ml
$NaHCO_3$ (10%, w/v)	0.30 ml
Selenite-tungstate solution	0.01 ml
Vitamin solution	0.01 ml
MOPS buffer, 10 mM, pH 7.2, filter sterilized	0.05 ml
Yeast autolysate	0.20 ml
Cofactors solution	0.10 ml
Dithiothreitol, 0.1 M, filter sterilized	0.05 ml

Trace element solution SL-10	
HCl (25%: 7.7 M)	10.00 ml
$FeCl_2 \times 4\ H_2O$	1.50 g
$ZnCl_2$	70.00 mg
$MnCl_2 \times 4\ H_2O$	100.00 mg
H_3BO_3	6.00 mg
$CoCl_2 \times 6\ H_2O$	190.00 mg
$CuCl_2 \times 2\ H_2O$	2.00 mg
$NiCl_2 \times 6\ H_2O$	24.00 mg
$Na_2MoO_4 \times 2\ H_2O$	36.00 mg
Distilled water	990.00 ml

First dissolve $FeCl_2$ in the HCl, then dilute in water, add and dissolve the other salts. Finally, make up to 1,000.00 ml.

Selenite-tungstate solution

0.5 g NaOH, 3 mg $Na_2SeO_3 \times 5\ H_2O$, 4 mg$Na_2WO_4 \times 2\ H_2O$, 1 l distilled water.

Vitamin solution	
Vitamin B_{12}	100.00 mg
p-Aminobenzoic acid	80.00 mg
D(+)-Biotin	20.00 mg
Nicotinic acid	200.00 mg
Calcium pantothenate	100.00 mg
Pyridoxine hydrochloride	300.00 mg
Thiamine-HCl × 2 H_2O	200.00 mg
Distilled water	1,000.00 ml

Yeast autolysate

Suspend 5.6 g of dry baker's yeast (Sigma, cat. no. YSC-1) in 20 ml distilled water and allow cells to autolyze for 24 h at 56°C. After, cell debris is removed by centrifugation (20.000 × g for 15 min), and the amber-colored supernate is neutralized with 5 M NaOH, the solution in filter sterilized, and stored under N_2.

Cofactor solution (μg/ml)	
Pyridoxal-HCl	250.00
Pyridoxal phosphate	250.00
Calcium folinic acid	50.00
β-NAD	50.00
Coenzyme A	50.00

FAD	50.00
Nicotinamide	25.00
Folic acid	2.50
Riboflavin	0.50
Haemin (dissolved separately in 10 mM NaOH)	65.00
Thiamine pyrophosphate	2,500.00

Adjust pH to 7.0 with 1 M NaOH against indicator paper, filter sterilize.

For Treponema azotonutricium DSM 13862
Prepare medium under 80% N_2 and 20% CO_2 gas mixture and supplement 5 ml of medium with 0.15 ml of a mixture of disaccharides containing maltose, cellobiose, sucrose, and trehalose (5 mM each), sterilize by filtration and store under N_2.

DSMZ-Medium 169	
Solution A	
Glucose	2.00 g
Yeast extract (Difco)	4.00 g
$CaCl_2 \times 2 H_2O$	0.04 g
$MgSO_4 \times 7 H_2O$	0.50 g
Cysteine-HCl × H_2O	0.50 g
Agar (Bacto, Difco)	10.00 g
Distilled water	960.00 ml

Adjust pH to 7.2 with KOH.

Solution B	
$NaHCO_3$	1.00 g
Distilled water	20.00 ml
Solution C	
0.5 M K-phosphate buffer, pH 7.4	20.00 ml

Solution A (without cysteine) is boiled for few minutes to remove dissolved oxygen, and cooled to room temperature under a stream of 100% nitrogen gas. Then, cysteine is added and the pH adjusted to 7.2. The three parts are sterilized separately each by autoclaving under 100% nitrogen atmosphere and combined thereafter. Final pH of the complete medium is 7.5.

8. Physiology

The central region of the paunch is anaerobic because of the high oxygen consumption rates within the gut periphery (Brune et al., 1995; Berchtold et al., 1999). Like other intestinal spirochetes, the spirochetal isolates from the termite gut are anaerobic bacteria.

The hydrolysis of polysaccharides is initiated by the termite's own glycosylases secreted in the salivary glands and the midgut (Nakashima et al., 2002; König et al., 2007). Poly- and oligosaccarides reaching the hindgut are fermented by the hindgut microbes mainly to acetate and in lower amounts to propionate and butyrate. In the digestion of cellulose and hemicellulose flagellates, bacteria and yeasts are involved (Table 3; Schäfer et al., 1996; Wenzel et al., 2002; cf. König et al., 2002, 2006). The gut spirochetes produce acetate, the main carbon source of termites, by homoacetogenesis ($4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$) and fermentation of oligosaccharides and sugars (Fig. 4). The dominance of homoacetogenesis over methanogenesis from H_2 and CO_2 in the termite gut can be explained by the high abundance of acetogenic spirochetes (Breznak, 2006; Pester and Brune, 2006).

They contribute to the nitrogen requirement of termites by fixing dinitrogen, which often feed on substrates such as wood with a low nitrogen content. The physiological characteristics of *Treponema primitia* and *T. azotonutricium* were recently summarized by Breznak (2006).

Spirochaeta coccoides strain SPN1 (Dröge et al., 2006) is a strictly anaerobic organism. It exhibits no catalase activity and is not able to grow under either oxic and microaerophilic conditions. *Sp. coccoides* utilizes no glucose, but is able to grow with yeast extract as sole carbon and energy source. In addition to yeast extract, it also ferments different carbohydrates. Fastest growth and highest cell yield are observed by addition of maltose or maltotriose. From both, yeast extract and fermentable sugars, strain SPN1 produces formate, acetate, and ethanol as main end products (Fig. 4). Maltose was fermented to ethanol (2.2 mol/mol maltose), formate (2.9 mol/mol), and acetate (1.4 mol/mol). *S. coccoides* shows glycolytic activities, which are involved in the further breakdown of oligosaccharides derived from cellulose and hemicellulose in the termite gut. Similarly, different rumen bacteria like *Ruminococus flavefaciens* use cellobiose but not glucose (Bryant, 1984). However, turnover rates of injected glucose in agarose-embedded hindguts (<0.5 nmol/termite/h), determined by Tholen and Brune (2000), may be a hint that free glucose is not a very important intermediate in the termite hindgut.

Formally, the degradation of lignocellulose can be divided into three steps, a hydrolytic step, a fermentative step, and a methanogenic/acetogenic step (Table 3; König et al., 2002, 2006). Spirochetes are involved in both, the hydrolytic step of oligosaccharides and the fermentative step. During fermentation, *Treponema saccharophilum* (Paster and Canale-Parola, 1981, 1985) incorporates about 15% of the carbon source into cell carbon. If this is also true for *S. coccoides,* the carbon recovery would be about 99%. Most spirochetes ferments carbohydrates via the Embden–Meyerhof pathway to pyruvate, which is metabolized to acetate, ethanol, CO_2, and H_2 by means of a clostridial-type clastic reaction (Fig. 4; Canale-Parola, 1984; Salmassi and Leadbetter, 2003). This pathway could be also proposed for *Treponema azotonutricium*, isolated from the termite *Zootermopsis angusticollis* (Lilburn et al., 2001), which produces acetate, ethanol, H_2, and CO_2 in a molar ratio of about 3:1:5:4 from maltose (Graber et al., 2004). The formation of formate in case of *S. coccoides* indicates some modifications in this pathway, probably including a pyruvate:formate lyase. The formation of formate in addition to ethanol and acetate is known from several spirochetes isolated from bovine rumen fluid such as *Treponema saccharophilum* (Paster and Canale-Parola, 1981, 1985). However, the ratio of fermentation products produced by *S. coccoides* differs from that proposed for *T. saccharophilum,*

Table 3. Intestinal isolates involved in the different stages of lignocellulose degradation in the termite gut.

	Substrate	I. Stage Hydrolytic Microorganism (Polysaccharide hydrolysis)	II. Stage Oxidative/Fermentative Microorganisms (Oligosaccharide hydrolysis)	III. Stage Acetogenic, Methanogenic, Sulfate-reducing Bacteria	
Wood Mechanical degradation to µm- particules by termites	**Cellulose** (34–62%)	**Cellulase** *Flagellates*: *Trichomitopsis termopsidis*, *Trichonympha sphaerica*	**β-Glucosidase**		
		Bacteria: *Alcaligenes*, *Azospirillum*, *Bacillus*, *Brevibacillus*, *Cellulomonas*-related spp., *Clavibacter*, *Clostridium*, *Corynebacterium*, *Klebsiella*, *Kocuria*, *Paenibacillus*, *Microbacterium*, *Micromonospora*, *No cardioforme*, Rhizobia, *Ochrobactrum*, *Paenibacillus*, *Sphingomonas*, *Spirosoma*-related spp., *Streptomyces*	*Actinobacteria*, *Arthrobacter*, *Au-reobacterium*, *Bacillus*, *Citrobacter*, *Enterobcater*, *Enterococcus*, *Klebsiella*, *Lactococcus*, *Nocardia*, *Ochrobactrum*, *Pseudomonas*, Rhizobia, *Rhodococcus*, *Serratia*, *Streptomyces* Spirochetes		***Food source of termites*:** **Acetate, Propionate, Butyrate, Microbial cells**
		Yeasts (*Cryptococcus*, *Filobasidium*)	Yeasts		
	Hemicellulose (14–32%)	**1.4-β-Xylanase1.3-β-Galactanase**	**β-D-Galactosidase** **α-L-Arabinosidase** **β-L-Xylosidase**	$2H_2 + O_2 \Rightarrow H_2O$ $Lactate \Rightarrow Acetate + CO_2$ *Desulfovibrio*	***Excreted end products*:** CO_2 , H_2 , **CH_4** **Microbial cells**
		Actinobacteria, *Acinetobacter*, *Bacillus*, *Flavobacterium*-related spp., *Ochrobactrum*, *Paenibacillus*, *Pseudomonas*, *Streptomyces*	*Actinobacteria*, *Acinetobacter*, *Arthrobacter*, *Aureobacterium*, *Bacillus*, *Burkholderia*, *Citrobacter*, *Cellulomonas*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Nocardia*, *Ochrobactrum*, *Pseudomonas*, *Paenibacillus*, *Rhizobium*, *Serratia*, *Staphylococcus*, *Streptomyces*	$4H_2 + 2CO_2 \Rightarrow Acetate + 2H_2O$ *Acetonema*, *Clostridium*, *Sporomusa*, Spirochetes $4H_2 + CO_2 \Rightarrow CH_2 + 2H_2O$ *Methanobrevibacter*	
		Yeasts (*Candida, Debaryomyces, Pichia, Sporothrix*)	Yeasts		

Table 3. (continued)

	Substrate	**I. Stage Hydrolytic Microorganism (Polysaccharide hydrolysis)**	**II. Stage Oxidative/Fermentative Microorganisms (Oligosaccharide hydrolysis)**	**III. Stage Acetogenic, Methanogenic, Sulfate-reducing Bacteria**
	Aromatic compounds (5%)		*Acinetobacter*, *Arthrobacter*, *Alcaligenes*, *Aureobacterium*, *Bacillus*, *Burkholderia*, *Klebsiella*, *Listeria*, *Nocardia*, *Ochrobactrum*, *Pseudomonas*, *Rhizobia*, *Serratia*, *Streptomyces*	
	Lignin (18–39%)	*Streptomyces*		**Excreted: Modified lignin**
N_2-fixation			*Enterobacter, Desulfovibrio, Rhizobia, Klebsiella,* Spirochetes, *Citrobacter*	

Spirochetes are involved in stages II and III and dinitrogen fixation.

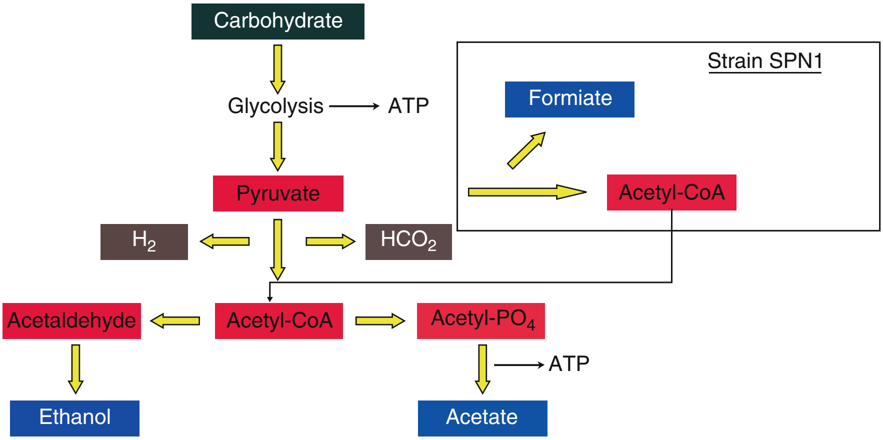

Figure 4. Fermentation of sugars by spirochetes. The pathway of *Spirochaeta coccoides* strain SPN1 is indicated in the box.

which produces formate, acetate, and ethanol in molar ratio of nearly 2:1:1 (Paster and Canale-Parola, 1985). Fastest growth of *S. coccoides* was obtained at the same temperature at which the termites were grown. The pH-range for growth of *S. coccoides* corresponded to the physicochemical conditions in the gut. The pH-value of the hindgut of lower termites is in the range between 6.0 and 7.5, whereas in higher termites parts of the midgut have an alkaline pH-value up to 10.4 (Bignell and Anderson, 1980).

Treponema isoptericolens strain SPIT5 (Dröge et al., 2008) is a chemoheterotroph and shows a fermentative metabolism. It grows on a variety of carbohydrates including arabinose, xylose, fructose, cellobiose, maltose, trehalose, and maltotriose. *S. isoptericolens* requires yeast extract (at a minimum concentration of 0.5 g/l) and a cofactor solution, previously described by Leadbetter et al. (1999), for growth. At higher concentration, yeast extract can serve as sole carbon and energy source. This is most probably caused by the relatively high trehalose content of the yeast extract, which is used for cultivation and growth studies. Analysis of yeast extract solutions with trehalase (Sigma) and HPLC revealed a trehalose content of about 85 mg per gram dry powder, resulting in a final concentration of about 0.5 mM trehalose in the medium, which was used for the isolation of strain SPIT5^T (containing 2 g/l yeast extract). Growth of strain SPIT5^T was improved by the addition of peptone, but in contrast to yeast extract it could not be used as a sole growth substrate. Maltose was fermented to ethanol as the main liquid fermentation product. Small amounts of acetate were also detected but no hydrogen. Strain SPIT5 possesses enzymatic activities of α-L-arabinosidase, β-D-cellobiosidase, β-D-fucosidase, α-D-galactosidase, α-D-glucosidase, β-D-glucosidase, and β-D-xylosidase. Not detected were activities of β-D-galactosidase, β-D-glucuronidase, and α-D-mannosidase. The tested enzymatic activities seemed to be cell-bound, because no glycolytic activity was found in the supernant of the growth medium.

9. Genomics

Investigation on the genome of the spirochete isolates from the termite gut is scarce. The genome size of *T. primitia* (3.5 Mb) and *T. azotonutricium* (3.9 Mb) is similar to that of free-living spirochetes, while host-associated species have a smaller genome (Breznak, 2006). The small subunit rRNA-encoding gene occurs only in two copies. Metagenomic and functional analysis showed that 68% of the marker genes in the metagenomic DNA belonged to treponemes (Warnecke et al., 2007).

10. Perspectives

The microbiota of the termite gut is dominated by spirochetes. The termite gut harbors a vast variety of spirochete morphotypes as well as different rRNA clones (species) not found elsewhere in nature except in wood-feeding cockroaches. Only a few spirochete species have been obtained in pure culture, and their energy pathways have been investigated. Further studies are required to learn more about this unique symbiotic association in respect to coevolution of spirochetes and their hosts as well as their different functions in the food chain of lignocellulose degradation.

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Biodata of **David Iluz**, author of ***"The Plant–Aphid Universe"***

Dr. David Iluz is a lecturer in Bar Ilan University, Ramat Gan, Israel. He obtained his Ph.D. from Bar Ilan University in 1998 *summa cum laude,* and spent his postdoctoral fellowship at Hebrew University, Israel. Dr. Iluz worked on the photoacclimation of marine and freshwater phytoplankton (Lake Kinneret, Mediterranean, Red Sea). The focus of his work has been the study of bio-optical parameters in different biogeochemical provinces of Israel's water bodies and their relevance for estimating primary production. He developed algorithms to determine pigment concentration and composition from upwelling light, which can be estimated from underwater in situ light spectra or by satellite remote sensing.

Additional projects combining his interests in archeology and biology were the study of the chemistry and molecular biology of pigments obtained from scale aphids. He identified a species of Kermes oak coccid that grew in Israel, the aphid from which the precious Biblical scarlet dye [*tola'at shani*] was extracted, providing new insights on the ancient dye trade.

Dr. Iluz participated in several research expeditions to Red Sea coral reefs, collected and analyzed bio-optical data from the First Israel-Eritrea Joint Cruise, the GAP-IOLR cruise in the Eastern Mediterranean, and in the First Israel-Seychelles Joint Cruise to the Indian Ocean. He has published 25 scientific papers and is the recipient of several international and Israeli grants.

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THE PLANT–APHID UNIVERSE

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1. Taxonomy

Aphids belong to the suborder Sternorrhyncha, which contains the aphids, whiteflies, psyllids, and scale insects, groups which are included in the order Hemiptera (Table 1). Sternorrhyncha refers to the rearward position of the mouthparts relative to the head.

Found worldwide, all members of this group are plant feeders, use phloem sap as their dominant or sole food source (Dolling, 1991), and several of their species are major crop and ornamental pests. Many exhibit adaptations to parasitism such as modified morphology and/or life cycles, flightless morphs, parthenogenesis, sexual dimorphism, and even eusociality (McGavin, 1992, 1993; Moran, 1992).

Aphids are small plant-feeding insects, members of the superfamily Aphidoidea. About 4,400 species of ten families are known and they vary in length from 1 to 10 mm (McGavin, 1993). The scale insects are an important group of about 8,000 species of aphids that are generally classified as the superfamily Coccoidea. Scale insects vary dramatically in their appearance from very small organisms (1–2 mm) that occur under wax covers, shiny pearl-like objects (about 5 mm), and creatures covered with mealy wax. Adult females are almost always immobile (aside from mealybugs) and permanently attached to the plant they have parasitized (Ben-Dov, 2005).

This review deals with the insects that belong to the suborder Sternorrhyncha, mainly aphids and scale insects. Here, we also use the word aphid for scale insects because of our focus on their ecology and similar biological functions.

Aphids are among the most destructive insects on Earth (Ben-Dov, 2005; McGavin, 1992; Miller et al., 2002). Around 250 species are serious agriculture and forestry pests as well as an annoyance for gardeners (Rutledge et al., 2004). Aphids are distributed worldwide, but are most common in temperate zones. They can migrate great distances, mainly through passive dispersal riding on winds. For example, the currant lettuce aphid (*Nasonovia ribisnigri Mosley*) is believed to have spread from New Zealand to Tasmania in this way (Courtney,

Table 1. Scheme of taxonomic affinities for the suborder Sternorrhyncha Kingdom: Animalia; phylum: Arthropoda; class: Insecta; order: Hemiptera; suborder: Sternorrhyncha.

Class:		Insecta	
Order:		Hemiptera	
Suborder:		Sternorrhyncha	
Superfamily:	**Aleyrodoidea (>1,500)**	**Aphidoidea (>4,400 species)**	**Coccoidea (>8,000 species)**
Family:	Aleyrodidae (whiteflies)	Aphididae (aphids and plant lice) Adelgidae (pine aphids and spruce aphids) Eriosomatidae (gall-making aphids and woolly aphids) Phylloxeridae (spruce aphids)	Aclerdidae (aclerdid scales) Asterolecaniidae (pit scales) Coccidae (soft scales, tortoise scales, and wax scales) Conchaspididae (false armored scales) Dactylopiidae (cochineal insects and cochineal scales) Diaspididae (armored scales) Eriococcidae (eriococcid scales) Kermesidae Kermidae (gall-like coccids) Margarodidae (cottony cushion scales, giant coccids, and ground pearls) Ortheziidae (ensign coccids) Pseudococcidae (mealybugs)

2007; Suwanbutr, 1996). Aphids have also been spread by human transport of infested plant materials (Fig. 1).

Many aphids are named after the plants that they destroy, for example, cotton aphid and apple aphid. Aphids damage plants by sucking the juices from buds, leaves, shoots, or roots. They exude a sugary liquid called honeydew (see below, Fig. 2), which sticks to the plant and causes additional damage due to the growth of various harmful fungi and molds (Hajek and Stleger, 1994). Some kinds of aphids cause the formation of galls (see Raman, present volume) on plants (Cook, 2008). Aphids can also spread plant diseases, and are major vectors in the spread of viral plant epidemics (Anderson et al., 2004).

Plant phloem saps are rich in sugars and poor in amino acids and nitrogen. This leads to aphids excreting large amounts of sugary liquid, known as honeydew, because the amount of sap they have to drink in order to get sufficient nitrogen in their diet results in them acquiring far more sugar and liquid than they can utilize (Wilkinson and Douglas, 1996). Honeydew can often be seen on the lower faces of the leaves of infested trees, giving them a sticky coating.

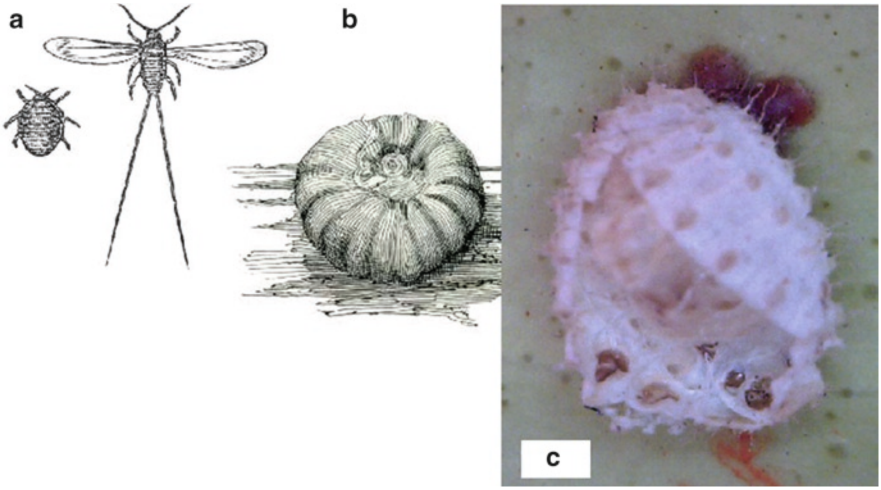


Figure 1. Different types and morphologies: (**a**) female (*left*) and male (*right*) cochineals; (**b**) Rosette lac scale*;* *Paratachardina decorella* (*Hemiptera*: *Kerridae*); (**c**) scale aphid.

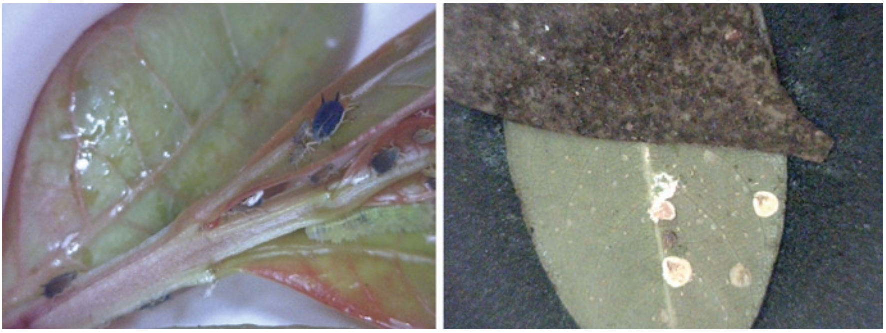


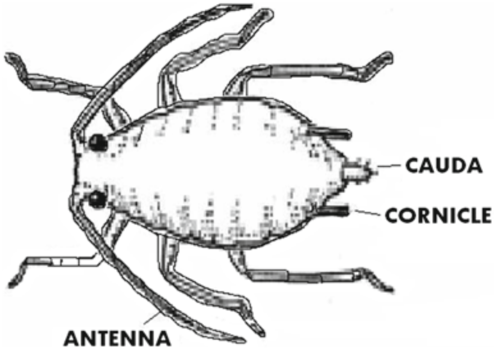
Figure 2. *Left*: honeydew and aphids; *right*: soot-covered laurel leaves and scale aphids on the dorsal face of leaf.

This is then fed on by other insects such as the common wasp, *Vespula vulgaris,* and the brown hairstreak butterfly, *Thecla betulae*, and a yeast-like fungus makes the leaves look black, as if they were covered in soot (Shah and Pell, 2003).

2. Anatomy

Most aphids have soft, green bodies, but other colors are also common, such as black, brown, and pink. Aphids have antennae with as many as six segments (McGavin, 1993; Miller et al., 2002). Aphids feed through sucking mouthparts

Figure 3. The aphid anatomy.



called stylets, enclosed in a sheath called a rostrum, which is formed from modifications of the mandible and maxilla. They have long, thin legs and two-jointed, two-clawed tarsi (McGavin, 1993) (Fig. 3). Most aphids have a pair of cornicles (or siphunculi), abdominal tubes through which they exude droplets of a quick-hardening defensive fluid (Stroyan, 1997) containing triacylglycerols called cornicle wax. Other defensive compounds can also be produced by some types of aphids (McGavin, 1993).

Aphids have a tail-like protrusion called a cauda above their rectal apertures. They have two compound eyes and an ocular tubercle behind and above each eye, made up of three lenses (called triommatidia) (Dixon, 1998; Mutti et al., 2006). Many aphid species are monophagous (that is, they feed on only one plant species). Others, like the green peach aphid (*Myzus persicae*), feed on hundreds of plant species across many families (Jansson and Smilowitz, 1986).

3. Reproduction as a Function of Adaptation to Seasons and Climate Changes

Some aphid species have complex, specialized reproductive patterns, while others have fairly simple lifecycles. Adaptations include having alternating sexual and asexual reproductive phases, creation of eggs or live nymphs, and switches between woody and herbaceous types of host plant at different times of the year (McGavin, 1992, 1993) (Fig. 4).

Many aphids undergo cyclical parthenogenesis. In the spring and summer, mostly or only females are present in the population. The overwintering eggs that hatch in the spring result in females, called fundatrices. Reproduction is typically parthenogenetic and viviparous. Females undergo a modified meiosis that results in eggs that are genetically identical to their mothers (parthenogenetic) (Jahn et al., 2005; Moran, 1992). This process iterates throughout the summer, producing multiple generations that typically live 20–40 days. Thus, one female hatched in spring may produce many billions of descendants. For example, some species of cabbage aphids (like *Brevicoryne brassicae*) can produce up to 41 generations of

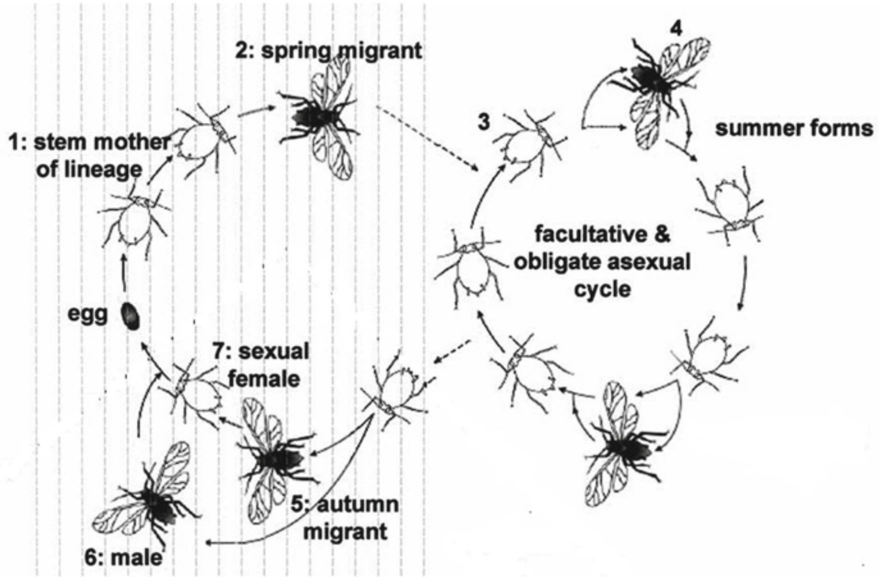


Figure 4. Schematic representation of facultative (holocyclic) and obligate (anholocyclic) asexual life-cycles in a hypothetical aphid species (figure adapted from Field and Blackman, 2003). A sexual egg hatches to produce the stem mother (1) of the facultative asexual lineage. After the spring migrant (2), this can then be maintained as apomictic parthenogens in summer forms (3 and 4), provided the right environmental conditions are met. In facultative asexuals, summer forms can be induced to produce the autumn migrant (5) and male (6) in appropriate conditions. These conditions will also induce the autumn migrant to give birth to the sexual female (7). Sexuals mate, forming a sexual egg.

females, or more than 1.5 × 10^{27} offspring, if they would all live (Hughes, 1963; Lamb, 1961; van Emden and Bashford, 1969).

In autumn, aphids undergo sexual, oviparous reproduction. A change in photoperiod and temperature, or perhaps, a lower food quantity or quality, causes females to parthenogenetically produce sexual females and males. The males are genetically identical to their mothers, except that they have one less sex chromosome. These sexual aphids may lack wings or even mouthparts (McGavin, 1992). Sexual females and males mate, and females lay eggs that develop outside the mother. The eggs endure the winter and emerge the following spring as winged or wingless females. In warm environments, such as in the tropics or in a glasshouse or greenhouse, aphids may go on reproducing asexually for many years (Stroyan, 1997). Some species produce winged females in the summer, sometimes in response to low food quality or quantity. The winged females migrate to start new colonies, often of quite a different kind, on a new plant (McGavin, 1992).

Parthenogenesis is beneficial for spreading rapidly when conditions are optimal, broadcasting one genotype, suited for these conditions. However, any adverse conditions are met by sexual reproduction, producing few, genetically diverse, hardship-resistant offspring that are likely to survive until favorable conditions return. Parthenogenesis enables aphids to reproduce rapidly and efficiently as long as the nutritional conditions are favorable; however, aphids, as well as other herbivorous insects, are often nitrogen-limited for most of the growing season. Although some types of essential amino acids are prerequisites for aphid reproduction, the phloem sap of the host plant contains high levels of sugars, but low concentrations of these amino acids, except in developing leaves (Dixon, 1998; Wilkinson and Douglas, 2003).

A number of studies have been conducted to clarify how aphids optimize their nutrition support a high reproductive rate. There are four possible adaptations for achieving high fecundity: host alternation, group feeding, intracellular symbiosis with Buchnera, and gall formation. Host-alternating aphid species use two different taxa of plants, the so-called primary and secondary host plants, which ensure complementary growth of the population, consequently evading a decline in the nutritional levels of their primary host, in the case of deciduous trees (Dixon, 1998).

Some aphids have “telescoping generations.” The parthenogenetic, viviparous female has a daughter within her, which is already parthenogenetically producing her own daughter. Thus, a female’s diet can affect the body size and birth rate of more than one generation (daughters and granddaughters) (Jahn et al., 2005; Nevo and Coll, 2001). Cyclical parthenogenesis predominates in aphids. The capacity for parthenogenesis was probably acquired by a common sexual ancestor, some 250 million years ago (Dixon, 1998; Hales et al., 1997). However, only a few percent of *species have lost the sexual phase and become strict obligate parthenogens* (Dixon, 1998; Moran, 1992).

In most aphids, only cyclical parthenogens are able to produce cold-resistant diapausing eggs, providing a major short-term ecological advantage of sex. This should result in climatic clines, with sexual lines predominating in cold regions and being replaced by asexual lines where winters are mild (Rispe et al., 1998). This is supported by empirical studies that show clines in aphid reproductive mode in spite of strong dispersal and gene flow (Dedryver et al., 1998, 2001; Simon et al., 2002).

Aphids are affected by inclement weather phenomena (Dicke and Sabelis, 1988; Starý and Lukásova, 2002), such as precipitation (Brust, 2006), temperature (Lamb, 1961; Powell and Parry, 1976), and wind (Jones, 1979).

Aphids can be easily killed by unfavorable weather, such as late spring frosts (Krupke et al., 2007). Excessive heat kills the symbiotic bacteria that some aphids depend on, thus making the aphids infertile. Rain prevents winged aphids from dispersing, and knocks aphids off plants, thus killing them from the impact or by starvation (Brust, 2006; Hughes, 1963; Starý and Lukásova, 2002).

4. Evolution

Although fossil evidence suggests that the Aphidoidea diversified concomitant with the first angiosperms in the lower Cretaceous (approx. 140 million years ago), it is likely that the family is much older. For example, three groups, the Prociphilini

(Pemphigidae), Mindarus (Mindaridae), and Neophyllis (Drepanosiphidae), have what are probably ancient ties to conifers. Therefore, aphids probably had already appeared around 280 million years ago, in the early Permian period. They probably fed on plants like Cordaitales and Cycadophyta. Most aphid families became extinct at the Cretaceous-Teriary (K/T) boundary along with the dinosaurs. The majority of extant taxa can be found in the family Aphididae, which underwent a striking radiation in the Miocene (approx. 5–26 million years ago) (Heie, 1994; Richards, 1966; Shcherbakov and Wegierelc, 1991; Stadler and Dixon, 2005). The oldest known aphid fossil is of the species *Triassoaphis cubitus* Evans from the Triassic (Johnson et al., 2001). The number of species was small, but increased considerably with the appearance of angiosperms. The diversifying angiosperms allowed aphids to specialize, leading to the evolution of their numerous species. Organs such as the cornicles did not appear until the Cretaceous period (Heie, 1994).

5. Relations with Ants

Some species of ant "farm" aphids, transport them from plant to plant, protect them on the plants they eat, and feed on the honeydew that the aphids release from the termination of their alimentary canals. In many cases, the ants stimulate the aphids to secrete honeydew (see Fig. 5).

The trophobiotic ant–aphid relationship is a classic example of a mutualistic interaction, with a number of different levels of mutual dependence (Cherix, 1987; Hölldobler and Wilson, 1990; Matsuura and Yashiro, 2006; Nixon, 1951;

Figure 5. Aphids and worker ants in the process of "milking" their aphid herd; they ferociously guard these 'cattle' herds from any attack by predators, such is the value these "farmers" place upon their livestock. http://www.anthillwood.co.uk/antfarmers.htm.

Offenberg, 2001; Stadler and Dixon, 1999, 2005; Stadler et al., 2003). Based on their relationship with ants, aphids are divided into two main groups: myrmecophilous (tended by ants) and nonmyrmecophilous (unattended). As predators and aphid tenders, ants have a great influence on aphid fauna. The myrmecophilous species of aphids excrete sugar-rich honeydew and possess specific adaptations for interacting with ants (Nixon, 1951; Stadler and Dixon, 2005).

Myrmecophilous aphids and coccids show behavioral and structural modifications to life with ants. When an ant encounters such an insect, it usually strokes it with its antennae. This induces the aphid or coccid to suppress its usual defensive behavior of kicking out, running away, dropping off the plant, or clamping down. Instead, it raises its abdomen and exudes droplets of honeydew, which the ants then imbibe. Ants gain food from the association, for honeydew is rich in carbohydrates and also contains some amino acids, amides, proteins, minerals, and B vitamins (Carroll and Janzen, 1973; Hölldobler and Wilson, 1990; Way, 1963). At times, ants also gain protein, by preying on aphids or coccids. Benefits to the aphids or coccids include improved hygiene via removal of cast skins, dead aphids, and honeydew (Banks, 1958; Seibert, 1992; Way, 1954); direct increases in development rate, adult body size, fecundity, and reproductive rate (Banks, 1958; El-Ziady, 1960; El-Ziady and Kennedy, 1956); and protection from enemies (Banks, 1962; Bartlett, 1961; Jiggins et al., 1993).

Honeydew is the reason that ants are associated with aphids. In fact, many species of ants are so addicted to this sweet drink that they will protect the aphids from various predators and move them to new plants if the one they are on starts to wilt (Skinner and Whittaker, 1981; Vepsäläinen and Savolainen, 1994). The benefits and costs of these relationships for aphids are closely associated with the behavior of the ants. Some ants even go as far to build small shelters over species that feed near the base of the plant and to keep root-aphids inside their own nests (Jahn et al., 2005; Way, 1963). The ant *Lasius fuliginosus* workers carry newly hatched aphid fundatrices (parthenogenetic viviparous female aphids emerging in spring from the overwintered eggs and giving birth to one or more generations of fundatrigeniae (which succeed one another on the primary host)) from the base of oak trees where they overwinter as eggs, to new-growing leaves at the top of the tree, as soon as they hatch. The common meadow ant *Lasius flavus* has a particularly close relationship with the root aphids as it even collects their eggs in the autumn and early winter and stores them in its nests; then, in spring, the eggs are moved to suitable chambers so that plant roots are available for them as soon as they hatch. In effect, these ants treat the aphids as well as they treat their own brood (Anderson and McShea, 2001). Matsuura and Yashiro (2006) investigated the behavior of ants kept with aphid eggs in Petri dishes to examine whether the ants recognize the aphid eggs and tend them or only provide a refuge for the aphids. Workers carried almost all of the aphid eggs into the nest within 24 h. The ants indiscriminately tended the aphid eggs collected from their own colonies and from other ant colonies. The ants cleaned the eggs and piled them up in the nest. Egg tending by ants dramatically increased aphid egg survival rates. Starving the ants showed no significant effect on aphid-egg survivorship. Without the ants, aphid eggs were rapidly killed by fungi.

The interaction between aphids and ants has been going for a long time. Some ants are almost dependent on aphids for food while others, such as *Protrama* spp., are obligate myrmecophiles, and do not excrete honeydew unless stimulated to do so by ants (Cherix, 1987; Skinner and Whittaker, 1981).

The number of ants associated with a given species of aphid and the number of aphid species associated with a given species of ant varies from place to place: up to 17 different aphid species have been found in *Lasius niger* nests. Sometimes, different ant species living in similar habitats will foster different aphid species, i.e., *Lasius niger* with *Anoecia corni* and *Lasius flavus* with *Anoecia nemoralis*. However, aphid species that have evolved close relationships with ants may have broader environmental tolerances than their hosts, hence, the aphid *Forda formicaria* is attended by *Lasius* spp. in the lowlands and by *Formica* spp. in the highlands. Ants gain much nutrition from their relationships with aphids and the honeydew excreted by them, in some cases such as *Aulacorthrum* sp. can suffice as their complete food. Generally, the amount of proteins and amino acids excreted in honeydew varies among aphid species and host plant. However, as ant attendance stimulates aphids to feed at a rate 2–3 times their normal rate, far beyond the concomitant increase in growth rate, it follows that the reduction in nutrients resulting from aphid digestion is greatly lessened. Also, as adult ants do not need proteins but only sugars, honeydew is a perfect food for them. Ants are not always "good guys" though, like us with cows: if aphid numbers build up, the ants quite happily kill a few off and feed them to their larvae (Dixon and Hemptinne, 2001). It is known that the behavior of ants towards their aphid partners can depend on the availability of alternative food sources (Offenberg, 2001).

The protection that ants give the aphids they tend is not always efficient, and varies according to the type of predator or parasite. Generally, the ants are better at dealing with ladybird larvae and anthocorid bugs than with lacewing and hoverfly larvae. They not only remove the larvae, sometimes killing them, but also remove the eggs of hoverflies and ladybirds (Dixon and Hemptinne, 2001).

An interesting variation in ant–aphid relationships involves lycaenid butterflies (such as the Sievers blue butterfly and the Japanese copper butterfly) and the Myrmica ants. For example, *Niphanda fusca* butterflies lay eggs on plants where ants tend herds of aphids. The eggs hatch as caterpillars, which feed on the aphids. The ants do not protect the aphids from the caterpillars, but carry the caterpillars to their nest. In the nest, the ants feed the caterpillars, which produce honeydew for the ants. When the caterpillars reach full size, they crawl to the colony entrance and form cocoons. After two weeks, butterflies emerge and take flight (Stadler and Dixon, 2005).

Aphids with a conspicuously high reproductive rate by parthenogenesis frequently form a large colony on a host plant. Thus, they need to disperse to new host plants when the host plant quality declines because of their feeding on it. In many aphid species, a wing polymorphism is found, and alatae (winged forms (are produced under conditions of low host-plant quality (Dixon and Glen, 1971; Mittler and Sutherland, 1969; Sutherland, 1969b) or high aphid density (crowding) (Johnson, 1965; Lees, 1967; Mittler and Kurkel, 1971; Shaw, 1970; Sutherland, 1969a; Sutherland and Mittler, 1971). Furthermore, it has been reported recently that alatae are also produced in response to the presence of natural enemies (Dixon and Agarwala, 1999; Kunert and Weisser, 2003; Sloggett and Weisser, 2002; Weisser et al., 1999).

To elucidate the potential for colony growth and the dispersal of aphids in relation to ant attendance, mobility, tolerance of starvation, colony growth, and dispersal were examined by Tokunaga and Suzuki (2008) in the ant-tended *Aphid craccivora Koch* and the non-ant-tended *Acyrthosiphon pisum* Harris, under the absence of predators and ants. The differences in ant attendance have been influential in the development of morphological and behavioral traits concerning dispersal ability, and consequently, different potentials for reproduction and dispersal have developed in ant-tended aphids and non-ant-tended aphids (Tokunaga and Suzuki, 2008).

The ant-tended *Aphid craccivora* can gain protective service from ants attracted to its honeydew (Katayama and Suzuki, 2002, 2003). In spite of this loose relationship, *A. craccivora* aphids rarely show escape responses in the presence of their natural enemies such as ladybird larvae, even when ants are absent. On the other hand, *Acyrthosiphon pisum* aphids, which are not tended by ants, tend to drop from a host plant to escape predation (Losey and Denno, 1998; Nault et al., 1973).

Among such behavioral traits, one of the major responses is dropping from the host plant, triggered by an alarm pheromone (Bower et al., 1972; Chau and Mackauer, 1997; Dill et al., 1990; Dixon, 1998; Edwards et al., 1973; Losey and Denno, 1998; Montgomery and Nault, 1977; Nault et al., 1973; Roitberg and Myers, 1978). However, dropping has great potential costs (Dill et al., 1990), such as risks of mortality from desiccation on the ground (Dill et al., 1990; Roitberg and Myers, 1978, 1979) and from predation by ground-foraging predators (Bryan and Wratten, 1984; Loughridge and Luff, 1983; Winder, 1990).

Pasteels (1976, 2006) listed the chemicals that regulate the interactions between aphids, ladybirds, and ants, illustrating the astonishing refinement of some regulatory mechanisms. They were classified as pheromones (a chemical compound produced and secreted by an animal, which influences the behavior and development of other members of the same species), allomones (a defensive chemical produced by a plant: a chemical substance produced by a plant in response to attack by other organisms), kairomones (a chemical substance produced and released by a living organism that is beneficial to the receiver and disadvantageous to the donor), or synomones (a mutually beneficial signal chemical: a chemical released by members of one species that affects the behavior of another species and is beneficial to both parties), according to whether or not partners were of the same species, and how benefits and losses were distributed between partners (Dicke and Sabelis, 1988).

The use of chemical signals allows aphid parasitoids or ladybirds to squeeze into ant–aphid mutualistic associations (Pasteels, 2006).

6. Mutualism with the Intracellular Bacteria *Buchnera aphidicola*

Most aphids host intracellular bacteria of the genus *Buchnera*. The bacteria are transmitted "vertically" to the offspring via the aphid ovary, and the association is obligate for both the partners: bacteria-free aphids grow poorly and produce few or no offspring, and *Buchnera* are unknown apart from aphids and apparently unculturable (Douglas, 1998, 2003; Moran and Degnan, 2005). The biology of *Buchnera* has been reviewed by Douglas (2003). The key information relevant in the present context is that *Buchnera* sp. is a coccoid proteobacterium that dominates the microbiota of aphids, accounting for >90–99% of all microbial cells in the aphid tissues. *Buchnera* is obligately intracellular, restricted to the cytoplasm of specialized insect cells (Fig. 6).

Mutualistic bacterial symbionts play a central role in host ecology by provisioning rare nutrients and thus enabling specialization on restricted diets (Baumann et al., 1997). Among such symbionts, genomic studies are most advanced for *Buchnera*, the obligate symbiont of aphids, which feed on phloem sap. The contents of the highly reduced *Buchnera* genomes have verified their role in aphid nutrition. The bacterium synthesizes essential amino acids that lack in the aphid diet. In addition, thereby, the bacteria serve as a "sink" for excess ammonia, and without it, concentrations of enzymes that participate in the acceleration of the evacuation of ammonia in the aphid would increase, resulting in the loss of this essential building block of amino acids and proteins (Douglas, 1998).

The intracellular location of the symbionts requires that the host supplies the bacteria with energy, carbon, and nitrogen. One amino acid, glutamine, is

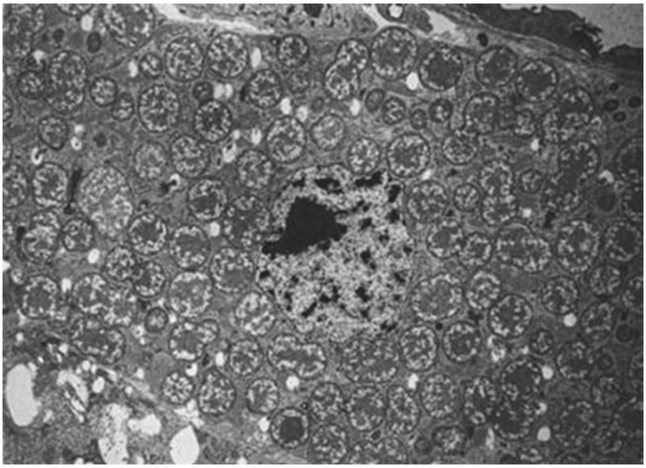


Figure 6. Symbionts (*Buchnera aphidicola*) within a bacteriocyte of a pea aphid (*Acyrthosiphon pisum*). The central object is the host nucleus. Buchnera cells are round and packed into the cytoplasm.

found in the phloem. It was shown that glutamine is ingested by the aphid and transported to the cells in which the symbionts are housed (bacteriocytes). In the bacteriocytes, glutamine is converted to glutamic acid, which, in turn, is taken up by the bacterial symbiont. The nitrogen from glutamic acid is then used through transamination, to synthesize other amino acids, which are ultimately utilized by the host animal. This cycling of amino acids permits the growth and reproduction of aphids (Douglas, 1998).

The symbiont genome also reflects this biosynthetic activity. *Buchnera aphidicola*, the symbiont of the aphid *Schizaphis graminum*, carries a plasmid of two genes that are important in tryptophan synthesis. Each bacterium contains three or four such plasmids, each containing four tandem repeats of these genes (Wernegreen and Moran, 2001).

The sequence of the symbiont genome was determined. Its analysis revealed that bacteria carried the genes required for the biosynthesis of amino acids that the host could not synthesize, but lacked those needed for the biosynthesis of non-essential amino acids (Baumann et al., 1999). The symbiont also lacked many other genes that are commonly found in free-living or facultative intracellular bacteria. This suggests that the symbionts and host have coevolved to such an extent that they can only live in each other's presence (Baumann et al., 1999; Shigenobu et al., 2000).

Until recently, virtually all research on the microbial symbiosis in aphids have focused on the γ-proteobacterium assigned to the genus *Buchnera* (Gherna et al., 1991). The accessory bacterial taxa generally have a broader tissue distribution in aphids than *Buchnera*, and can be transmitted among aphids both vertically and horizontally (Duchaud et al., 2003; Hypsa and Dale, 1997; Lefevre et al., 2004; Subandiyah et al., 2000; Swofford, 1998). The pea aphid, *Acyrthosiphon pisum*, has also been reported to bear Spiroplasma (Maddison and Maddison, 2000), Erwinia (Montllor et al., 2002), and *Staphylococcus* spp. (Mira and Moran, 2002).

The interaction of the two partners dates back 150–250 million years, and both have become so dependent on each other that, under natural conditions, they cannot exist independently (Douglas, 1998). Before 150–250 million years ago, when this mutualism was probably established, a *Buchnera* ancestor infected an aphid ancestor. An important aspect in the evolution of this symbiosis is the vertical transmission of the symbionts (from parent to offspring), indicating coevolution of the bacteria and host. This coevolution is apparent in the similarity between the branching patterns of the aphid phylogenetic tree and those of the symbionts (Baumann et al., 1995b).

7. Relationships with Predators and Parasites

Aphids are relatively common and, in the right circumstances, they occur in large numbers. For this reason, there are quite a number of animals that use them as a food source. Many specialize on aphids, while others are general predators that take advantage of aphid colonies when they can.

Figure 7. Aphids in different stages (sloughs, young and adults). Ladybird (adult and egg), larva of hoverflies (Syrphinae) feed on aphid.

Aphids, except scale insects, are soft-bodied, and have a wide variety of insect predators (Fig. 7). Insects that attack aphids include predatory ladybird beetles (Coleoptera: Coccinellidae) (Sato et al., 2008), hoverfly larvae (Diptera: Syrphidae), parasitic wasps (Oliver et al., 2003), aphid midge larvae, aphid lions (Williams, 1999), crab spiders (Eubanks and Denno, 1999), and lacewings (Neuroptera: Chrysopidae) (Dixon, 2000; Elliott et al., 2002; Sato et al., 2008).

Aphids are also often infected by bacteria (Lacey and Shapiro-Ilan, 2008), viruses (Agarwala et al., 1998), and fungi. Fungi that attack aphids include *Neozygites fresenii*, *Entomophthora*, *Beauveria bassiana*, *Metarhizium anisopliae*, and entomopathogenic fungi such as *Lecanicillium lecanii* (Shah and Pell, 2003). Aphids brush against the spores, which then stick to the aphid, germinate, and penetrate the aphid's skin. The fungus grows in the aphid's hemolymph. After about 3 days, the aphid dies and the fungus releases more spores into the air. Infected aphids are covered with a woolly mass that progressively grows thicker until the aphid is obscured. Often, the visible fungus is not the type of fungus that killed the aphid, but a secondary one (Brust, 2006) (Fig. 2).

The scale insects' waxy covering makes them quite resistant to pesticides, which are only effective against the first-instar nymph *crawler* stage (Levitin and Cohen, 1998).

The cabbage aphid (*Brevicoryne brassicae*) stores and releases chemicals that produce a violent chemical reaction and a strong mustard-oil smell in order to repel predators. Aphids are also known to defend themselves from attack by parasitoid wasps by kicking (Pontoppidan et al., 2001).

Some species of aphids interact with plant tissues, forming a gall (Akimoto, 1985), an abnormal swelling of plant tissue. Aphids can live inside the gall, which provides protection from predators and external elements. A number of galling aphid species is known to produce specialized “soldier” forms, sterile nymphs with defensive features that defend the gall from predator invasion (Brust, 2006; Stroyan, 1997).Galls play a major role in improving the quality and quantity of phloem sap by drawing photosynthesized products from the surrounding leaves to the gall (Inbar et al., 1995; Larson and Whitham, 1991).

In *S. chaetosiphon* galls, *R. insertum* colonies propagated more rapidly and the second generation grew larger and more fecund than on ungalled leaves. The amount of amino acids exuding from cut galled leaves was fivefolds of that in ungalled leaves; however, there was no significant difference in the amino acid composition between galled and ungalled leaves. Koyama et al. (2004) suggested that *S. chaetosiphon* galls function to promote the breakdown of leaf protein, leading to an increased performance of gall-inhabiting aphids (about galls, see Raman, present volume).

8. Relationship with Humans

Aphids include some destructive pests of agricultural crops, such as citrus (Fig. 8), coconut, grapevine, mango, oil palm, deciduous fruit trees, forest trees, and ornamentals. In recent years, it has become increasingly obvious that introduced species have a major economic and aesthetic impact on plants (Miller et al., 2002). Millions of dollars of agricultural losses are attributed directly and indirectly to aphids every year. Damage is not only related to their direct mechanical feeding and subsequent transmission of numerous plant diseases, but is also tied to the enormous costs for pesticide application for aphid control, the development of resistant crops, and changes in farming practices (Miller et al., 2006). On the other hand, several species are beneficial insects, as they produce components of economic importance (Ben-Dov, 2005).

Figure 8. Right: *Lapidosaphes beckii* on citrus leaves. Left: Scale insects secrete waxy scales that serve as a protective covering.

8.1. DESTRUCTIVE PESTS OF AGRICULTURAL CROPS

About 10% of ~4,400 aphids' species are pests, but only about 250 are considered serious pests (Rutledge et al., 2004). Perhaps, the most important aphid pest is *Myzus persicae*, the peach–potato aphid. *Myzus persicae* is a green or slightly reddish aphid, which has peach as its primary host and a wide range of secondary hosts, including many Brassicas. *Myzus persicae* is cosmopolitan in temperate climates, occurring in USA and a fair portion of Europe, including the UK. Though it seldom occurs in numbers large enough to cause direct damage from feeding pressure, it is capable of transmitting/spreading over 100 viruses, including the potato leaf roll and Y viruses of potatoes, mosaic, yellow net, and yellows viruses of sugar beet, cauliflower mosaic, plum pox, cucumber mosaic, lettuce mosaic, and turnip mosaic (Pickett et al., 1997; Rutledge et al., 2004).

Some additional serious aphid pests are: the black bean aphid *Aphis fabae,* which is a major pest that can, if unchecked, cause losses of up to 46% in broad bean crops. Its primary host is the spindle tree *Euonymous europaeus*. It does not reach epidemic levels every year, and it is possible to forecast the likelihood of infestation and the need to spray pesticides by monitoring the numbers of aphids flying away from the primary host in spring. For this to work properly, numerous suction traps need to be operated around the country. Forecasting likely pest levels is an important science because it can greatly reduce the amount of pesticides that need to be applied (Banks, 1958, 1962; Jiggins et al., 1993).

The pea aphid *Acyrthosiphum pisum* is a large green aphid found on many leguminous plants. It transmits lucerne mosaic virus, pea leaf roll virus, pea enation mosaic virus, and pea mosaic virus in the UK and pea enation mosaic virus in the USA. The pea aphid has only one host; in colder areas, eggs are laid by sexually mated females in overwintering forage crops and hatch early in the spring to find new colonies, which, as they grow in size, produce winged individuals that disperse to other crops. In warmer climes, overwintering is by asexual females and no sexuals occur (Sloggett and Weisser, 2002).

The cabbage aphid *Brevicoryne brassicae* is a serious pest of the major cabbage crops: cabbages, cauliflowers, and brussel sprouts. Like the pea aphid, the cabbage aphid is a one-host species spending all its life on Brassicas. The main overwintering form is eggs from sexual matings, but, in warmer years, asexual females may successfully overwinter as well. The eggs hatch in February or March, hence, the species gets off to an early start. The main cause of its pest status is the transmission of the virus' cauliflower mosaic and turnip mosaic (Pontoppidan et al., 2001).

Plants with aphid damage can exhibit a variety of symptoms, such as decreased growth rates, mottled leaves, yellowing, stunted growth, curled leaves, browning, wilting, low yields, and death. The removal of sap creates a lack of vigor in the plant, and aphid saliva is toxic to plants. Aphids frequently transmit disease-causing organisms such as plant viruses to their hosts. Cotton aphids (*Aphis gossypii*) often infect sugarcane, papaya, and groundnuts with viruses (McGavin, 1993).

Aphids contributed to the spread of late blight fungus (*Phytophthora infestans*) among potatoes in the Great Irish Potato Famine of the 1840s (Nichols, 2007). The coating of plants with honeydew can contribute to the spread of fungi that can damage plants (Reynolds and Volk, 2007). Honeydew produced by aphids has been observed to reduce the effectiveness of fungicides by obstructing their absorption (Dika and Van Pelt, 1992). The damage to plants, and in particular, commercial crops, has resulted in considerable resources and efforts being spent attempting to control aphid activities (McGavin, 1993). The study of chemical ecology, particularly involving pheromones and other semiochemicals (chemical control of behavior) that influence insect behavior, promises methods of pest control as alternatives to the exclusive use of broad-spectrum toxicants. Semiochemicals, when employed alone, often give ineffective or insufficiently robust pest control. Use of semiochemicals should therefore be combined with other approaches in integrated management strategies. The main components of such strategies are pest monitoring to allow accurate timing of pesticide treatments; the combined use of semiochemicals, host-plant resistance, attraction of pests to traps, and manipulation of pest behavior; and selective insecticides or biological control agents to reduce pest populations. The objective is to draw together these approaches into a push–pull or stimulo-deterrent diversionary strategy (SDDS). In an SDDS, the harvestable crop is protected by host-masking agents, repellents, anti-feedants, or oviposition deterrents. At the same time, aggregative semiochemicals, including host–plant attractants and sex pheromones, stimulate colonization of pests on trap crops or entry into traps where pathogens can be deployed. Because the individual SDDS components are not, in themselves, highly efficient, they do not select for resistance as strongly as conventional toxicant pesticides, thereby making the SDDS intrinsically more sustainable (Pickett et al., 1997).

The current control of aphid pests still involves large amounts of pesticides in some countries, but other more ecologically friendly methods have been used in other places for some time. These generally involve biological control, mostly with a range of Hymenopteran parasites, genetic manipulation of crops for aphid resistance, and forecasting to allow for pre-emptive spraying in smaller amounts. These methods are being studied and will be more functional once detailed information is made more easily available to the forecasters. Also, it is important that farmers be educated on the alternatives to chemical sprays.

8.2. BENEFITS TO HUMANS

Some types of aphids are economically valuable, such as the cochineal (Fig. 9), Polish cochineal, and lac scales (Fig. 9). The cochineal insect yields a dye (Mendez et al., 2004) and the lac insect is a source of shellac (Miller and Kosztarab, 1979; Miller et al., 2004).

Cochineal, a red or purple dye obtained from the dried females of the coccid *Dactylopius coccus* Costa, is an insect living on cladodes of prickly pears

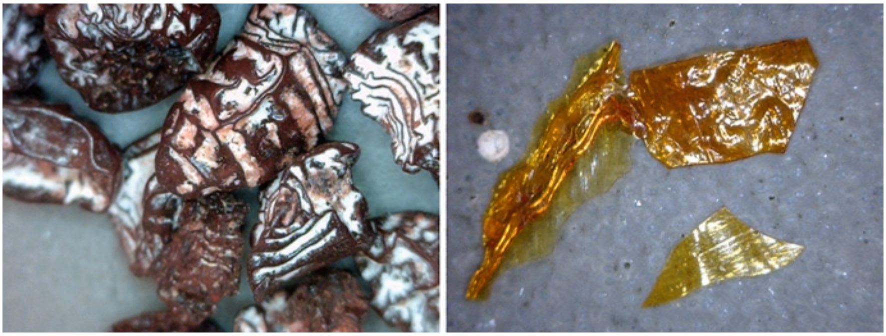


Figure 9. Dry Cochineal aphid, *Dactylopius coccus* Costa (left) and shellac from lac aphids (right).

(*Opuntia ficus indica*). The dye was formerly used as a food coloring, and for this purpose, has been largely replaced by synthetic dyes. It is still a source of carmine and is currently used as a dye in cosmetics, medicines, and biological-laboratory staining fluids (Mendez et al., 2004). *Dactylopius coccus* is native to tropical and subtropical South America and Mexico, where their host cacti grow natively. During the colonial period, it was in high demand. It was so economically important that it generated the third highest income for New Spain after gold and silver exportations (Brana, 1964). In recent years, it has become commercially valuable again, though most consumers are unaware that the phrases "cochineal extract," "carmine," "crimson lake," "natural red 4," "C.I. 75470," "E120" (Chimenos et al., 2003), and even "natural coloring" refer to a dye that is derived from an insect. One reason for its popularity is that, unlike many commercial synthetic red dyes, it is not usually toxic or carcinogenic (Barayovits, 1978; Sandberg, 1997). However, as of 2005, the market price of cochineal was between US $50 and 80 per kg, while synthetic raw food dyes are available at prices as low as US $10–20 per kg (Sandberg, 1997).

Today, it is used as a fabric and cosmetics dye, as a natural food coloring, and for oil paints, pigments, and watercolors. Natural carmine dye used in food and cosmetics can render it unacceptable to vegetarian (or vegan) consumers, and many Muslims and Jews consider carmine-containing food forbidden (*haraam* and *treif*, respectively) because the dye is extracted from insects (Mendez et al., 2004; Sandberg, 1997).

Carmine is one of the very few pigments considered safe enough for use in eye cosmetics. A significant proportion of the insoluble carmine pigment produced is used in the cosmetics industry for hair- and skin-care products, lipsticks, face powders, rouges, and blushes. A bright red dye and the stain carmine used in microbiology are also often made from the carmine extract. The pharmaceutical industry uses cochineal to color pills and ointments (Sandberg, 1997). Nevertheless, at least some people have been reported to have negative responses to it. Therefore, when used as a food additive, the dye must be mentioned on packaging labels.

Sometimes, carmine is labeled as *E120*. An unknown percentage of people have been found to have allergies to carmine, ranging from mild cases of hives to atrial fibrillation and anaphylactic shock. Carmine has been found to cause asthma in some people. Cochineal is one of the colors that the Hyperactive Children's Support Group recommends be eliminated from the diet of hyperactive children.

Lac is a resinous substance produced by the lac insect and used to make shellac. Shellac is used as a finish and for making lacquer and polishes. It is also used to stiffen felt hats and as an electrical insulator. Lac may be formed into cakes or sheets. The cakes are called button lac or garnet lac (Azouka et al., 2007). The lac insect is *Laccifer lacca* of the superfamily Coccoidea.

Infestation of a variety of Chinese trees by Chinese sumac aphids (*Melaphis chinensis Bell*) can create a "Chinese gall," which is valued as a commercial product. As "Galla Chinensis," these galls are used in Chinese medicine to treat coughs, diarrhea, night sweats, dysentery, and to stop intestinal and uterine bleeding. Chinese galls are also an important source of tannins (Stroyan, 1997).

9. Aphids in the Bible

Utilization of natural colors occupied an important place throughout ancient cultures, including those of Egypt, Mesopotamia, Greece, and Rome. The "Scarlet Dye" – "shani" in Hebrew – was used in the Holy Land during the Biblical period. According to the descriptions of Roman authors (e.g., Dioscorids IV, 48; Plinius XVI, 12, 32) and later illustrations, there is no doubt that we are dealing with a dye produced from a coccid aphid species (Donkin, 1977). Most researchers believe that the source of the original Scarlet Dye is the Armenian cochineal or *Kermococcus vermilis* (*Kermes vermilis*) (Donkin, 1977; Sandberg, 1997), and, therefore, the dye must have been imported because this species was not found in the Holy Land. Recently, Amar et al. (2005) have identified the dye produced from the local *Kermis echinatus* (Fig. 10), a parasite of the common Israeli oak *Quercus calliprinus* as the "shani" used during the Second Temple period (ca. 100 BC–AD 70).

In the Old Testament, the scarlet dye was mentioned 25 times, alone or along with other precious and expensive pigments (e.g., in the book of Exodus and the second book of Samuel 1, 24), including the blue and purple dyes obtained from marine snails. Shani was used in the Holy Land during the Biblical period for both secular (such as coloring expensive weaves) and ritual (e.g., in the temple for leper purification rites and as a component of the purifying ritual with red cow ash) purposes (Leviticus 14, 6: Numbers 19, 6).

Identity of the "Manna" that the Israelites ate in the desert engaged the imagination of many researchers (Ben-Dov, 1988; Bodenheimer, 1947; Danin, 1972; Schmitschek, 1980) in last few hundred years. Is it possible that the phenomenon mentioned in the Bible, i.e., "Bread dropped from the sky" in the Sinai Desert, is the "manna" produced by coccids?

Sweet exudations come from insects (*Aphidida*, *Coccida*, *Psyllida*, *Coleoptera*) or plants (fungi, lichens, trees). Schmitschek (1980) suggested that the bread

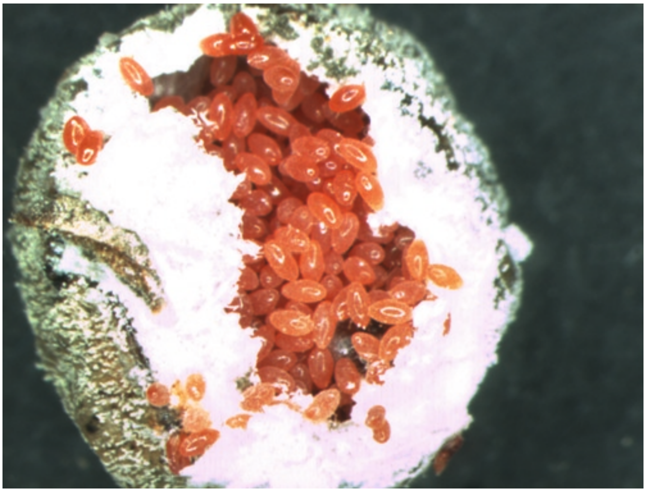


Figure 10. The coccid scale *Kermes echinatus* with eggs.

dropped from the sky in the Sinai Desert, as described in the Bible, was manna produced by coccids, particularly *Trabutina mannipara* Ehr. that grows on tamarisk (*Tamarix nilotica*). The mealybug *Trabutina mannipara* (Hemprich and Ehrenberg, 1829) was described and illustrated (Ben-Dov, 1988), and a neotype female was selected from topotypic material collected in the Sinai Peninsula. A lectotype female was designated for *Trabutina palestina* (Bodenheimer, 1947), which is shown to be a junior synonym of *T. mannipara* (Ben-Dov, 1988).

In the Mideast, people still collect the sweet excretions of scale insects that feed on tamarisk. They call it "man" and it is, most likely, the manna described in the Old Testament (Hölldobler and Wilson, 1994).

10. Summary

Aphids belong to the suborder Sternorrhyncha, which comprises the aphids, whiteflies, psyllids, and scale insects, groups which are included in the order Hemiptera. They probably had already appeared around 280 million years ago, in the early Permian era. All these taxa feed on phloem sap, thus, several species are major crop and ornamental plant pests. Many exhibit adaptations to parasitism, such as modified morphology and/or lifecycles, flightless morphs, parthenogenesis, sexual dimorphism, and eusociality. Aphids are adversely affected by inclement weather phenomena such as precipitation, temperature, and wind.

Aphids have diverse relationships with other organisms; the ant–aphid relationship is a classic example of a mutualistic interaction. Aphids are divided into two main groups: myrmecophilous (tended by ants) and nonmyrmecophilous (unattended). As predators and aphid tenders, ants have a great influence on the aphid fauna. The myrmecophilous aphids excrete sugar-rich honeydew and possess specific adaptations for interacting with ants. Another mutualism is with the intracellular bacterium *Buchnera aphidicola*. *Buchnera* is obligately intracellular, restricted to the cytoplasm of specialized aphid cells, and plays a central role in host ecology by provisioning rare nutrients and thus enabling specialization on restricted diets. Aphids have also developed commensalismic relationships with plant tissues, forming protective galls.

Aphids have a wide variety of insect predators; ladybird beetles, hoverfly larvae, parasitic wasps, aphid midge larvae, aphid lions, crab spiders, and lacewings. They are also often infected by bacteria, viruses, and fungi.

Relationship with humans can be useful in a few specialized cases; some types of aphids are economically valuable, such as the cochineal, Polish cochineal, and lac scales. On the other hand, aphids are among the most destructive agricultural pests on Earth, and are major vectors in the spread of viral plant epidemics.

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Bio-data of **Anantanarayanan Raman**, author of ***"Insect–Plant Interactions: The Gall Factor"***

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INSECT–PLANT INTERACTIONS: THE GALL FACTOR

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1. Introduction

Insects[1] interact with plants as pollinators, vectors of microbes, and gall inducers. Consequent to pollinating role of insects, plants achieve a positive outcome (pollination, fertilization, and fruit set) and consequent to the action of vectorial insects, plants achieve a negative outcome (expression of a disease caused by the vectored microbe). Consequent to gall induction, plants experience a modest level of stress, whereas the inducing insects gain shelter and nutrition. The overall metabolism of the gall-bearing plants remains unaffected, except for the stress-related changes at and near the locations where galls develop (Raman et al., 2006). Gall-bearing plants, rarely, either suffer extensive damage or experience death. Recent outbreaks of gall-inducing *Leptocybe invasa* (Hymenoptera: Eulophidae) (Mendel et al., 2004; Jacob and Kumar, 2009) on diverse species and subspecies of *Eucalyptus* (Myrtaceae) and that of the gall-inducing *Quadrastichus erythrinae* (Hymenoptera: Eulophidae) (Kim et al., 2004; Yang et al., 2004; Faizal et al., 2006; Uechi et al., 2007) on different species of the garden ornamental *Erythrina* (Fabaceae) in different parts of the world are evoking serious concern. Cursory evaluations by managers of forests and recreational gardens indicate that *L. invasa* and *Q. erythrinae* affect the performance and productivity of their respective hosts, similar to what has been shown with *Ophelimus eucalypti* (Hymenoptera: Eulophidae) infesting *Eucalyptus saligna* (Myrtaceae) in New Zealand (Withers et al., 2000; Raman and Withers, 2003; La Salle, 2005). Heavy build-up of populations of *Q. erythrinae* has been recorded to induce death of trees of *Erythrina* in Hawaii (Heu et al., 2006). One established example of a gall-inducing insect being a serious pest of rice is *Orseolia oryzae* (Diptera: Cecidomyiidae), the Asian

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*Fondly dedicated to Odette Rohfritsch, my teacher and mentor, who upholds and champions the Küster – Maresquelle – Meyer legacy in the study of galls.

[1] The term ‘insects’, in this chapter, occasionally, would refer to eriophyid mites as well.

Figure 1. "Silver shoots" (galls, the pale, tubular structures on the shoot terminals) on *Oryza sativa* (Poaceae) induced by *Orseolia oryzae* (Diptera: Cecidomyiidae). (Photo courtesy: Dr K L Heong, International Rice Research Institute, Manila, The Philippines.)

rice gall midge, which induces silver shoots (galls) on rice shoots in many Asian countries (Fig. 1). In India alone, *O. oryzae* has been recorded to inflict an average annual yield loss of 500,000 ts of grain (0.8% of the total production; value *c.* US$80 m) (Bentur et al., 2003; Vijaya Lakshmi et al., 2006). *Orseolia oryzivora* (Diptera: Cecidomyiidae), the African rice-gall midge, inflicts considerable damage to rice crop in Africa (Harris and Gagné, 1982; Nwilene et al., 2006). Several species of gall-inducing Cecidomyiidae (Diptera) affect the performance and productivity of *Mangifera indica* (Anacardiaceae) in mango-cultivating regions of the world (Raman 2007b), although not to the extent and magnitude of *O. oryzae* on *O. sativa*. Besides such negative relationships – measured in terms of damage to economically important plants and loss of the commercial product in such plants – gall-inducing insects are useful to humans, because the galls they induce are useful in medicine and industry, and even as food (see Raman et al., 2005a).

More importantly, because of the high level of specificity of gall-inducing insects to their hosts, many of them are currently being explored for their role as biological-control agents of invasive weeds (Florentine et al., 2001; Muniappan and McFadyen, 2005; Muniappan et al., 2009).

In the context of biological control, gall-inducing Lepidoptera (Raman and Dhileepan, 1999; Florentine et al., 2005; Raman et al., 2006) and Coleoptera (Florentine et al., 2002; Raman et al., 2007) are considered to be more efficient because of their tunneling habit in the axial organs of plants, than the gall-inducing Hemiptera and Diptera (Cecidomyiidae).

Galls have been a part of human culture and civilization (Berlin and Prance, 1978). In the medieval times, insect-induced galls were used in medicine and in the extraction of coloring materials; in the recent past, they have been used as a key source for tannins and in ink production (Williams, 1994). A useful summary under the headings "Injurious gall insects" (page 9) and "Certain galls have value" (page 10) by Felt (1940) are relevant even today. In short, considerable medicinal value is currently attributed to insect-induced galls; for example, those induced on the leaves of *Quercus infectoria* by *Cynips tinctoria* (Hymenoptera: Cynipidae), distributed from the Middle-East to Malaysian Peninsula, are being investigated for their potential anti-inflammatory capability and α-glycosidase inhibitory activity, and their role as efficient antioxidants (Hwang and Kong, 2000; Pin et al., 2006; Kaur et al., 2008).

Some species of *Acacia* (e.g., *A. collinsii*, *A. cornigera*, *A. sphaerocephala*) in tropical America, Africa, and Asia bear enlarged stipular spines that house colonies of ants (*Pseudomyrmex ferruginea*; Hymenoptera: Formicidae). These swollen spines occur in pairs resembling bull's horns and hence called "bullhorns" (Carroll and Janzen, 1973). The bullhorn Acacia – *Pseudomyrmex* interactions exemplify symbiosis: the ants protect *Acacia* from other herbivores and even snip leaves and shoot terminals of other plants that competitively grow on and around *Acacia*; *Acacia* not only shelter ants in their swollen spines, but also provide them with nourishment through nectar produced by the glands located at the leaf bases (Hocking, 1970). The bullhorn *Acacia* generates yellowish "pear"-shaped bodies, known as the Beltian bodies, at the tip of some of its leaflets, on which the ants feed. The Beltian bodies include cells rich in proteins and essential lipids (Palmer and Brody, 2007). The swollen spines of *Acacia* cannot be considered galls because these are genetically programmed structures and do not arise in response to either chemical or physical stimuli from *Pseudomyrmex*.

Only scanty information exists on the overall chemistry of galls. The inner gall, in a majority of insect-induced systems, that encloses the gall-inducing insect, is generally rich in lipids, proteins, and hydrolyzable sugars, whereas the outer gall would include secondary plant metabolites in plenty. In a range of galls induced by species of *Pontania* (Hymenoptera: Tenthredinidae) on different species of *Salix* (Salicaeae), a variety of phenolic compounds, such as flavonoids, salicylates, cinnamic-acid derivatives, condensed tannins, and nontannin phenolics occur (Nyman and Julkunen-Tiitto, 2000). That the tannin compounds play an inhibitory role on the digestive enzymes of the inhabiting larva and thus impact on the survival and growth of the gall-inducing insect (Cornell, 1983) is well known. Because insect-induced galls exist in natural conditions, they function as ideal microhabitats for pathogenic fungi and bacteria; it has been shown that galls induced by *Smynthurodes betae*, *Forda riccobonii*, and *Slavum wertheimae* (Hemiptera: Pemphigidae) arising on the leaves of *Pistacia atlantica* (Anacardiaceae) include high levels of pathogenesis-related (PR) proteins as a measure of self-defense against the air-borne microbial pathogens (Inbar et al., 2003). Elevated levels of sterols such as ponasterone A and ecdysterone occur in the galls induced by *Taxomyia taxi* (Diptera: Cecidomyiidae) on the shoot-apical meristems of *Taxus baccata* (Coniferales: Taxaceae) (Lovett, 1980). Gall induction entails amendments to pigmentation patterns as well; for example, in the galls induced by *Eriophyes tiliae* var. *rudis* on leaves of *Tilia cordata*, different carotenoids such as α-cryptoxanthin, isocryptoxanthin, lutein occur as novel compounds, while β-carotene was much lower in gall-bearing leaves than the normal leaves of comparable age and 4-keto-α-caroetene was much higher in gall-bearing leaves than the normal leaves (Czeczuga, 1975). Elevated levels of different minerals are also known in different galls induced by Hymenoptera (Bagatto and Shorthouse, 1997; Soon et al., 2007) and Lepidoptera (Florentine et al., 2005).

The ability to induce galls is a specialized habit within the broad context of insect phytophagy. This habit in insects is considered to have arisen from leaf-mining Diptera (e.g., Agromyzidae) and Microlepidoptera (e.g., Nepticulidae, Stigmellidae) (Raman et al., 2005a), which, over time, are purported to have sought "new" food sources in concealed, and therefore, "protected" environments (Crane and Jarzembowski, 1980). In such a context, the origin of leaf-mining habit from mycophagy needs to be factored (Labandeira, 1998). A contrasting proposition is that gall-inducing habit originated from tunneling Lepidoptera (e.g., Tortricidae) and Coleoptera (e.g., Curculionoidea) (Scott et al., 1994; Labandeira and Phillips, 2002). What is critical, however, will be to recognize that gall-inducing insect taxa, which remain scattered among diverse and unrelated natural orders, and which induce galls on host–plant species belonging to unrelated families, have arisen many times and through different evolutionary routes (Cook and Gullan, 2004; Korotyaev et al., 2005; Roskam, 2005). Physiological differences in the behavior of the inducing insect and susceptibility level of the plant have also contributed to the variety of galls we see today.

Gall-inducing ability among insects is mostly confined to species of Thysanoptera, Hemiptera, Coleoptera, Diptera, Lepidoptera, and Hymenoptera, and to those of Eriophyoidea in Acarina. Even within these orders, the capability to induce galls does not occur uniformly in all species. For example, in Thysanoptera, gall-inducing capability occurs among taxa belonging to Tubulifera and almost none in the Terebrantia (Ananthakrishnan and Raman, 1989). Gall-bearing plants also do not reveal any discernible pattern because galls induced by closely related insect taxa develop on plants of unrelated families. In the temperate latitudes, a majority of insect-induced galls occur on species of

Fagaceae, Salicaceae, Convolvulaceae, Malvaceae, Capparidaceae, Rosaceae, Asteraceae, and Euphorbiaceae (Meyer, 1987). In the tropical and subtropical world, species of Fabaceae, Moraceae, Lauraceae, Myrtaceae, Combretaceae, Dipterocarpaceae, Anacardiaceae, and Asteraceae bear the greatest numbers (Docters van Leeuwen-Reijnvaan and Docters van Leeuwen, 1926; Mani, 1964; Raman et al., 2005b).

2. Species Richness and Patterns of Adaptive Radiation

Global richness of gall-inducing insects is estimated at 133,000 species (Espírito-Santo and Fernandes, 2007). Gall-inducing insects are highly evolved taxa with sophisticated biology and physiology that enable them to utilize their host plants more efficiently and resourcefully than their nongall-inducing relatives (Raman et al., 2005a; Schaefer et al., 2005; Shorthouse et al., 2005). Although a majority of the gall-inducing insects are restricted to particular plant species, and even to specific plant organs (Abrahamson et al., 1998; Raman et al., 2005a), a few induce galls on plant species that are closely related to their preferred hosts (Raman, 1996; Raman et al., 1996; Gagné, 2004; Wool, 2005; Raman, 2007a). In the Cecidomyiidae, for example, most gall-inducing gall midges are either monophagous or narrowly oligophagous (Gagné, 2004); however, a few species of Asphondyliina and Schizomyiina have been found, in recent years, to be polyphagous (Yukawa et al., 2003; Uechi et al., 2002, 2004; Tokuda et al., 2005).

Host shifts in naturally occurring gall-inducing tephritid populations of North America and Europe have been explained by the evolution of sympatric host races – due to changes either in the preference in feeding and/or oviposition sites or by developing "new" physiological adaptations to new host plants or through assortative mating (Abrahamson and Weis, 1997; Korneyev et al., 2005). Differences in the temporally regulated flowering and leafing phenologies in susceptible host plants could also be playing a role in isolating gall-inducing insect populations, thus enabling divergence and diversification by genetic drift (Stone et al., 2001). The generic understanding, as of today, is that host shifts and radiation in gall-inducing insects are more complex than what has been understood in nongall-inducing insects. Such a complexity is attributed to composite relationships of gall-inducing insects with plants and the spread of gall-inducing insects through different biogeographical realms, influenced by the abundance and richness of plant species (Raman et al., 2009a).

Among the nearly 25 species of *Mangifera indica*-infesting Cecidomyiidae (Raman, 2007b), only *Procontarinia matteiana* induces galls of similar morphology on many varieties of *M. indica* in the Indian subcontinent, although the levels of susceptibility among the different varieties vary: 75% susceptibility in the variety "Alphonso"; 20% susceptibility in the variety "Benisan" (Jhala et al., 1987). *Procontarinia matteiana*, the only taxon that occurs in regions outside the Indian subcontinent, is known in South Africa and variations similar to those *M. indica* varieties in levels of susceptibility to *P. matteiana* infestation are known (Schoeman et al., 1996). Among the varieties of *M. indica* populations tested in South Africa for *P. matteiana* infestation, the varieties "Keitt," "Kent," and "Irwin" are partly susceptible manifesting aborted galls, most likely because of secondary resistance in these varieties causing mortality of early-instar larvae (Githure et al., 1998).

The biology of *P. matteiana* in two principal *M. indica*-growing bioregions (Indo-Gangetic plains, India, 25–27°N; South Africa 24–26°N) offers some insights into its capabilities to adapt to local climatic conditions and to demonstrate different behaviors (see Raman et al., 2009a), which is similar to the behavior of the gall-inducing *Andricus kollari* (Hymenoptera: Cynipidae) introduced into south-western Britain in the nineteenth century and now spread throughout the British Isles (Schönrogge et al., 1999). Although many of the behavioral traits are similar between the Indian and South-African populations of *P. matteiana*, the striking similarity is in the incidence of two annual generations (February–March; October–November), notwithstanding the fact that the locations are at identical latitudinal coordinates. However, the incidence of extended larval periods and delayed adult emergence in one of the three annual generations in the Indian populations of *P. matteiana* remains to be explained.

In brief, host shifts, and subsequent speciation, among the gall-inducing insects occur because of host–plant traits (e.g., host–plant chemistry), competitive interactions of the gall-inducing insect selecting the "best" host resources, and the potential for escape from natural enemies (Abrahamson et al., 1994). Yet, it is hard to list reasons why specific host shifts happen most of the time, especially because host shifts are common only among the related and closely related plants. Co-speciation and host tracking among host-shifting patterns, shown in gall-inducing species of *Kladothrips* (Thysanoptera: Phlaeothripidae) on species of *Acacia* (Mimosaceae) in Australia (McLeish et al., 2007) offer insights in this context. Further, Price's (2005) commentary on the patterns of adaptive radiation among gall-inducing sawflies of North America, cynipid gall wasps of Europe, gall midges of Japan, and aphids of the Middle-East sheds some clarity.

3. Gall and Its Induction

A gall is a cumulative expression of a suite of adaptations achieved by the host plant for accommodating the inducing insect. In principle, the gall provides nutrition and shelter to the inducing arthropod; in the galls induced by Eriophyoidea, Thysanoptera, and Hemiptera (Aphidoidea, Coccoidea), besides the gall-founding female, her progeny also finds shelter and nutrition, whereas among the gall-inducing taxa of the higher orders, Diptera and Hymenoptera, only a single immature will live in the gall. To realize a gall, the insect activates a perturbation in the growth mechanisms of the plant organ and alters the differentiation processes in that organ, thus modifying that organ's (or a part of) architecture to its advantage (Raman, 2003). A gall, which is induced by an insect, is invariably symmetrical – displaying either radial or bilateral symmetry. Classical definitions of a gall (e.g., Mani, 2000) would include any generic, anomalous growth on plants such as those incited by bacteria, fungi, nematodes, and such a definition would also include growths induced by insects. However, contemporary definitions of a gall would exclude plant growths incited by bacteria and fungi, because their actions normally result in amorphous growths, and therefore are to be called "tumors," whereas those induced by insects mostly result in symmetrical structures, and therefore, are "galls."

The capability to inhibit normal growth and differentiation and to induce new growth by establishing new polarity points and gradients in their host plants is common to all gall-inducing insects. Not only a vast majority of galls are symmetrical, but also they include novel patterns of differentiation (see Meyer and Maresquelle, 1983; Meyer, 1987). Since insects derive their nutrition from gall tissue, the gall becomes a sink for different nutrients and energy that will be used by the insect for its development (Raman and Abrahamson, 1995; Raman, 2003). A majority of gall-inducing insects stimulate the host–plant tissue to develop into galls by their feeding action (Rohfritsch, 1992; several chapters in Raman et al., 2005b), whereas species of Hymenoptera induce galls by oviposition (Bronner, 1973; Rey, 1992; several chapters in Raman et al., 2005b). Even the vascular tissues can be modified by gall induction, so that they supply nutrients and water subserving the needs of the inducing insect (Meyer, 1969a; Wool et al., 1999; Raman et al., 2006).

Insects belonging to the same order usually induce galls similar in shape and structure: for example, almost all the known species of *Gynaikothrips* (Thysanoptera: Phlaeothripidae) induce leaf-fold galls (Ananthakrishnan and Raman, 1989; Mound and Morris, 2005) and those of *Aceria* (Acarina: Eriophyidae) induce pouch galls on leaves (Channabasavanna, 1966; Oldfield, 2005). However, such a behavior is less common among gall-inducing species in the advanced families such as the Cecidomyiidae (Diptera) and Cynipidae (Hymenoptera) because a diverse range in gall morphologies exists even among those induced by different species of the same genus (Meyer, 1969b, 1987; Roskam, 2005; Yukawa and Rohfritsch, 2005; Csóka et al., 2005). The feeding action of insects (viz., insertion of mouth-parts and sucking by those of hemispheroid stock; biting and chewing by those of coleopteroid stock) and the secreted salivary chemicals are the two key factors that trigger growth of galls. Feeding action inflicts wound and the secretion of salivary chemicals alters the subcellular chemistry in host tissues; both of these, occurring concurrently, stress the host–plant cells. As a neutralizing response to the stress, the plant translocates different photoassimilates to the site where the insect feeds. Continued feeding activity by the insect induces the plant to respond, resulting in a gall.

A majority of the gall-inducing insects that bear minute and relatively short mandibles (e.g., Cecidomyiidae, Cynipidae, Eulophidae, Eriophyoidea, and Thysanoptera) generally induce galls by stinging 1–2 cells on the host–plant organs and initiating cell irritation, which responds with gall growth (see

Rohfritsch, 1980–1981). The neonate nymphs of *Trioza jambolanae* (Hemiptera: Psylloidea: Triozidae) insert their short stylets through stomatal aperture and initiate gall development on the leaves of *Syzygium cumini* (Myrtaceae) by feeding on the substomatal parenchyma (Raman, 1991). Gall-induction capability in Adelgidae (Hemiptera), shown in many European taxa (Meyer, 1962; Rohfritsch, 1966a, b, c, 1967, 1976, 1977), is uniquely different. The Adelgidae, by sucking and accompanied with salivary secretion, induce galls on the vegetative buds with "cavities" for the juveniles that include well-defined nutritive tissue, between primordial leaf bases (Meyer, 1987); Meyer (1987) indicates that the nymphs migrate, dictated by their "instincts," to the cavities that would develop in the "galled" bud. The gall-founding adult female of *Adelges cooleyi* (Aphidoidea: Adelgidae) upon settling on the vegetative buds of a hybrid spruce (*Picea glauca* x *P. engelmannii*) discharges a chemical stimulus, which gets transported over a distance of about 30 cm and modifies the vegetative bud to develop into a gall (Sopow et al., 2003). A strikingly different process occurs in the fir-cone-like galls induced on the vegetative buds of *Mangifera indica* (Anacardiaceae) by *Apsylla cistellata* (Hemiptera: Calophyidae: Apsyllinae) (White and Hodkinson, 1985) in the northern plains of India (22–28°N; 76–92°E) (Fig. 2) (Raman et al., 2009a). The feeding action of the first-instar nymphs of *A. cistellata* "triggers" gall development, although the nymph remains partly embedded within the egg shell and feeds on the same leaf where the mother oviposited (Singh et al., 1975). These galls develop through the modification of axillary buds as the first-instar nymphs feed on leaves and only the second-instar nymphs migrate to the already-established galls.

4. Gall Initiation and Growth

Gall development generally includes two phases: initiation and growth, the latter including increment in mass and qualitative differentiation. The earliest event in gall initiation is the isolation and insulation of one cell or a group of 2–3 cells in the attacked plant organ (e.g., leaf) from their normal course of differentiation (similar to the behavior demonstrated in vegetative plant cells designated to become reproductive cells; Bhojwani and Soh, 2001) inciting metaplasia in the one or 2–3 cells coming under the direct feeding or oviposition impact of the insect (Rohfritsch, 1978, 1980–1981; Meyer and Maresquelle, 1983; Raman et al., 2005b). A chemical "stimulus" – which could be either "high-molecular weight proteins" (Carango et al., 1988) or "bruchins" (Doss et al., 2000) or "mitogenic lipids" (Farmer, 2000) – from the insect triggers division activity in the metaplasied cell(s), which indicates the earliest visible sign of gall initiation. This stimulus continues to activate and regulate the novel patterns of differentiation that occur subsequently at the site of metaplasia (Harper et al., 2004). In the galls on *Rosa spinosa* (Rosaceae) induced by *Diplolepis spinosa* (Hymenoptera: Cynipidae), genes homologous to "nodC" ("nodC" is the gene known in *Rhizobium* group that

Figure 2. Fir-cone-like galls induced on the vegetative axillary buds of *Mangifera indica* by *Apsylla cistellata* (Hemiptera: Psylloidea: Calophyidae). (Photo courtesy: Dr B K Das, Bidhan Chandra Krishi Viswavidyalaya, Kalyani, West Bengal, India.)

induces nodules on legumes) have been characterized; as of now, our understanding remains that such genes possibly act as signal molecules in the interaction triggering gall induction (Schonrögge et al., 1998).

As in the known anomalous plant growths, be it a gall or a tumor, auxins (e.g., indole-acetic acid, IAA) play a key role (see Hori, 1992 for several classical references; Mapes and Davies, 2001a). An imbalance arising because of the stress induced by the physical action (viz., wounding, sucking) and salivary secretions either triggers new growth because of synthesis of growth promoters or enhances the vulnerability of plant cells to growth promoters that are already present at the induction site. Such an imbalance results in a "combined" function of different growth promoters (e.g. auxins and kinins, Byers et al., 1976), which triggers growth. However, the question "how symmetry in gall shape, either radial or bilateral, is achieved?" remains unanswered: not only that the "stimulus" from the insect spreads uniformly, but also that at some point of time, even while including a living insect, growth of the gall ceases. As of now, growth in galls, based on the "auxin" theory, is explained by two contrasting hypotheses: one by Hartley (1999) and another by Miles (1999), which explain the pathways of IAA synthesis in galls. The Hartley hypothesis (1999) builds on the premise that insect-induced galls are similar to any microbe-induced abnormal growth (e.g. tumors induced by *Agrobacterium tumefaciens*, Proteobacteria: Rhizobiaceae), in that both systems large quantities of phenolic compounds get accumulated as the abnormal system grows. Synthesis and accumulation of phenolic compounds is the defense reaction of the host plant to attack by the invading organism. However, in the bacteria-induced tumors, phenolic compounds are implicated to have two other roles: they are the indicators of vulnerability levels in host plants and they neutralize manipulation of the plant consequent to attack by altering gene expression of the host plant and by changing hormone activity. This enables interpreting growth in insect-induced galls. Phenolic compounds abound in gall tissues; moreover, gall tissues also show high activities of phenyl-ammonia lyase, the enzyme, which catalyzes the first committed step in phenolic biosynthesis. Such events disappear when the inducing insect is either dead because of natural causes or removed under experimental conditions. Since some of the phenolic compounds are well-known growth promoters (e.g., Yoshioka et al., 2004), it is implicated that phenolic compounds promote cell division and gall growth by interacting with the existing plant hormones (IAA) and/or IAA–oxidase, which are especially abundant in meristematic tissues (where galls are mostly induced). Nonetheless, the unexplained context here is whether one or more chemical signals, which are specific in a particular "gall inducer–host plant" situation, operate linking phenolic substances with the "IAA–IAA-oxidase" complex. Alternatively, what kind of interaction occurs at the plant–cell level? Does any kind of "immune" system work and mediate the interactions? The Miles hypothesis (1999) explains that salivary components (e.g., amino acids) are similar in both gall-inducing and nongall-inducing, but plant-feeding insects. IAA precursors (e.g., tryptophan) in insect saliva are of negligible quantities when compared with those found in plant systems in general. Moreover, the suggestion that salivary proteinases are utilized to release plant-bound IAA is disputable, because the saliva of gall-inducing aphids does not include proteinases. According to Miles (1999), vigorous uptake of oxygen occurs in the gall tissues; such an uptake coupled with "wounding" (consequent to feeding action) stimulates plant-bound auxin activity. Use of oxygen in plant tissues under insect attack will be so intense that IAA–oxidase activity, which regulates accumulation of IAA, is deprived of oxygen. Such an oxygen-deprivation enhances the synthesis and accumulation of IAA at insect-feeding sites, triggering growth at the involved meristems. Salivary oxidases could be playing a role in the disruption of the IAA-oxidase pathway – a supposition to be established.

The Hartley and Miles hypotheses provide an explanation to growth in galls, based on the role played by IAA. Nonetheless, the question "what specific factor triggers cell enlargement, which works synchronously with cell multiplication, in gall development?" remains unanswered. Several studies implying the role of cytokinins in insect-induced galls are available (Byers et al., 1976; Hori, 1992; Mapes and Davies, 2001b), whereas growth promoters (auxins and/or kinins, either individually or in combination) have a definite role in gall growth involving cell enlargement and cell division. However, the most critical element that has not been alluded to is the precise trigger mechanism involving a single host–plant cell or the group of 2–3 cells that receive the initial "stimulus" directly from the insect. As demonstrated in many well-studied gall systems (Rohfritsch, 1992), the earliest and most critical event in gall development is metaplasia; all other subcellular events in gall development occur later (Meyer and Maresquelle, 1983; Meyer, 1987). However, as of now, what we do not know are the nature and types of subcellular hormone receptors and transporters, and gene switches in that metaplasied cell (or the group of 2–3 cells), and activate growth subsequently.

5. Gall Shapes and Morphogenetic Responses in Plants: Examples from the Indian Subcontinent

Galls present unique shapes entailing unique types of growth and patterns of differentiation that do not occur in the normal morphogenesis of the involved organ. As referred to earlier, a "gall" is a cumulative but substantively symmetrical expression of response to insect-induced perturbation. Several extraordinary examples illustrating the altered differentiation pathways resulting in "novel" expressions have been documented (e.g., Meyer and Maresquelle, 1983; Meyer, 1987; Rohfritsch, 1992; Dreger-Jauffret and Shorthouse, 1992; Stone and Schönrogge 2003).

Several fascinating examples of galls exist in the Indian subcontinent (Mani, 2000; Raman, 2007a), such as the cylinder–piston galls on the leaflets of *Acacia ferruginea* (Mimosaceae) induced by *Contarinia manii* (Diptera: Cecidomyiidae) (Fig. 3) (Rohfritsch, 1971a; Gagné, 2004; Harris, 2010) (Fig. 3), sea-urchin-shaped galls on the vegetative shoot-apical meristems of *Hopea ponga* (Dipterocarpaceae) by *Mangalorea hopeae* (Hemiptera: Beesoniidae) (Fig. 4) (Raman and Takagi, 1992), and the fir-cone-like galls induced on the vegetative shoot apical meristems of *Mangifera indica* by *Apsylla cistellata* (Singh, 2003; Raman et al. 2009a).

Although the galls on *H. ponga* and *M. indica* are induced by hemipteran insects, the final gall shapes are strikingly different: galls of *H. ponga* are woody, nearly spherical, and bear several stiff, spiny, multicellular appendages between which the nymphs (progeny from the gall-founding female) live, whereas those of *M. indica* develop similar to fir cones, not woody, but with vertically arranged chambers for sheltering and nourishing the nymphs. Galls on both *H. ponga* and *M. indica* continue to grow until the immatures of *M. hopeae* and *A. cistellata* inhabit the locations "meant" for them in their respective galls. Nonetheless, given that the vegetative axillary meristem is the site of infestation for both *M. hopeae* and *A. cistellata* and that both the insects belong to Hemiptera, what is striking is

Figure 3. Cylinder–piston galls induced on *Acacia ferruginea* by *Contarinia manii* (Diptera: Cecidomyiidae); each gall involves two oppositely lying leaflets. (Photo courtesy: Dr S Amerjyothy, Presidency College, Madras, India.)

Figure 4. Sea-urchin-shaped galls induced on the axillary vegetative mersitems of *Hopea ponga* by *Mangalorea hopeae* (Hemiptera: Coccoidea: Beesoniidae).

that both galls are markedly different in their mechanics of development. Even rudimentary galls such as those induced by whiteflies (Hemiptera: Aleyrodidae), which are poor gall-inducing taxa (Byrne, 2005), present difficult morphogenetic questions. For example, the soft, parenchymatous galls on the leaves of *Achyranthes aspera* (Amarantaceae) induced by *Bemisia tabaci* (Hemiptera: Aleyrodidae) (Jesudasan and David, 1986; Mani, 2000) are characteristically scarlet-red because of anthocyanins. Identical pigmentation manifests as faint striations on the normal bristly bracts of *A. aspera*. It remains to be explained why the leaf, under the feeding pressure of *B. tabaci*, expresses the pigmentation, which exists normally only at sites unaffected by *B. tabaci* action in the same plant.

Plant morphogenesis entails signal trafficking and crosstalk across all levels of organization to coordinate metabolic and genomic networks (Farnsworth, 2004). Inherited genetic traits obviously play a role, followed by that played by the correlating morphogenetic factors (Hable et al., 1998). The genomic networks and inherited genetic traits, acting in conjunction, enable the expression of the phenotype, and in the present context, the gall. Galls are the best modified geometrical natural structures that arise solely through the trigger messages received from an alien organism, viz., an insect (Maresquelle, 1980–1981). How do logical circuits and signal-activated subsystems work in galls, such as those induced on *Acacia ferruginea*, *H. ponga*, and *M. indica*? Articulated step-by-step explanation of the developmental process, which commences from either a single or a group of metaplastic cells, gets transmitted through subsequent growth promoter-mediated cell expansion, until the commitment of the metaplastic cell and those in its neighborhood enabling the start of "novel" cell-cycle patterns and cell multiplication is needed. Although the events are similar to those occurring in tumors of primary and secondary origins, the difference is that the processes identified in galls are highly regulated. Moreover, what mechanism facilitates intercellular auxin transport? For each of these, a genomic subsystem obviously exists and mediates. We now know that in normal developmental process in plants, meristems control patterns both at a macroscale and at a fine scale. Structural specification at each scale is efficient only when pertinent gene activity and mediation occur concurrently in both of the complementary scales of development. In the fir-cone-like galls of *M. indica* and sea-urchin-shaped galls of *H. ponga*, gall induction incites perturbation through an asynchrony initiated between the macroscale and fine-scale differentiation processes. What we know today, with reasonable clarity, is that the "stimulus" from the insect (physical injury [wounding] and salivary chemicals) initiates perturbation, but what we do not know is at what temporal point during gall initiation, does the asynchrony between macroscale and fine scale get activated? The more intriguing question is, in spite of the asynchrony at the two scales, how does the gall achieve symmetry at the conclusion of its growth, which matches precisely with the lifecycle of the inducing insect? What factors trigger the vegetative meristem of *M. indica* to deviate from the normal differentiation pathway in leaf ontogenesis and phyllotaxy, but to differentiate microphyll-like structures (as in many lower Pteridophyta), which enclose and provide nutrition to the developing insect stages? On the contrary, why does the apical meristem of *H. ponga*, under gall development, follow a completely different pathway of *H. ponga*, compared with that of *M. indica*, involving a total suppression of leaf development, but the "soft" trichomes of the normal plant bud develop into "stiff" spines? The most obvious in the two compared gall systems is that the gall – in its totality – accommodates the progeny, a trait that is shared by diverse species of Adelgidae (Hemiptera: Aphidoidea) that induce galls on different Coniferales in Europe (Rohfritsch, 1966c, 1977; Meyer, 1987; Rohfritsch and Anthony, 1992). Hormones modulate complex suites of ecologically relevant traits through differentially modulated signal transductions. Given that changes in the function of one hormone can result in multiple changes in plant traits (Fleming, 2005), the role of abscisic acid (ABA) is gaining relevance in interpreting growth in insect-induced galls (De Bruyn et al., 1998; Florentine et al., 2002), because of greater levels of ABA detected in gall tissue, and especially the tissue that suffers anoxia. However, the precise role of ABA in the regulation of subcellular osmotic stability and/or lability and membrane integrity in galls remains to be verified. The specific role of cytokinins, which promote cell division, stimulate growth, and enhance gall-tissue capacity in becoming nutrient sinks (more specifically that of nitrogen), needs to be interpreted better in galls.

6. Why Galls Develop?

In spite of a reasonable understanding of the mechanisms that regulate gall initiation and growth, what remains unexplained and daunting till date is why insects induce galls (Price et al., 1987). In the words of Schaefer et al. (2005), "A gall is a remarkable biological phenomenon. Induced by one organism and produced by another wholly unrelated, a gall is an expression of complex collaboration. … a gall is a *new* structure, developed in or on the plant at the 'urging' of the arthropod to the benefit of the latter and, perhaps, with little harm to the former." Galls vary in their external shape, internal structure, and overall design; gall-inducing insects and gall-susceptible plants vary in their respective phylogenetic positions. Obviously, the relationship between the inducing insect and the susceptible plant has neither easily nor quickly evolved, because available evidences (refer to Table 1 in Schaefer et al., 2005) indicate that the capability to induce galls has arisen only occasionally in major phyletic lines; however, in the long history of gall-induction, capability (e.g., from the late Carboniferous, Labandeira and Phillips, 2002) has arisen often enough that it must confer considerable adaptive value on the inducing insect on the one hand and, therefore, may not be seriously maladaptive to the plant (Raman, 2003; Schaefer et al., 2005). Two preconditions appear to have determined the insect's capability to turn into a gall inducer: (1) the insect must have been closely and specifically associated with the particular plant species, and (2) the insect must have been small (rather minute). The smallness of the insect appears critical because larger insects, if they were to induce galls, would have induced large galls, which under the conditions of biological pressure, would have been self-defeatingly fatal. A small insect will induce a gall small enough to be completed within a growing season, and thus be able to ensure a continuous supply of food and adequate protection the insect requires during its immature stages. A gall ensures at least two of the most critical biological necessities: nutrition and shelter; shelter refers to a protected environment from the third-trophic level organisms and exigencies of weather (Stone and Schönrogge, 2003). In the following section, we shall explore the "nutrition" hypothesis that drives gall induction by insects.

7. Adaptations in the Plant Towards Nutrition of the Insect

One purpose of the gall is to provide nutrition to the inducing insect, and to achieve that purpose, the gall system is well structured (Wool et al., 1999) with a nutritive tissue (Lacaze–Duthiers, 1853) – a "special" tissue that usually develops lining the inner surface of the larval chamber (Fig. 5). The nutritive cells in galls induced by Cecidomyiidae and Cynipidae are considered typical: their nutritive tissue is characterized by abundance of cytoplasm, reduced or fragmented vacuoles, hypertrophied nuclei and nucleoli, and abundance of cytoplasmic organelles. Nutritive cells in insect-induced galls usually include hypertrophied and lobed nuclei, hypertrophied and vacuolated nucleoli, abundant ribosomes (often clustered as polysomes) that remain bound to a well-developed ergastoplasm, numerous plastids, and mitochondria that occur clustered around the nucleus, and numerous autophagic vacuoles (Bronner, 1992).

Figure 5. Gall (slit horizontally) on the leaves of *Eucalyptus saligna* induced by *Ophelimus eucalypti* (Hymenoptera: Eulophidae) in New Zealand; nutritive cells (the glistening golden-yellow layer) lining the larval chamber and the young larva feeding on them.

Although many differences exist in the morphologies of mouthparts and actions among the immature stages of gall-inducing insects, Rohfritsch (1992) considers the feeding action of the Cecidomyiidae as the basic model for the feeding actions of gall-inducing Hemiptera, Thysanoptera, and Acarina. Immature stages of these groups suck plant sap by wounding host–plant tissues: "subtle" feeding action occurs in the Cecidomyiidae, Hemiptera, and Acarina, and "harsh" feeding action occurs in Thysanoptera. Thysanoptera pierce the plant tissue with their asymmetrical mouthparts (Chisholm and Lewis, 1984), and then suck cell contents. In that process, they damage the epidermal and uppermost parenchyma cells of the host leaf (Raman and Ananthakrishnan, 1983a, b, 1984). The gall-inducing taxa of Cecidomyiidae, Hemiptera, Thysanoptera, and Acarina regurgitate their saliva mixed with the plant fluids they sucked. Most importantly, gall-inducing taxa falling under the Cecidomyiidae model require predigested food, which is available to the feeding immatures in the host–plant cells by the action of diverse hydrolases from both the plant and the insect (Bronner, 1977). The larvae of gall-inducing taxa of the Tenthredinidae (Hymenoptera) have larger mandibles than the insects of other groups referred before and they consume plant cells by chewing them; neither a well-defined nutritive tissue develops nor these larvae require predigested food. Feeding patterns of gall-inducing Tenthredinidae predispose those of gall-inducing Coleoptera and Lepidoptera. Rohfritsch (1992) prefers to refer to the nutritive tissue in the galls induced by Cynipidae (Hymenoptera) as the most "perfect" among insect-induced galls that characteristically include an inner functional tissue and an outer storage tissue. In galls with actively feeding larvae, in the galls induced by Cynipidae, a well-structured sclerenchyma layer differentiates, which is implicated to be a transfer tissue of solutes and water (Fourcroy and Braun, 1967; Bronner, 1977).

Cells of the nutritive tissue include high concentrations of starch and lipids, soluble sugars and proteins, in addition to a battery of different hydrolyzing enzymes (Raman and Ananthakrishnan, 1983c; Bronner, 1992; Raman, 1993; Mani and Raman, 1994). Starch invariably occurs in cell layers away from the feeding larva. Lipids usually occur proximally to the feeding larva. In the galls induced by Hymenoptera, lipids occur as phospholipids (Bayer, 1983). Carbohydrates and lipids exist in definite patterns in the nutritive tissue and such patterns reflect the relationship between the tension arising consequent to the feeding action of the larva and the metabolic response of the host plant. When the larva is either removed or killed, the carbohydrate – lipid patterns change, rather dramatically. The nutritive tissue is also the seat of intense enzyme activity (Bronner, 1977) and new proteins that mediate lipid biosynthesis (Harper et al., 2004). At times of maturity of the inducing larvae, the larval chambers include phenolic materials (Shorthouse, 1975; Abrahamson et al., 1991; Raman 1993). Gall tissues also include a variety of minerals (Bagatto and Shorthouse, 1994; Florentine et al., 2005; St John and Shorthouse, 2000).

Using labeled carbon (^{14}C), in 1969, Jankiewicz et al. demonstrated that photo-assimilates translocate to gall locations in the *Cynips (Diplolepis) quercusfolii*

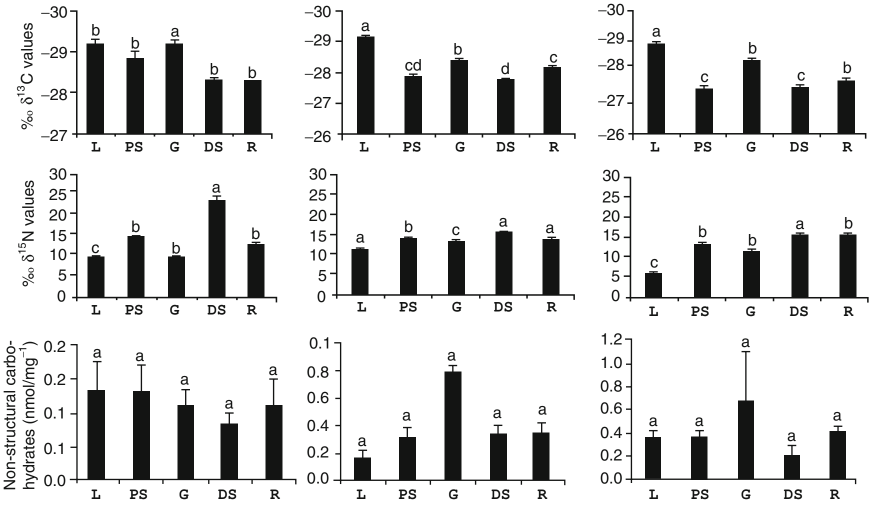

Figure 6. Mean values (+ SE of mean) of $\delta^{13}C$ and $\delta^{15}N$, and levels of total nonstructural carbohydrate (TNC) from the leaf (L), proximal stem (PS), gall (G), distal stem (DS), and root (R) from different developmental stages of *Parthenium hysterophorus*. Same letters indicate that means are not statistically different (Tukey's HSD test, $P > 0.05$). Carbon values (*top row*) ($\delta^{13}C$, ‰) from rosette, preflowering, and flowering stages (*left to right*). Nitrogen values (*middle row*) ($\delta^{15}N$, ‰) from rosette, preflowering, and flowering stages (*left to right*). TNC levels from rosette, preflowering, and flowering stages (*left to right*).

(Hymenoptera: Cynipidae) induced gall-bearing leaves on oak (Fagaceae). Kirst and Rapp (1974) using ^{14}C in the *Mikiola fagi* (Diptera: Cecidomyiidae) – *Fagus sylvatica* (Fagaceae) gall system substantiated the translocation of photo-assimilates to gall sites and established the term "nutrient sinks" for galls. Subsequently, much work has progressed demonstrating the nature of galls as sinks for nutrients and energy using more sophisticated measurement techniques such as stable-isotope ratio mass spectroscopy (Raman et al., 2006) (Fig. 6). A recent study of Diamond et al. (2008) unequivocally proves that the nutrition hypothesis offers a convincing explanation to the adaptive nature of galls.

8. Rapid Differentiation of the Nutritive Tissue and Measures of Susceptibility

Differentiation of a nutritive tissue is an early indicator of susceptible response (Rohfritsch, 1988; Ollerstam et al., 2002; Raman et al., 2009b). The subcellular responses in leaf tissues of three subspecific taxa of *Vitis* (*Vitis riparia* x *V. rupestris* cv. C–3309, *V. vinifera* x *V. labrusca* cv. Weisse Amerikaner, and *V. vinifera* cv. Portugieser Weissherbst) tested for gall-development response to *Daktulosphaira vitifoliae* (Hemiptera: Phylloxeridae) present varying pictures. Within 3–6 h of feeding by *D. vitifoliae*, the fifth- and sixth-layer mesophyll cells of cv. C–3309 differentiate activated, metaplasied cells, which develop into nutritive tissue in the next 24–48 h, establishing well-defined galls in 21 days. The nutritive tissue in *Vitis riparia* x *V. rupestris* cv. C–3309 show neither lignification nor accumulation of phenolic materials, similar to the structure of the nutritive tissue in the galls of *Picea excelsa* (Coniferales: Pinaceae) induced by *Adelges abietis* (Hemiptera: Adelgidae) (Rohfritsch, 1977, 1988). On the contrary, within 3–6 h of feeding by *D. vitifoliae*, the fifth- and sixth-layer mesophyll cells of *V. vinifera* cv. Portugieser Weissherbst exhibit a hypersensitive response and those of *V. vinifera* x *V. labrusca* cv. Weisse Amerikaner show a "mixed" response, integrating features of a nutritive tissue and that of hypersensitive reaction. No galls eventuated on the leaves of either cv. Portugieser Weissherbst or cv. Weisse Amerikaner.

The 24–48 h old nutritive tissue in the susceptible taxon (cv. C–3309) displayed signs of vigorous protein synthesis and cell autophagy and lysis, whereas the 24–48 h old mesophyll tissue in the resistant taxon (cv. Portugieser Weissherbst) showed a hypersensitive response including degeneration of the membrane system, hypertrophy and degeneration of organelles, pit fields, and plasmodesmata blocked by an electron-dense substance; phenolic materials accumulated in those cells directly under the feeding impact of *D. vitifoliae* and thickening of walls occurred in cells in the immediate vicinity, followed by swelling of membrane-bound organelles and inclusion of degenerated organelles in autophagic vacuoles. Degenerated chloroplasts included many plastoglobuli indicating degeneration of thylakoids consequent to subcellular stress, leading to localized apoptosis. Stressed mesophyll cells in cv. Portugieser Weissherbst appeared intensely stressed and dying with irregularly occurring abnormal wall thickenings and the slackened cell membranes.

In the leaf tissues of *Salix viminalis* (Salicaceae), a hypersensitive response occurred in 12 h after attack by the neonate larvae of *Dasineura marginemtorquens* (Diptera: Cecidomyiidae) (Ollerstam et al*.,* 2002). In cv. Portugieser Weissherbst – *D. vitifoliae* interactions, the pattern of response was broadly similar to that reported in *D. marginemtorquens* – *S. viminalis* interactions that a hypersensitive response occurred in the leaves of cv. Portugieser Weissherbst, but in 3–6 h.

9. Conclusion

A gall is a complex system; it is a subtle product of the coordinated effort of two unrelated genomes – an insect and a plant. In a majority of investigated instances, it involves only two participants. The insect is so specialized that it invariably remains committed to that particular plant taxon and effectively utilizes food resources by inducing the gall (Raman, 1988). A few of the galls induced by certain Cecidomyiidae include a third, active participant, a fungus – better known as "ambrosia galls" (see Roskam, 2005; Yukawa and Rohfritsch, 2005; Rohfritsch, 2009). Dozens of papers have referred to the complex arthropod community associated with galls, which includes a variety of parasitoids, predators, inquilines, and even casual visitors. Indeed, one proposal is that the gall-inducing behavior among Insecta has arisen as an effort to protect themselves from predators and parasitoids. For a better understanding of this proposal, the reader is referred to other papers (e.g., several papers in Ozaki et al., 2006).

To conclude, a gall is an organized, elegantly geometrical entity, which mostly arises as a sequel to the feeding stimulus of certain insect taxa. In a majority of the known instances, no gall-inducing insect inflicts any substantial damage to the host plants. The behavioral modifications that arise in both the plant and the insect consequent to gall-inducing interaction are remarkably intricate and revealing, which involve highly complex, but well-synchronized steps. Gall-inducing organisms – from the primitive eriophyid mites to the advanced cynipids – display a sophisticated biology and physiology and utilize their hosts more efficiently and resourcefully than their nongall-inducing relatives.

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PART 3:
POLLINATION AND SEED DISPERSAL

Rostas
Tautz
Dag
Shafir
Iluz

Biodata of **Michael Rostás** and **Jürgen Tautz**, authors of ***"Ants as Pollinators of Plants and the Role of Floral Scents"***

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ANTS AS POLLINATORS OF PLANTS AND THE ROLE OF FLORAL SCENTS

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Ants are considered to be classical nectar thieves. In recent years, however, limited evidence has suggested that flower-visiting ants can contribute to pollination. In this chapter, we review why pollination by ants is rare and under what circumstances it may occur. Furthermore, we elaborate on the role of floral scents for foraging ants.

1. Introduction

The pollination of flowers by insects is one of the most well-known mutualisms in nature. Angiosperm plants are characterized by reproductive organs that are conspicuously colored and scented, they offer nectar, and they are in many ways morphologically adapted to pollinating insects (Proctor et al., 1996). The degree of this mutualism, as well as, how strongly both sides interact varies, depending very much on the plant and animal species involved (Schoonhoven et al., 2005). Among all insects, members of the Hymenoptera order have evolved the strongest adaptations to pollination. In particular, honeybees, bumblebees, and solitary bees rely exclusively on nectar and pollen provided by flowers. Honeybees, for instance, have evolved specialized legs that rake pollen grains from the whole body and collect them in pollen baskets on the tibia. Their fur consists of hairs with hooks and teeth that allow the transport of many pollen grains, and they regulate their body temperature, enabling flight in conditions too cold for most other insects. In honeybees, the ability to learn and distinguish visual patterns and floral scents is well known, and helps to maintain flower constancy for a certain length of time such as one or more foraging trips (Tautz, 2008).

Other Hymenoptera also visit plants but their dependence on flowers is less extreme (although fig wasps are a clear exception). Wasps of the sub-order Apocrita are the majority in this order, being generally parasitoids or predators that feed on arthropods during their larval development (Kugler, 1970). However, as adults, they are usually active foragers and require nectar or honeydew as a source of energy. Owing to their short mouth parts, flowers that allow for easy access to nectar are preferred, such as Apiaceae or Euphorbiaceae. Social wasps are particularly attracted to tubular flowers, often brownish in color, which are considered to be adapted to this category of pollinators. These wasp plants may use scent to selectively lure their pollinators (Shuttleworth and Johnson, 2009) and even use deceit to ensure visitation. Flowers of *Epipactis helleborinae,* for example, enhance their attractiveness by emitting green leaf volatile compounds that are normally released by vegetative tissue upon caterpillar feeding. The summoned wasp in search for herbivores is rewarded with nectar only (Brodmann et al., 2008). Another orchid species, *Chiloglottis trapeziformis,* attracts males of the wasp *Neozeleboria cryptoides* by mimicking the female's sex pheromone that consists of only one very specific compound. During pseudocopulation with the flower, wasps pollinate the orchid (Schiestl et al., 2003).

Probably, the most important Hymenopterans from an ecological point of view are the ants. Their preferred diet depends very much on the species considered. While some are notorious predators, others collect seeds and nectar or use plant leaves to cultivate fungi for food (Hölldobler and Wilson, 1990). Ants inhabit almost all terrestrial ecosystems and 10–15% of the animal biomass is thought to consist of this insect group (Beattie and Hughes, 2002). Flowering plants (Angiospermae), on the other hand, are by far the most dominant taxon of the terrestrial plants. It is therefore not surprising that a plethora of interactions between ants and plants have evolved, which can be facultative or obligate, antagonistic or mutualistic (Rico-Gray and Oliveira, 2007). Well known are the symbiotic associations between myrmecophytic plants and ants, such as *Acacia* and *Pseudomyrmex* (Heil and McKey, 2003). These trees house obligate mutualistic ants in swollen thorns and feed them with extrafloral nectar and lipid-rich food bodies. In exchange, the plants are defended against herbivores and encroaching vines. Myrmecochory, the dispersal of seeds by ants, is another example for a mutualistic ant–plant interaction. A special part of the diaspore, called eleiosome, contains attractive lipids and encourages ants to carry the seed back into its nest.

While pollination remains the best-studied relationship between insects and higher plants, ants in contrast to bees are not well known for being significant pollinators. In this chapter, we explore the role of ants as flower visitors and potential pollinators. Furthermore, we pay some attention to what is known about ants using environmental volatiles such as floral scents for food location.

2. Ants as Pollinators

Frequently, workers of many ant species can be observed to visit flowering plants and collect nectar. Thus, it seems surprising that the pollination of flowers by this visitor group has rarely been reported (Proctor et al., 1996). While two thirds of all angiosperms are pollinated by insects, only about 30 reports suggest pollination by ants (Peakall et al., 1991; Schoonhoven et al., 2005) and even less have attempted to confirm ant pollination experimentally (Table 1). Various non-exclusive reasons have

Table 1. Plant species that have received experimental support for ant pollination.

Plant species	Test for fruit or seed set	Test for seedling germination	Reference
Polygonum cascadense (Polygonaceae)	No	No	Hickman, 1974
Hutchinsia [= *Thlaspi*] *alpina* (Brassicaceae)	No	No	Petersen, 1977
Diamorpha smallii (Crassulaceae)	No	No	Wyatt, 1981
Scleranthus perennis (Caryophyllaceae)	No	No	Svensson, 1986
Epipactis palustris (Orchidaceae)	Yes	No	Brantjes, 1981
Microtis parviflora (Orchidaceae)	Yes	No	Peakall and Beattie, 1989
Leporella fimbriata (Orchidaceae)	Yes	No	Peakall, 1989
Hormathophylla spinosa (Brassicaceae)	Yes	No	Gomez and Zamora, 1992
Blandfordia grandiflora (Lilliaceae)	Yes	No	Ramsey, 1995
Bordera pyrenaica (Dioscoreaceae)	Yes	No	Garcia et al., 1995
Alysum purpureum (Brassicaceae)	Yes	No	Gomez et al., 1996
Sedum anglicum (Crassulaceae)	Yes	No	Gomez et al., 1996
Retama sphaerocarpa (Fabaceae)	Yes	No	Gomez et al., 1996
Frankenia thymifolia (Frankeniaceae)	Yes	No	Gomez et al., 1996
Arenaria tetraquetra (Caryophyllaceae)	Yes	No	Gomez et al., 1996
Paronychia pulvinata (Caryophyllaceae)	Yes	No	Puterbaugh, 1998
Lobularia maritima (Brassicaceae)	Yes	Yes	Gomez, 2000
Euphorbia cyparissias (Euphorbiaceae)	Yes	No	Schurch et al., 2000
Fragaria virginiana (Rosaceae)	Yes	No	Ashman and King, 2005
Epipactis thunbergii (Orchidaceae)	Yes	No	Sugiura et al., 2006
Neottia listeroides (Orchidaceae)	Yes	No	Wang et al., 2008
Trinia glauca (Apiaceae)	Yes	No	Carvalheiro et al., 2008
Euphorbia seguieriana (Euphorbiaceae)	Yes	Yes	Rostás, 2009, unpublished

been put forward to explain the small number of ant-pollinated plant species, but rigorous tests for many of these arguments are still missing.

On the insect side, it has been assumed that ants, due to their small size, are able to exploit nectar without touching the anthers or stigmas of a flower, and thus act as nectar thieves. Additionally, a smooth cuticle and frequent grooming may prevent pollen attachment (Barth, 1991). While this might be true for some ant species, others are as large and hairy as many important winged pollinators such as solitary bees. Besides, ants have often been reported to carry pollen (Peakall et al., 1991). It has been put forward that ants do not carry pollen far enough as they are not winged, and therefore do not contribute to out-crossing (Schubart and Anderson, 1978). Peakall et al. (1991) demonstrated that the foraging patterns of ants (and thus pollen transport) may follow a leptokurtic distribution with short mean distances, similar to the movements of many winged pollinators.

Therefore, the authors argue that ants may well be able to effect out-crossing. Nevertheless, it has likewise been confirmed that within-plant movement prevails and that individual foragers exclusively visit the same individual plant for an extended period (Fowler, 1983). This strict *Ortstreue* (Hölldobler and Wilson, 1990) to single plants was observed, e.g., in individually marked ants of *Tapinoma erraticum* and *Formica emarginatus* visiting nectaries of *Euphorbia seguieriana,* where they are the most abundant visitors (Rostás, unpublished data).

In the 1980s, it has been suggested that the most likely reason for the paucity of ant pollination is the presence of metapleural gland secretion on the integument of many ant species (Beattie et al., 1984). The secretion derived from these paired glands is actively distributed over the bodies of adults and larvae, and due to its antimicrobial properties, protects the bearer from pathogenic fungi and bacteria (Fernandez-Marin et al., 2006; Hölldobler and Engel-Siegler, 1984; Poulsen et al., 2003). An important side-effect of metapleural gland secretion is its strong impact on the viability of pollen grains (Beattie et al., 1984; 1985; 1986). Interestingly, such a strong reduction of pollen viability cannot be found in honeybees and wasps, although the high densities of individuals in their nests also produce a favorable microclimate for pathogens (Harriss and Beattie, 1991). Nevertheless, the secretion's biocidal activity varies in potency among ant species, and sufficient numbers of vital pollen may still be left sticking on the ant surface to allow for pollination and seed set (Garcia et al., 1995; Gomez and Zamora, 1992; Hull and Beattie, 1988; Ramsey, 1995). Pollinia of the ant-pollinated orchid *Microtis parviflora*, e.g., did not loose germinability after contact with the cuticle of *Iridomyrmex gracilis* (Peakall and Beattie, 1989). It remains to be elucidated whether ant-pollinated plants are characterized by pollen that is more resistant to metapleural gland secretion. Alternatively, it could be an adaptation to pollination if ants had secretions that would be effective against microbes, but benign to pollen. In any case, it seems that orchids are better suited for ant pollination as only the small area of the pollinia's stalks gets attached to the ant's cuticle. It is also within this plant family that a case of strong specificity has been reported: *Leporella fimbriata* is pollinated by pseudocopulation with males of the ant species *Myrmecia urens* (Peakall, 1989).

Apart from the above-mentioned arguments that focus on ant traits, several adaptations on the plant side may prevent ant pollination. Ants are considered to be typical nectar thieves or even robbers, which can reduce a plant's reproductive success by damaging the anthers and pistils or chasing away other potential pollinators (Galen and Butchart, 2003; Gaume et al., 2005). As early as 1879, the Austrian botanist Kerner von Marilaun described numerous morphological and chemical barriers intended to keep ants from visiting flowers. Examples of slippery stems, sticky blossoms and trichomes, extrafloral nectarines, or repellent-containing floral tissues have been listed. It has been suggested that floral nectar may contain deterrent allelochemicals to ward off ants (Janzen, 1977). After all, plants from 21 families are known to possess floral nectar with secondary metabolites (Adler, 2000), and recent evidence suggests a complex ecological role for secondary

Figure 1. *Formica* spp. worker covered with yellow pollen from *Euphorbia cyparissias* flowers. (Credit: C. Müller.)

nectar constituents. Nectar compounds such as nicotine have been shown to shorten the duration of feeding by pollinators like hummingbirds and hawkmoths, which results in enhanced flower visitation rates (Kessler and Baldwin, 2007). Still, studies in tropical and desert habitats could not confirm the repellency hypothesis, and it was proposed that other mechanisms must be present to keep ants away from flowers (Fowler and Whitford, 1982; Guerrant and Fiedler, 1981).

3. The Role of Floral Scents

Floral volatiles play an important role in attracting many kinds of insect pollinators and it seems nearby to assume that floral scent is pivotal for foraging ants, too. Interestingly, the relationship between volatiles and ants is quite ambiguous. In contrast to the dominant hymenopteran pollinators, such as bumblebees and honeybees, visual orientation is less important in ants, while tactile and chemical cues are primary sources of information (Gronenberg, 1996). Their antennae are in constant contact with the substrate, other nest mates, or a potential food source, and thus, ants experience a continuous input of more or less volatile signals (Gronenberg and Hölldobler, 1999). It is well known that a great number of pheromones mediate communication between nest mates (Hölldobler and Wilson, 1990).

Less studied is the question of how ants make use of volatile cues from the environment (Ehmer, 1999). Only recently, olfactometer experiments unequivocally showed that individual ants perceive and learn single odor compounds and can navigate topochemically in their environment (Dupuy et al., 2006; Helmy and Jander, 2003). Ants may also use volatiles to locate food sources. *Crematogaster scutellaris* recognizes the odor of its prey, the fig wasp, outside of the fig (Schatz et al., 2003). Other examples are the European fire ant *Myrmica rubra* that is attracted by plant-derived terpenes emanating from the fecal shields of *Cassida* larvae (Müller and Hilker, 1999), or *Lasius niger* that uses chemical cues to recognize aphids that are attended by nest mates (Glinwood et al., 2003). However, in the latter case, it is unclear whether the ant worker senses a pheromone that is transferred onto the aphid by another worker or whether the signal is truly aphid-derived. Foragers of the leaf-cutting ants *Atta cephalotes* and *Acromyrmex octospinosus* orient upwind to odor stimuli to reach the proximity of a food source (Littledyke and Cherrett, 1978). Anemotactic and olfactory orientation has also been found in the desert-dwelling ant *Cataglyphis fortis*, a species well-known for its visual capabilities. Using video-tracking, Wolf and Wehner (2000) showed that workers were able to pinpoint food sources by zigzagging up-wind, similar to butterflies in search for a mating partner. Volatiles are not only used for locating far away food sources, but they can also be used by recruited nest mates as a decision criterion. The leafcutter ant *Acromyrmex lundi* learns the specific odor of food items that are brought into the nest. Once the recruited worker ant has arrived at the location of the food source, they choose those items that bear the same odor as the template they got to know in the nest (Roces, 1990, 1994).

Rather than attracting ants for pollination, studies by Stäger (1931) and van der Pijl (1955) suggested that floral scent could have deterrent effects on ants. This notion was also supported by more recent experiments. Some *Acacia* are associated with mutualistic ants species, offering them domatia and food. In return, the trees are patrolled for detrimental herbivores. A potential conflict arises if the aggressive ants scare away pollinators as well. This is avoided by floral volatiles that repel the ants from the trees' inflorescences (Ghazoul, 2001). Further support for floral volatiles functioning as repellents comes from a study that screened the responses of non-mutualistic ants to floral bouquets from 32 plant species and to isolated volatile compounds Junker and Blüthgen (2008). In the ant *Camponotus floridanus*, 20 out of 30 plant species were repellent, while eight out of 26 plants repelled *Lasius fuliginosus.* Junker and Blüthgen (2008) concluded from their study that ants could have been a selective force in the evolution of floral scent with volatiles functioning as a plant defense against antagonistic visitors in the first place. Bees, on the other hand, being specialized in collecting nectar and pollen, have adapted to floral compounds and use them for orientation. From these results, it is clear that ants do perceive and respond to volatiles from the environment. However, whether ants use floralscent compounds for locating nectar as well is still unclear because the experimental setup of the study by Junker and Blüthgen allowed only for testing repellency, not attractiveness.

Interesting insights come from a study by Kessler and Baldwin (2007) who showed that foragers of *Solenopsis xyloni* readily consume the scented nectar of *Nicotiana attenuata* flowers. The complete volatile blend emitted by the nectar had a neutral effect on ants. However, when testing single volatile nectar compounds, *S. xyloni* was attracted to certain chemicals, repelled by others and remained neutral towards some. It is thus conceivable that distinct scent compositions could be attractive to ants and that plants relying on ants for pollination should be devoid of repellent compounds. Furthermore, the role of associative learning should not be underestimated because sugar can reinforce the response to an unconditioned stimulus such as floral volatiles (Dupuy et al., 2006).

4. How Common Is Ant Pollination?

In spite of only a few documented reports on ant pollination and the many obstacles described earlier that may hinder this kind of interaction, it has been argued that pollination by ants may be more widespread (Gomez and Zamora, 1992). The reason for this argument is that the relationship between plants and pollinators is characterized by two components: a qualitative and a quantitative part (Fenster et al., 2004). Arguments seeing ants exclusively as antagonists of plant sexual reproduction focus on the pollinator's quality, while neglecting the quantitative side. Qualitative aspects emphasize a pollinator's efficiency to deposit the right pollen on the corresponding stigma during each visit to a flower. Ants may not be very efficient pollen vectors for various reasons: they do not fly, they may not carry much pollen, metapleural secretion can reduce pollen viability, and in some cases, they can damage a flower's reproductive organs. However, often it has been overlooked that ants are social insects and thus recruit their nest mates whenever they find a good food source. This resource will be exploited by many individuals, until it has vanished. The high frequency of flower visitations (quantitative aspect) may thus compensate for less favorable qualitative ant traits (Gomez and Zamora, 1992; Schürch et al., 2000). Nevertheless, this argument is not without limitations: due to the ant's foraging strategy, i.e., visiting the same resource for many times, geitonogamy (pollen carry-over from one flower to another on the same plant) could be a problem, especially in plant species that prevent selfing and where individuals consist of many inflorescences and flowers. Thus, it is not surprising that a considerable number of ant-pollinated plants rely on facilitated autogamy, i.e., flowers are self-compatible, but selfing is accomplished by insect vectors. In this light, one needs to be cautious with current evidence for ant pollination. Even studies that confirmed ant pollination from assessing seed or fruit set in pollinator exclusion experiments, did not measure the viability of the F1 generation. The only exceptions where seedling germination was assessed are studies on *Lobularia maritima* and *Euphorbia seguieriana* (see Table 1). In these experiments, plants were caged in different ways. Access to the inflorescences was selectively denied to ants, winged insects, none, or both (Fig. 2). In *E. seguieriana*,

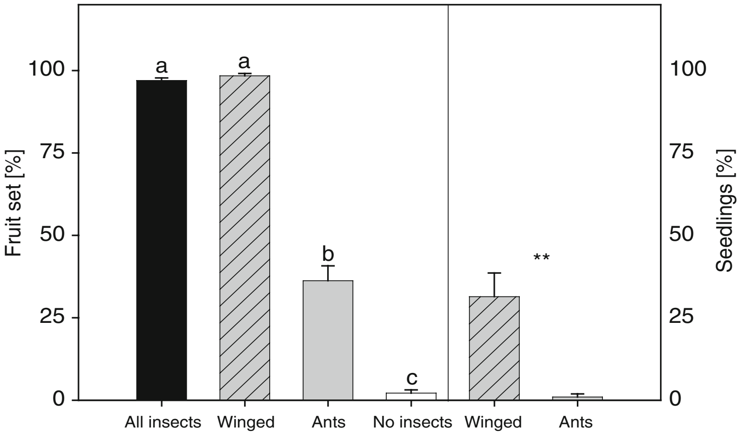

Figure 2. *Left*: Percentage of *Euphorbia seguieriana* plants that set fruit in a pollinator exclusion experiment. *Right*: Percentage of germinated seedlings. "All insects" = Flowers accessible to all visitors, "Winged" = Only winged insects had access, "Ants" = Only ants had access to flowers, "No insects" = All visitors were excluded. Bars and whiskers represent means ± standard error. Different letters indicate statistically significant differences. $^{**}P < 0.01$.

ants accounted for more than 60% of all flower visits, and plants that were pollinated exclusively by ants produced significantly more fruits than plants from which all insects were excluded. However, while there was no negative effect on the germination rate of ant-pollinated seedlings in *L. maritima*, the proportion of seedlings from ant-pollinated *E. seguieriana* plants was close to zero. A plausible reason for this observation is that individual ants visited only one individual plant for many days in a row. Thus, the ants deposited pollen on different flowers from the same plant, which resulted in geitonogamy. The seeds produced were probably sterile due to inbreeding depression. We need to be aware that ant-assisted selfing may also have occurred in other cases where pollination by ants has been reported. Unless shown experimentally that plants produce viable progeny, all reports on ant pollination should be treated with caution. Plant species that can reproduce by facilitated autogamy may be the exception.

In the 1970s, Hickman was one of the first to propose that pollinating ants and plants could have coevolved (Hickman, 1974). Using the knotweed *Polygonum cascadense* as a model, a list of traits that should be characteristic for an ant pollination syndrome was postulated. Accordingly, plants are rather short and preferentially grow in dry and warm habitats with an abundance of foraging ants. Ant-pollinated plants are expected to occur in high population densities and are rather small with easily accessible flowers. Such flowers offer only very little nectar and thus promote movement between flowers while discouraging other insects with higher energy demands than ants. Synchronously blooming flowers must be few per plant because many attractive flowers will support geitonogamy rather than out-crossing, and as ants are inappropriate for carrying large qua ntities of pollen, volume and/or size of pollen must be small to avoid grooming. Finally, Hickman suggested that seeds should be few per plant, as each seed requires at least one pollen transfer. Indeed, many of Hickman's traits can be found in the plant species listed in Table 1, and most of them do live in dry habitats. However, systematic studies still need to demonstrate the existence of an ant pollination syndrome. Given that plant traits of the postulated syndrome, such as being short with many small flowers are rather adaptations to their abiotic environment (Gomez et al., 1996), it will be necessary to demonstrate that certain plant traits have really evolved as an adaptation to ants.

5. Conclusions

Despite the fact that increasingly more studies with rigorous exclusion experiments have demonstrated a beneficial role for ants in plant reproduction, generalizations on the significance of ant pollination may be premature. On the one hand, ants acting as pollinators may be underestimated, in particular, in habitats where they are very abundant. This suggests that the quantitative aspect of pollen vectoring needs to be considered more closely. On the other hand, measuring seed set may not be sufficient to prove that plants benefit from ant visitation, especially in plants relying on allogamy. Future studies need to show that ants have a positive effect on plant fitness by demonstrating that ant-pollinated plants also produce significant numbers of viable seedlings. The role of floral scent acting as repellents to discourage nectar thievery or alternatively as attractants to facilitate ant pollination awaits further clarification.

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CROP POLLINATION IN MODERN AGRICULTURE

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1. Introduction

Approximately two thirds of all flowering plants are pollinated by insects. This "service" is not free. In return for pollen transfer, plants provide food for their partners in the form of nectar and pollen. Pollen is the nutritional source of protein, fatty acids, lipids sterols, vitamins and minerals (Todd and Bretherick, 1942) while carbohydrates, the basic source of energy, are supplied mostly by the nectar (Baker and Baker, 1983). Because each of the two parties can only barely survive, if at all, without the other, this is an exemplary case of mutualism. Associations from which both partners benefit are widespread, but that between angiosperms and insect pollinators is probably the most spectacular and large-scale example of mutualism in the living world (Schoonhoven et al., 1998). Animal-pollinated flowers advertise themselves by presenting various stimuli – visual, olfactory and tactile, simultaneously. The conspicuousness of the advertisement depends on the flowers' color, size and shape, as well as the strength of their volatile emissions, and the perception of these traits by pollinators (Kevan, 2005).

The current review covers developments in crop pollination in the past few decades. It summarizes data from studies performed worldwide on crops' dependence on insect pollination. It describes the latest findings regarding management of the major crop pollinator – the honeybee, and "alternative" pollinators that have come into limited commercial use, but might become important in the future. The effects of two major components in modern agriculture – pesticides and the use of genetically modified (GM) crops, on pollinators are summarized, and a brief overview is provided of recent progress in using pollinators not only for disseminating pollen but also for disseminating biological agents.

2. The Economic Value of Crop Pollination

Animal pollination is important for the sexual reproduction of many crops (McGregor, 1976; Free, 1993; Delaplane and Mayer, 2000), as well as of most wild plants (Kearns et al., 1998; Ashman et al., 2004). In Europe, Williams (1994) assessed the pollinator needs of 264 crop species and concluded that 84% of them depend, to some extent, on animal pollination. For tropical crops, Roubik (1995) provided a detailed list of 1,330 species, 70% of which appear to have at least one variety for which production is improved by animal pollination. In their review, Klein et al. (2006) reported that fruit, vegetable or seed production from 87 of the leading global food crops depends upon animal pollination, while that from 28 crops does not. Honeybees (*Apis mellifera*) remain the most economically valuable pollinators of crops worldwide (Watanabe, 1994). In the USA alone, the marginal increase in value attributable to honeybees – that is, the value of increased yield and quality achieved through pollination by honeybees, was $14.6 billion in 2000 (Morse and Calderone, 2000). In Canada, it was estimated at $440 million in 1990 and $780 million in 1998 (www.honeyconcil.ca). In the EU, it was estimated at $4.25 billion (Borneck and Merle, 1989), in the UK – £200 million (Carreck and Williams, 1998), in Australia – $0.6–1.2 billion in 1989 and $2.4 billion in 2001 (Gordon and Davis, 2003), and in Israel – $230 million in 1998 (Dag and Regev, 1999). The second most important commercial pollinator is the bumblebee (*Bombus* spp.) (Fig. 1). The main crop pollinated by these insects is tomato, comprising about 95% of all bumblebee sales worldwide and involving over 40,000 ha of greenhouse culture (Velthuis and van Doorn, 2006). The main bumblebee species used for greenhouse pollination in Eurasia is *Bombus terrestris*. In North America, similar considerations led to choosing *Bombus impatiens* for areas east of the Rocky Mountains and *Bombus occidentalis* for areas west of the Rockies. In East Asia, *Bombus ignitus* has been selected, while in China, both *B. ignitus and Bombus lucorum* are preferred (Velthuis and van Doorn, 2006). In contrast, only one species of honeybee, *Apis mellifera* L., is used worldwide, with variations in genetic strains based on the lines available in a particular country.

Figure 1. Bumblebee (*Bombus terrestris*) visiting almond (*Prunus dulcis*) flower.

3. Honeybee Management for Crop Pollination

As already mentioned, the honeybee is the major crop pollinator worldwide. However, it forages on a wide range of host plants. It continuously monitors, samples and collects information on the most rewarding food source available and has a highly developed system for recruiting nest mates to these sites. Thus, under certain conditions, maintaining honeybees on target crops is extremely difficult (Jay, 1986) (Fig. 2).

3.1. TIMING OF COLONY INTRODUCTION TO THE CROP

The timing of colony introduction to a crop, in relation to the latter's blooming stage, strongly influences the number of bees visiting that crop. Many reports have shown that placing hives in the midst of the pollinated crop before the main bloom has taken place leads the bees to abandon the crop in favor of competing flowers in the vicinity with which they establish constancy (Mayer et al., 1986; Free, 1993). Therefore, it was recommended to delay the placement of bee colonies until after bloom had started (Humphry-Baker, 1975; Kevan, 1988). Although the introduction of colonies at the right time exposes the bees to massive blooming in the target crop, they still tend to gradually widen their forage area, and may even end up abandoning the target crop altogether (Free et al., 1960). To overcome this problem, Al-Tikrity et al. (1972) suggested the introduction of additional colonies at a later date: thus the new bees are first attracted to the target crop bloom, before discovering the competing bloom. This sequential introduction was attempted in

Figure 2. Honeybee (*Apis mellifera*) visiting onion (*Allium cepa*) flower.

pear orchards in Washington (Mayer, 1994) and was found to raise the number of bees in the orchard (albeit only for 1 day) and the consequent fruit set. More pronounced results were obtained in pear by Stern et al. (2004) and in Japanese plum by Sapir et al. (2007). In addition to reducing the effect of competing flora, sequential introduction of colonies also improved mobility of the bees in the target crop and hence, increased cross-pollination level, which is particularly important in self-incompatible crops (Stern et al., 2001, 2004; Sapir et al., 2007). However, this technique is not always helpful; in cranberry, Shimanuki et al. (1967) found no advantage in placing honeybee colonies before rather than at peak bloom. In apple (Stern et al., 2001), the positive effect of the sequential introduction of honeybee colonies on fruit set and yield was also attributed to increased pollination effectiveness of naïve bees. On the flowers of some apple cultivars, such as “Delicious”, there are gaps at the base of the stamens that enable “sideworking” honey bees to obtain nectar without contacting the flower’s anthers and stigmas (Roberts, 1945; Thomson and Goodell, 2001; Schneider et al., 2002) (see Fig. 3). As a result, the pollination rate of “Delicious” apples is particularly low when compared with other cultivars (Stern et al., 2007). Robinson and Fell (1981) found that after a single honeybee visit to a “Delicious” flower, fruit set was 8% when the visit was by a “sideworker”, compared to 50% when the visit was by a “topworker”. Collecting nectar from the side, without touching the anthers and stigma, is very convenient for the bee, but takes time to learn (DeGrandi-Hoffman et al., 1985). The proportion of “topworkers” was considerably higher in apple orchards in which colonies were introduced sequentially in comparison to instances where colonies were introduced once, at the beginning of flowering. These effects were observed during only 2 days of full bloom, but led to increased cross-pollination and apple pollen

Figure 3. ‘Sideworking’ honeybee (*Apis mellifera*) visiting ‘Red Delicious’ apple (*Malus domestica*) flower.

collection (as assessed with pollen traps), and resulted in greater fruit set and yield (by 50–80%) (Stern et al., 2001).

3.2. ATTRACTANTS

Various products containing blends of worker bee pheromones and/or food additives have been sprayed on flowering crops over the years in attempts to attract pollinating honeybees and increase crop yield (Jay, 1986). Initially, sugar-based attractants were tested, but these were shown to actually diminish pollination efficiency because bees were diverted to collecting syrup off the foliage instead of pollinating the flowers (Free, 1965). Later on, other products became commercially available – "Beelure", "Bee-Scent", "Bee-Here", "Beeline", "Pollenaid", containing various components of worker bee Nasanov pheromone, sugars, and/or attractive oil. "Beeline" and "Bee-Scent" were found ineffective at increasing pollination level in cucumber and watermelon (Schultheis et al., 1994). "Beeline" was also found to be ineffective in apple and pear (Mayer and Johansen, 1982) and in red clover (Burgett and Fisher, 1979) pollination. Similarly, "Beelure" was found ineffective in apple (Rajotte and Fell, 1982). So, in general, unfortunately, none of these products or their individual components have proven effective in commercial pollination and they are not used on a commercial scale (Winston and Slessor, 1993). One of the latest products is "Fruit Boost", which contains a synthetic formulation of queen honeybee mandibular pheromone. This compound was found successful in increasing bees' pollination activity in pear, apple, cranberry and blueberry but not in sweet cherry (Winston and Slessor, 1993; Maumann et al., 1994). It was found that a combination of increased recruitment to queen mandibular pheromone-sprayed plots and greater time spent by foragers with increased flower visitation contributed to enhanced pollination of treated blooming crops (Higo et al., 1995).

3.3. GENETIC BACKGROUND FOR POLLINATION EFFICIENCY

Several honeybee genetic traits have been reported that may be related to their effectiveness as pollinators. These include a reduction in flight range, which reduces the impact of competing flora (Gary and Witherell, 1977), the tendency to collect pollen, which is an important characteristic in crops that are pollinated mainly by pollen gatherers (Hellmich et al., 1985; Calderone and Page, 1988; Gordon et al., 1995; Page, 1999), crop loading (Afik and Shafir, 2007), corbicular size (Milne and Pries, 1986; Milne et al., 1986), characteristics that affect flower handling (Alpatov, 1948; Mackensen and Nye, 1966), and preference for certain crops over competing flora (Nye and Mackensen, 1968, 1970; Basualdo et al., 2000, 2007; Dag et al., 2003, 2005; Afik and Shafir, 2007). Such traits, among others, may affect overall foraging preferences, though successful selection regimes may not always prove effective when applied commercially (Gary et al., 1978).

4. Pollination in Enclosures

Many high-value cash crops that were once grown exclusively in open fields are now grown in greenhouses and nethouses. This shift has occurred mainly to facilitate plant protection, enable out-of-season production, prevent environmental hazards and isolate plants for the production of true seeds (Dag, 2008). A wide variety of fruit and seed crops that are grown in greenhouses and nethouses are pollinated by insects (Sommeijer and Ruijter, 2000), mainly honeybees and bumblebees (Free, 1993; Velthuis and van Doorn, 2006). The main greenhouse crop pollinated by bumblebees is tomato (Velthuis and van Doorn, 2006). Most enclosure-grown crops other than tomato can be pollinated by either pollinator (honeybee or bumblebee), generally with similar levels of effectiveness, as was found with sweet pepper (Dag and Kammer, 2001), strawberry (Dag et al., 1995) and squash (Dag, 2006). Under enclosure conditions, humidity, temperature, carbon dioxide level and air-flow direction can be regulated, thus affecting flower rewards and pollinator efficiency (Dag and Eisikowitch, 1995, 1999, 2000). On the other hand, enclosures limit food availability – mainly pollen – which causes drastic brood reduction. This reduction may be overcome by providing a proper artificial diet (Kalev et al., 2002). Until recently, only vegetable crops were grown in enclosures; however, more and more fruit tree crops are now being shifted to this protected environment, creating new challenges in using pollinators effectively (Fig. 4).

Figure 4. Honeybee hive placed for pollination of avocado orchard under net.

5. Bees as Vectors of Biological Agents

In addition to pollen, bees can transfer fungal spores and bacteria among flowers of different plant species (Batra et al., 1973; Harrison et al., 1980; Sandhu and Waraich, 1985). This ability to vector microbes has been harnessed for biological control purposes, by using honeybees and bumblebees to transfer fungal, bacterial and viral inocula from the hive to flowers (Kevan et al., 2003). In practice, a dispenser (or "insert") is attached to the hive and loaded with a powder formulation of the desired agent (Fig. 5). The intent is that foragers exiting the hive will become dusted with the agent and deliver it to the target crop. The technique has been applied to control fire blight (*Erwinia amylovora*) in orchards (Thomson et al., 1992; Johnson et al., 1993; Vanneste, 1996; Matthews, 1997; Cornish et al., 1998) and gray mold (*Botrytis cinerea*) in strawberry and raspberry (Peng et al., 1992; Sutton, 1995; Yu and Sutton, 1997; Maccagnani et al., 1999; Kovach et al., 2000; Shafir et al., 2006). This technique was also evaluated for the dissemination of viruses to control *Heliothis* in clover (Gross et al., 1994), of insect-pathogenic fungi to control pollen beetles (*Meligethes aeneus*), and of *Beauveria bassiana* for the control of tarnished plant bug (*Lygus lineolaris*) in oilseed rape (Butt et al., 1998; Al Mazra'awi et al., 2006).

6. Alternative Pollinators

With regard to the more than 20,000 species of bees (Michener, 2000), it has been recognized that all but the parasitic ones play a role in pollination, leading to the production of seeds and fruits, and that the various morphological differences

Figure 5. 'Triwax' dispenser for disseminating biological agent via honeybees. The left-hand side of the device is inserted into the hive and foragers exiting the hive are dusted with the powder.

between the species (e.g., body size and absolute and relative tongue length) are related to a certain degree of specialization for the various flower types (Velthuis and van Doorn, 2006). Attempts have been made to use other bee species for crop pollination, but this is not an easy task. The bee species that visit and pollinate the flowers of the target crop must first be identified, then efficient ways to propagate promising species must be developed and finally, effective ways to combine crop plants and pollinator bees must be determined. To date, there are three species of solitary bees that are produced on a commercial basis to provide this kind of pollination service: *Nomia melanderi*, used in the USA and New Zealand for alfalfa pollination, *Megachile rotundata*, also used in several countries for alfalfa, and *Osmia cornifrons*, used in Japan and the eastern USA for apples and almonds (reviewed in Bohart, 1972; Torchio, 1987). A relatively new candidate for commercial rearing is *Osmia lignaria*, which is used in North America for a number of fruit crops, notably almonds and apples (Torchio, 1987; Bosch and Kemp, 2001). The mason bee *Osmia cornuta* appears to be an obvious candidate. This solitary bee occurs in Central and Southern Europe, Turkey and Northern Africa (Peters, 1977) and has been studied in different European countries for the pollination of various crops such as apple, pear, blackberry and red cabbage (Bosch, 1994; Bosch and Blas, 1994; Pinzauti et al., 1997; Ladurner et al., 2000; Vicens and Bosch, 2000a, b; Maccagnani et al., 2003). In an early review, Heard (1999) reported on ca. 90 crop species visited by stingless bees (Apidae: Meliponini). They were confirmed to be effective and important pollinators of nine species. Later, attempts were made to use stingless bees such as *Tetragonisca angustula* and *Nannotrigona testaceicornis* for pollination in enclosures in Costa Rica (Sánchez et al., 2001), *Nannotrigona perilampoides* for greenhouse tomatoes in Mexico (Cauich et al., 2004; Palma et al., 2008) and *Melipona quadrifasciata* for tomatoes in Brazil (Del-Sarto et al., 2005). Aside from stingless bees and the mason bee, in Australia intensive effort was made to find native bee species for pollination of greenhouse tomatoes as an alternative to introduced bumblebee species. One attempt (Hogendoorn et al., 2000) involved two species of carpenter bee, *Xylocopa aeratus* and *Xylocopa bombylans*. Two other attempts in Australia involved the blue-banded bees *Amegilla* (*Zonamegilla*) *holmesi* (Bell et al., 2006) and *Amegilla chlorocyanea* (Hogendoorn et al., 2006). In the US, Waller et al. (1985) experimented with *Xylocopa varipuncta* on cotton. Sadeh et al. (2007) demonstrated that the carpenter bee, *Xylocopa pubescens*, can effectively pollinate melon flowers in enclosures. However, at the present time, none of these bee species are used on a commercial scale.

7. Genetically Modified (GM) Crops

GM crops are becoming more and more abundant, and pollinators may be threatened by those belonging to the insect-resistance group (Jörg et al., 2008). GM crops that express Cry proteins derived from the soil bacterium *Bacillus thuringiensis*

(Bt) have been grown in several countries on steadily increasing acreages since their introduction in 1996. In 2005, transgenic varieties of cotton and maize expressing Bt proteins were grown on 32.1 million ha worldwide (James, 2005). The potentially negative effect of these GM crops on pollinators was highlighted by the case of Bt-maize pollen and the Monarch butterfly (*Danaus plexippus*). When pollen from Bt-maize was spread onto a plant fed to Monarch butterfly caterpillars in the laboratory, the caterpillars died (Losey et al., 1999). This study led to considerable debate over the environmental impact of Bt-maize. Follow-up studies to investigate the impact of widespread planting of Bt-maize on the Monarch butterfly essentially concluded that the impact of Bt-maize pollen from current commercial hybrids on Monarch butterfly populations is negligible (Hellmich et al., 2001; Zangerl et al., 2001). This was based on the low expression of Bt toxin genes in pollen from most GM maize cultivars and a lack of acute toxicity at the expected field rates, as well as limited overlap of pollen shed and larval activity. A number of studies have investigated the possible impacts of GM plants on bees. Direct toxicity is extremely rare and evidence from the most widely grown commercial crops has found no effect on honeybee colony performance (Malone and Pham-Delégue, 2001). At high doses, serine protease inhibitors have been shown to inhibit bee gut proteases, which may result in reduced adult longevity (Malone et al., 2000). In addition, GM crops expressing CrylAb protein (Bt) at high levels may affect honeybee food consumption or learning processes and thereby impact honeybee foraging efficiency (Ramirez-Romero et al., 2008). However, the expression level in pollen from GM plants is not likely to reach the high dose required for these effects. In one study, pollen expression of cowpea trypsin inhibitor (CpTI) reduced the ability of bees to learn a conditioned response to floral odor (Picard-Nizou et al., 1997), although other studies involving the expression of two other serine proteases (Girard et al., 1998) or the cysteine protease inhibitor oryzacystatin (Girard et al., 1998; Jouanin et al., 1998) found no effects on bees' learning or foraging behavior. The controversial findings regarding GM crop effects on pollinators has led to the development of a protocol for risk assessment of those crops to visiting pollinators (Rose et al., 2007).

8. Pesticide and Pollination

For the past 20 years, the effects of pesticides on beneficial arthropods have been the subject of an increasing number of studies, and the potential effects have been the subject of several reviews (Haynes, 1988; Thompson, 2003). Two groups of organisms, natural enemies and pollinators, have received the most attention in this regard (Richards, 1993; Van Driesche and Bellow, 1996). Methods to test the side effects of pesticides have been developed as a function of the beneficial insects and pesticides studied. In each country, regulatory insect risk assessment related to agrochemical use and registration follows specific guidelines (European Council Directive 91/414 in Europe, and the Federal Insecticide Fungicide and

Figure 6. Heavy honeybee losses due to poisoning by the pesticide Dichlorvos.

Rodenticide Act in the USA). For a long time, the classical laboratory method for estimating the side effects of a chemical on beneficial insects was to determine a median lethal dose (LD_{50}) or lethal concentration (LC_{50}). In the second step, the effects of pesticides on beneficial insects were further examined by running selectivity tests (pest/beneficial insect) to identify the products with the lowest nontarget activity (Croft, 1990). In addition to direct mortality (Fig. 6), other negative effects of pesticide application can appear. Insect growth regulators (IGRs) are commercial hormone mimics that disrupt molting (juvenile hormone or acdysone mimics), cuticle formation (chitin inhibitors), or more generally act on the endocrine system (Dhadialla and Carlson, 1998). For hymenopterous social pollinators, perturbations in larval development must be seen as a major threat for colonies. Reductions in broods and in the number of emerging Honeybees may be more damaging to colony health than the loss of foragers, because flexibility in the division of labor can replace foragers if there are sufficient brood and nurse bees (Thompson, 2003). IGRs can interfere with development, particularly when exposure occurs during the larval stage, but also in adults as found for *A. mellifera* and *Apis cerana* (Gupta and Chandel, 1995). For pollinators, visual learning of landmarks is important for spatial orientation. Honeybees use visual landmarks to navigate to a food source as well as to accurately communicate the flight distance and direction of that source to their nest mates (Von Frisch, 1967). A bee exposed to pesticide during a foraging trip may incorrectly acquire or integrate visual patterns, causing it to become disorientated and to get lost (Desneux et al., 2007). Nevertheless, only a relatively small number of studies have investigated this phenomenon, perhaps because of the difficulty involved in measuring parameters such as direction of flight or route time between flowers, and between the flowers and the hive. Techniques for the automatic tracking and identification of individual bees (Capaldi et al., 2000; Reynolds and Riley, 2002; Streit et al., 2003) have the potential to revolutionize the study of pollinators' behavioral ecotoxicology (Desneux et al., 2007). In addition to orientation ability, the general learning performance of pollinators can be affected by exposure to insecticides. Much has been done in this respect in honeybees because of the better understanding of their learning process and the importance of learning in the foraging process. Once memorized, odors play a prominent role in flower recognition during subsequent trips (Menzel and Müller, 1996). Under laboratory conditions, learning can be studied using a bioassay based on conditioning of the PER (Proboscis extension response) bioassay in restrained individuals (Takeda, 1961). The PER assay simulates the natural honeybee–plant interactions that take place when the bees land on flowers: the forager extends its proboscis as a reflex when the gustatory receptor set on the tarsi, antennae, or mouthparts are stimulated with nectar. This reflex leads to the uptake of nectar and promotes memorization of concomitant floral odors. The PER assay has been used with restrained worker bees to investigate the effect of pesticides on learning abilities (Weick and Thorn, 2002). Aside from toxicity, chemical compounds applied to pollinated crops need to be assessed for their repellency effect (Rieth and Levin, 1988).

9. Concluding Remarks

Pollination is a crucial step to achieving high yield and good-quality products in many crops. Populations of the major pollinator, the honeybee, as well as of other pollinators have seen a sharp decline in the last decade. If this process continues, growers might find themselves with low wild pollinator populations and no available commercial pollinators, which will lead to enormous economic losses. Research in crop pollination as well as in pollinator management must be intensively pursued to ensure that this important component in modern agriculture – crop pollination – continues to exist.

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BEE COGNITION AND CROP POLLINATION: PROVEN AND POTENTIAL APPLICATIONS

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1. Benefits of Bees to Agriculture

Make a list of all that you have eaten today. Most likely, your list does not include honey bee larvae – though this is a nutritious food that is a delicacy in some cultures. However, for most of us, about one third of the items on the list would not have been there if not for bees (Delaplane and Mayer, 2000). Clearly, if your list includes honey, you would recognize it as a bee product. But, the main benefit of bees to agriculture is through the pollination services that they provide. The direct benefits of these services would be clear to those who remember that fruits and nuts originate from flowers. Many of these depend on, or profit from, bee pollination. Equally important are indirect benefits of pollination of forage plants, such as alfalfa and clover, for feeding livestock. Pollination is needed in order to produce seeds from which more of these plants can grow.

There are other creative ways in which bees could be used in agriculture. For example, bees can efficiently transport biological control agents from their hive directly to flowers (Shafir et al., 2006). Their developed sense of smell could be used as a biological sensor of plant diseases (Rains et al., 2008).

In this chapter, I focus mainly on bee pollination. I discuss bee cognition and its relevance to crop pollination, and illustrate this relationship with a few examples. In some cases, an understanding of bee cognition has generated agricultural practices that are already being applied. In other cases, findings from basic research in bee cognition suggest potential applications in crop pollination, which remain to be applied.

2. Cognitive Ecology of Pollination

Bees make a living from collecting floral nectar and pollen as their main sources of carbohydrates and proteins, respectively. Unlike a goat, say, which can be enclosed in a particular pasture, bees are free to fly and choose between available flowers. Flight ranges vary between species, but honey bees, for example, can forage in a radius of several kilometers. Bees, of course, are not impressed by our reliance on

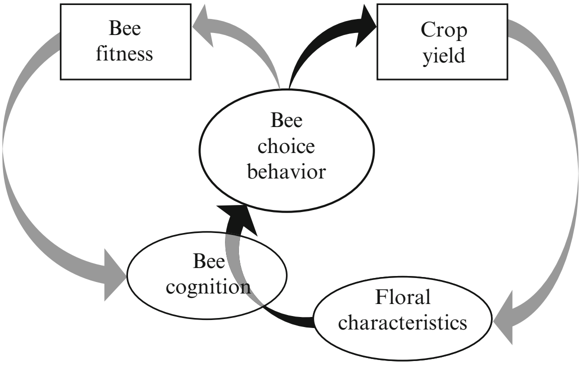

Figure 1. Schematic representation of the processes involved in cognition-mediated crop pollination. Bee choice behavior affects both its own fitness and crop yields. The objective values of the floral characteristics of the crop are filtered through cognitive processes in the bee's brain. This results in a subjective evaluation, which affects choice. In an evolutionary context, bee choice would affect plant fitness, and therefore, also the evolution of floral characteristics. And bee choice would affect bee fitness, and therefore, also the evolution of bee cognition. In an agricultural setting, these feedbacks (*light gray arrows*) are greatly weakened. (Modified from Shafir et al. 2003.)

their services – their foraging decisions are guided by their own needs. The choice behavior of the bees, therefore, affects both their own fitness and the yields of our crops (Fig. 1). Bees must perceive, evaluate, and make decisions about the floral sources available. Thus, crop pollination is cognition-mediated. This understanding is at the basis of the development of the field of the cognitive ecology of pollination (Chittka and Thomson, 2001).

3. Advances in Bee Cognition

Flowers usually consist of a display and rewards. The display acts as advertisement. Odor and visual cues help the bee locate the flower from a distance. At close range, these cues may also guide the bee to the floral rewards. The most common rewards are nectar and pollen. Research in bee cognition has advanced our understanding of how bees perceive odor and visual cues, how they evaluate nectar and pollen rewards, and the learning and memory involved in forming associations between cues and rewards.

3.1. ODOR PERCEPTION

The antennae of bees are covered with chemical receptors that are sensitive to a large array of odors. Odor processing occurs in the glomeruli of the olfactory lobes (Galizia and Menzel, 2001). Honey bees can discriminate between a large array of odors (Drezner-Levy and Shafir, 2007; Guerrieri et al., 2005), and odor perception and discrimination share many similarities with those in vertebrates (Giurfa, 2007). For example, bees can learn specific components of an odor mixture as well as the mixture itself (Deisig et al., 2003). Thus, adding a common component to two different mixtures reduces the ability to discriminate between them (Paldi et al., 2003). Olfactory discrimination can also be impaired by acute ingestion of ethanol (Mustard et al., 2008) and pharmacological manipulations (Hosler et al., 2000).

3.2. VISUAL PERCEPTION

Bees have a trichromatic color vision system, with peak spectral sensitivities in the UV, blue, and green regions (Peitsch et al., 1992). They can only detect objects from a short distance. A flower that differs from the background in the green region of the spectrum can be detected from a farther distance (when the visual angle of the object is >5º); they only use color to discriminate flowers from a close range (visual angle >15º) (Giurfa et al., 1996, 1997). Thus, they are myopic and would only detect a relatively large flower, 5 cm in diameter, having green contrast, from about 45 cm (Giurfa et al., 1996). Floral patterns also affect how well bees can detect them. Flowers with more contrast between dim and bright areas, for the green receptor, are detected from a longer distance (Hempel de Ibarra et al., 2001; Hempel de Ibarra and Vorobyev, 2009).

3.3. OPTIMAL FORAGING THEORY

Optimal foraging theory is concerned with how animals make foraging decisions based on fitness-maximizing criteria (Stephens and Krebs, 1986). Bees are a model organism for testing optimal foraging theory predictions for several reasons: (1) the energetic costs and benefits of foraging (especially for nectar) can be quantified, (2) a forager makes many repeated choices, and (3) foraging decisions are quite dissociated from other decisions (for example, about reproduction).

Bees generally prefer alternatives that are energetically more profitable. They probably cannot directly assess the nutritional value of pollen, though they prefer to collect pollen in an energetically efficient manner (Buchmann and Cane, 1989; Pernal and Currie, 2002; Waddington, 2001). Bees prefer nectar sources that provide greater volumes and sugar concentrations, and less handling and flight times (Seeley, 1995). However, preferences are often partial, and we are interested in understanding what factors affect the degree of preference. We realize that preferences are governed by subjective evaluations, and therefore, an emphasis is given to studying cognitive mechanisms involved in bee foraging decisions (Shafir et al., 2003; Waddington, 1997). Three phenomena have received particular attention: (1) discrimination between rewards based on relative differences following Weber's law of perception, (2) shifts in evaluation of rewards in different contexts, and

(3) sensitivity to variability in reward distributions. I start with discussing these decisions.

The foraging process involves additional decisions that are not about choice between floral alternatives. For example, nectar-collecting bees must decide how much to load their crops during each foraging bout. A honey-bee colony must allocate foragers between those collecting nectar and pollen. I will discuss these decisions later.

3.4. WEBER'S LAW

Weber's law describes a very general perceptual phenomenon in which discrimination between two stimuli depends on the relative, rather than absolute, difference in values between the stimuli. It also applies to the ability of bees to discriminate between nectar volumes and concentrations. Choice proportions are correlated to the difference between rewards divided by their mean value. By varying either flower-handling costs or nectar volume, Waddington and Gottlieb (1990) found that honey-bee choice proportions were affected by relative profitability differences between flowers. Honey bees more strongly prefer a 0.4 μL drop of nectar to no reward (0.4 – 0/0.2 = 2), than a 1.2 μL drop to a 0.4 μL drop (1.2 – 0.4/0.8 = 1), despite the magnitude of the difference between the two alternatives being twice as large in the latter case (Shafir et al., 2005). Bumblebees strongly prefer flowers offering nectar of sugar concentration of 20% than of 10% (20 – 10/15 = 0.67); they only weakly prefer flowers offering nectar of sugar concentration of 50% than of 40% (50 – 40/45 = 0.22) (Waddington, 2001).

3.5. CONTEXT-DEPENDENT CHOICE

What is the subjective value of a flower for a bee, with particular cost and reward characteristics? It would appear reasonable to assume that even though we may not always be able to calculate it exactly, such a value exists. But, the answer, it turns out, is that it depends. It depends on which flowers the bee had previously visited (background context), and which other flowers are present in the choice set with the particular flower (local context). Cognitive psychologists have demonstrated context-dependent choice in humans, thus challenging some basic axioms in rationality theory and classic economic theory. Similar context-dependent phenomena are prevalent in bees.

3.5.1. Background Context

When a honey bee forager returns to the hive from an attractive floral source, she conveys her subjective assessment of the source through the famous bee dance (Frisch, 1967; Seeley, 1995). More attractive sources elicit dances with a higher rate of turns.

Thus, in honey bees, we have a unique tool by which we can assess the individual's subjective evaluation of a foraging alternative.

Raveret-Richter and Waddington (1993) measured the dance rates of foragers trained to feed on sugar solution. For one group, sugar concentrations increased over time, and dance rates increased correspondingly. For another group, sugar concentrations decreased over time. Dance rates in this group decreased sharply. Most interestingly, dance rates for a 40% sugar concentration were almost half in the group that had previously experienced higher concentrations than for the group that had previously experienced lower concentrations.

A strong background context effect was also found by Afik et al. (2008). Avocado nectar, and consequently also honey, is relatively unattractive to honey bees and bumblebees (Afik et al., 2006a, b, 2007). Afik et al. (2008) first trained individual honey bees to feeders with either avocado or citrus honey. Then, the bees were offered a feeder with the other honey. All the bees that had first experienced avocado honey continued foraging from the citrus honey feeder, but most of the bees that had first experienced citrus honey stopped visiting the avocado honey feeder after one visit. Hence, previous experience with an attractive alternative (citrus) made a less attractive alternative (avocado) unacceptable. Without the prior experience, the relatively unattractive alternative was perfectly acceptable.

3.5.2. Local Context

If bees evaluate flowers only according to their objective characteristics, then given any set of, say, four flowers, it would be possible to rank them from most to least preferred. Shafir (1994) showed that this is not always possible. Honey bees were given a choice between pairs of artificial flowers that differed in nectar volume and handling cost. Bees generally preferred flowers that were less costly, though providing smaller rewards to flowers that were a bit more costly and provided somewhat greater rewards. However, given the choice between the two extreme flowers, some of the bees switched their preference and preferred the most costly flower providing the greatest reward to the least costly flower with the smallest reward. This inconsistency shows that bees do not assign fixed values to alternatives, but rather evaluate each alternative relative to the other available alternatives.

A local context effect was also shown in a similar study by Shafir et al. (2002). Honey bees were first given a choice between two artificial flowers, A and B, that differed in nectar volume and handling cost. One (A) was less costly than the other (B), but provided less nectar. Then, a third flower (C), which was less attractive than either of the other two flowers, was added to the choice set. For half of the bees, this flower (C1) was more costly than the other two flowers, yet provided the same nectar volume as the more rewarding of the other two flowers. For the other half of the bees, this flower (C2) provided less nectar than the other two flowers, but was as costly as the less costly of the other two flowers. As expected, in both cases, preference for the third flower was low. However, characteristics of this unattractive third flower affected the relative choice between the other two flowers. The presence of flower C1 increased the attractiveness of flower B relative to A, whereas the presence of flower C2 increased the attractiveness of flower A relative to B.

3.6. RISKY CHOICE

Foraging is a gamble. Floral resources are variable, and bees must make decisions in an uncertain terrain. Nectar volumes are especially variable, with some flowers offering large rewards, while others may be empty. Plant species differ in how variable their rewards are. Such differences are especially large for variability in nectar volume, and less so for variability in nectar concentration (Shafir et al., 2003). There may also be variability in other parameters that are important in the decision-making process, such as in flight or handling times. Bees tend to be risk-sensitive, that is, their foraging choices are affected by reward variability.

As a rule, when variability is in reward amount, bees prefer to avoid gambles; they are risk-averse. The degree of risk aversion depends on the values of several parameters that describe reward distributions (Drezner-Levy and Shafir, 2007; Perez and Waddington, 1996; Shafir, 2000; Shafir et al., 1999, 2005; Shapiro, 2000; Shapiro et al., 2001). Surprisingly, distribution variance and standard deviation are very poor predictors of choice behavior. Risk aversion increases the greater the coefficient of variation (CV = standard deviation/mean), especially for distributions that include empty flowers. Distribution skew may also affect risk attitude (Shafir et al., 2003). The independent effects of empty flowers and distribution skew remain to be tested (Drezner-Levy and Shafir, 2007).

In certain situations, risk attitude may shift towards risk proneness, a greater liking for the more risky alternative. The energy budget rule predicts such a shift for animals whose energy reserves are depleted (Stephens and Krebs, 1986). In a field study, Cartar (1991) found that bumblebees whose nectar reserves were depleted shifted to forage on a plant with greater reward variability. Animals are typically risk-averse when variability is in a hedonically positive outcome, such as amount of food, and are risk-prone when variability is in a hedonically negative outcome, such as delay to receiving food (Marsh and Kacelnik, 2002). There is much evidence in bees for the former situation; the latter has been less studied. When the low-value food reward is a rare occurrence, bees are risk-prone and prefer the (risky) alternative that rewards them more most of the time. However, this is affected by the perceptual accuracy in discriminating between the reward values (Shafir et al., 2008).

3.7. CROP-LOADING DECISIONS

Bees are central-place foragers. They do not immediately consume the nectar and pollen that they collect, but rather transport it back to their colony (social bees) or nest (solitary bees). As a bee loads up, it must decide at what point to stop and return home. The crop-loading decision has been studied most in honey bees. While a honey bee can load more than 70 μl in its crop, it often collects only about half that amount. Crop loads depend on the distance between the hive and the food source, the distance between flowers in a patch, nectar flow rate, ambient temperature, and genotype (Afik and Shafir, 2007; Kacelnik et al., 1986; Moffatt and Nunez, 1997; Schmid-Hempel, 1987; Schmid-Hempel et al., 1985).

3.8. COLONY-LEVEL DECISIONS

The vast majority of bee species are solitary. However, the most important pollinators in agricultural systems are honey bees, with their highly organized social system. Honey bees have division of labor, which includes task specialization among foragers, and a sophisticated communication system (Seeley, 1995). Honey bees allocate a greater proportion of pollen foragers relative to nectar foragers when the quality or quantity of their hive pollen reserves is reduced (Pernal and Currie, 2001). The disposition to collect pollen is partially genetically determined; the queen mates with more than ten drones and patrilines differ in foraging specialization (Fewell and Page, 2000; Oldroyd and Thompson, 2007; Page and Robinson, 1991).

Bumblebees are used for pollination of some specific crops, especially tomatoes in enclosures. A bumblebee colony develops from a solitary phase of a single queen to a social system with several hundreds of workers. Bumblebees have a more simple communication system by which a forager that finds a rich food source can alert its hivemates and cause more foragers to leave the hive in search of the floral odor that it carries (Dornhaus and Chittka, 1999, 2001).

4. Application of Bee Cognition in Crop Pollination

The previous section illustrates some of the recent advances in bee cognition research. Can this knowledge be applied to improve crop pollination? I believe that there is great potential, and suggest some possible directions. However, first I will discuss a proven application, that of sequential introduction of honey-bee colonies.

4.1. SEQUENTIAL INTRODUCTION OF HONEY-BEE COLONIES

Rosaceae fruit trees, such as apple, pear, plum, apricot, almond, and cherry, require cross-pollination between compatible cultivars, and in agricultural settings this service is provided mainly by honey bees (Delaplane and Mayer, 2000; Stern et al., 2007). However, these trees are often not the most attractive option for the bees. The colony's division of labor results in a certain proportion of the foragers being scouts that explore the area for attractive blooms. They evaluate the profitability of these alternatives, and recruit foragers via the dance communication system to the most attractive resources. This results in honey bees often abandoning the bloom of the Rosaceae fruit trees in favor of more attractive fruit trees or competing flora (reviewed in Stern et al. (2007)). This is a typical example in which the sophisticated cognitive abilities of the bees act to *our* disadvantage.

Al-Tikrity et al. (1972) offered a simple solution: to introduce additional colonies to the orchard at a later stage during the bloom. The new colonies are naïve to the available resources in the area and would start foraging on the nearby target fruit crops. This sequential introduction was first tried in pear orchards in Washington and was found to raise the number of bees in the orchard for the first day after the second introduction, which sufficed to increase subsequent fruit set (Mayer, 1994). This technique has been tested in several fruit trees in Israel, pear (Stern et al., 2004), apple (Stern et al., 2001), and plum, including even several sequential introductions (Sapir et al., 2007). Overall, sequential introductions resulted in more naïve bees foraging in the orchard for the first day or two after colony introduction. This resulted in more bees per tree, more bees moving between rows of different cultivars, more bees visiting flowers from the top in a manner that promotes pollination rather than learning to rob nectar from the side (in apples), with subsequent increased yields of between 50% and 100% (Stern et al., 2007). The sequential introductions technique is especially useful for situations in which the bloom is relatively short, yields are pollination limited, and naïve foragers are better pollinators. It has now been adopted as a routine management technique for Rosaceae fruit trees in Israel.

The idea of sequential introduction of honey-bee colonies originates from a basic understanding of bee learning and communication. Progress in the study of bee cognition raises new ideas for applications to improve crop pollination.

4.2. POTENTIAL APPLICATIONS

4.2.1. Increasing the Number of Bee Visits

As illustrated with the example of Rosaceae fruit trees, it is often desired to increase the number of bees visiting a target crop in order to improve pollination and subsequent yields. A general strategy in selection and breeding of crops includes promoting plant health and productivity. Entomological expertise is sought primarily for the purposes of developing crops that are resistant to insect damage (Sleper and Poehlman, 2006). It may prove worthwhile to incorporate more explicit measures of plant performance that increase its attraction to pollinators, such as the production of greater volumes of nectar and higher concentration. Furthermore, selection should take into account reducing the concentration of aversive substances in nectar, such as some minerals (Afik et al., 2006a) and secondary compounds (Irwin and Adler, 2008; London-Shafir et al., 2003).

In addition to increasing the actual profitability and attractiveness of the crop to the pollinator, a lesson from research on bee cognition is that it is important to increase *perceived* profitability. For example, crops with high perceived variability in floral rewards would be unattractive to bees. Selection of crops with reward distributions of lesser coefficient of variation, either by increasing their reward magnitude or decreasing their variability, would increase their perceived attractiveness. Another example relates to colonies that are kept in a distant apiary and brought into an orchard for pollination around first bloom. A prediction based on a background context effect is that colonies brought to pollinate a relatively unattractive crop, say, avocado, would be more attracted to the crop if they came from a poor rather than rich forage area. To achieve efficient pollination, however, it is important that the colonies nevertheless be strong, with a large work force. The local context can also be manipulated to increase the relative attraction of the target crop. Pear, for example, is relatively unattractive to bees owing to its low nectar concentration. Dispersing a plant among the pear trees in the orchard, which provides even lower nectar concentration, would increase the bees' evaluation of the pear nectar.

Bee activity could also be increased by pharmacological manipulations affecting the recruitment system. For example, dance behavior can be enhanced by administering the biogenic amine neuromodulator, octopamine (Barron et al., 2007) or cocaine, to honey bees (Barron et al., 2009). Octopamine may also shift foraging preferences towards lower nectar concentrations (Giray et al., 2007); this may increase the activity on crops with low nectar concentration. Bumblebee activity can be enhanced by exposing the nest to eucalyptol, the most active component of the bumblebee-recruitment pheromone (Granero et al., 2005).

Another strategy to increase bee activity in the target orchard would be to decrease the activity on competing flora. Colonies placed in an orchard send out scouts to explore distant alternatives. Scouts that find an attractive resource recruit other foragers by means of the bee dance. Gradually, the colonies abandon the target crop in favor of the attractive alternative. Disrupting the dance communication system would greatly reduce the number of bees abandoning the target crop. When hives are turned so that the combs are horizontal, rather than in their normal vertical orientation, dances are disoriented (Kirchner and Grasser, 1998; von Frisch, 1967). They no longer communicate distance and direction to the source. While recruits can still rely on odors carried by the dancer to locate the floral source, this becomes less effective for longer distances (Kirchner and Grasser, 1998). Furthermore, recruitment to sources in the vicinity of the hive is not affected by comb orientation. Thus, this technique may be used to maintain foragers in the target orchard and prevent recruitment to attractive distant alternatives.

4.2.2. Increasing Cross-pollination

It is not always sufficient to increase the number of bee visits on the target crop. Some crops and hybrid seed production require cross-pollination between different genotypes. Bees often preferentially visit the more profitable alternative.

If alternatives are similar in profitability, individuals may nonetheless develop constancy to one of them (Chittka et al., 1999). Such preferences require bees to be able to discriminate between alternatives, usually based on olfactory and visual cues. Reducing the ability of bees to discriminate between floral sensory stimuli and rewards would increase the probability that they would switch between the alternatives.

Discrimination between genotypes could be partially hampered by selection for genotypes with greater similarity in olfactory and visual stimuli as perceived in bee sensory space. Reduced discrimination could also be achieved by adding, through selection or genetic engineering, common components to the olfactory and visual displays of different genotypes. Based on Weber's law of relative discrimination, a similar effect could be achieved by increasing the overall intensity of stimuli that are similar between genotypes, so that those that differ become less apparent. Similarly, increasing the overall reward levels would reduce the effect of any existing differences in profitability between the alternatives. Finally, drugs could be administered that affect discrimination performance.

For any particular probability of a bee switching between genotypes during a single foraging bout, the chance for at least one switch is greater the greater the number of floral visits during that bout. Bees that collect larger crop loads would generally need to make more floral visits. Thus, increasing crop loads by manipulating the various parameters that affect crop-loading decisions, discussed earlier, should enhance cross-pollination.

4.2.3. Breeding of Selected Bee Lines

The genetic basis of honey bee behavior has been demonstrated in many cognitive tasks, including some that relate to foraging (reviewed by Oldroyd and Thompson (2007)). Differences between genetic lines have also been shown specifically in crop pollination (Afik et al., 2007, 2010; Dag et al., 2003, 2005; Gary and Witherell, 1977; Mackensen and Nye, 1966; Shimanuki et al., 1967). In studies that compare overall foraging or pollination performance, it is often not clear what trait exactly had been selected for. Such studies, in conjunction with recent advances in the study of the bee genome (Weinstock et al., 2006), could provide better insight into the precise traits under selection. Modern designs of bee-breeding programs (Laidlaw and Page, 1997; Oldroyd and Thompson, 2007) could be applied towards selection on many of the cognitive abilities reviewed in this chapter. There is great potential for applying a variety of techniques by which advances in our understanding of bee cognition could be implemented towards improving pollination and yields of agricultural crops.

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Additional projects combining his interests in archeology and biology were the study of the chemistry and molecular biology of pigments obtained from scale aphids. He identified a species of Kermes oak coccid that grew in Israel, the aphid from which the precious Biblical scarlet dye [*tola'at shani*] was extracted, providing new insights on the ancient dye trade.

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ZOOCHORY: THE DISPERSAL OF PLANTS BY ANIMALS

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1. Introduction

Zoochory is the dispersal of diaspores by animals. Animals can disperse plant seeds in several ways. Seeds can be transported on the outside of animals, a process known as epizoochory. Seed dispersal via ingestion by animals, or endozoochory, is the dispersal mechanism for most tree species. The *diaspores of* endozoochorous plants are eaten by animals and humans and the hard seeds or fruit stones pass through the intestinal canal without damage (Smallwood, 1984). Synzoochory is the dispersal of seeds (nuts), which are an attractive long-term, storable food resource for animals (e.g., acorns, hazelnuts, and walnuts): the seeds are stored some distance from the parent plant, and some escape being eaten if the animal forgets them. Synzoochory is mainly performed by ants and some birds. The dispersal process that involves birds carrying plant propagules in their bills is called stomatochory, a type of synzoochory (van der Pijl, 1982). Dyszoochory is the process whereby diaspores are destroyed (eaten and digested), but some of the seeds are accidentally dropped, thus contributing to regeneration (van der Pijl, 1982).

Endozoochory indicates the mutualistic relationship between animals and plants (Bascompte and Jordano, 2007). Synzoochory seems to indicate a predation relationship, but, in many cases, it has positive results on plants. In epizoochory, the plant benefits because its dispersal units are dispersed while the animal does not benefit but does not lose the seeds or fruits attached to its body; therefore, this relationship can be characterized as commensalism.

2. Epizoochory

Plant species transported externally by animals can have a variety of adaptations for dispersal, including adhesive mucus, and a variety of hooks, spines, and barbs, which are also used against predators. These fruits do not supply compensation and do not have means to exhibit and advertise. The success of long-distance dispersal is that the fruits meet with the dispersal animals randomly and the animals are not aware of the dispersal unit on their bodies (Sorenson, 1986). A typical

Figure 1. **(a)** *Trifolium angustifolium*; **(b)** *Xanthium strumarium* **L.**; **(c)** *Tribulus terrestris*; **(d)** *Neurada procumbens* on bike tires.

example of an epizoochorous plant is *Trifolium angustifolium* (Fig. 1a), a species of Old World clover that adheres to animal fur by means of stiff hairs covering the seed (Manzano and Malo, 2006). Several plants developed seeds and fruits that attach to fur. Xanthium and many of the Umbelliferae have spines and hooks that get entangled in hair and get carried by the unaware animal over great distances (Fig. 1b). *Tribulus terrestris*, *Xanthium strumarium* L., and *Neurada procumbens* have fruits that penetrate the soles of animals (even car and bike tires) and are dispersed by them (Fig. 1c, d).

Epizoochorous plants tend to be herbaceous, with many representative species in the Apiaceae and Asteraceae families (Sorenson, 1986). However, epizoochory is a relatively rare dispersal syndrome for plants as a whole; the percentage of plant species with seeds adapted for transport on the outside of animals is estimated to be below 5% (Sorenson, 1986). Nevertheless, epizoochorous transport can be highly effective if seeds attach to wide-ranging animals. This form of seed dispersal has been implicated in rapid plant migration and the spread of invasive species (Manzano and Malo, 2006). Growing recognition of the importance of long-distance dispersal (LDD) of plant seeds for various ecological and evolutionary processes has led to an upsurge of research into its underlying mechanisms. Nathan et al. (2008) summarized these findings by formulating six generalizations stating that LDD is generally more common in open terrestrial landscapes, and is typically driven by large and migratory animals, extreme meteorological phenomena, ocean currents, and human transportation, each transporting a variety of seed morphologies. LDD is often associated with the unusual behavior of the standard vector inferred from plant dispersal morphology, or mediated by nonstandard vectors.

Sorenson (1986) claims that in distributed habitats, the relative frequency of attached fruits is high; in desert habitats (semideserts), the frequency of attached fruits in plant populations is also high, but in true deserts, the amount of attached fruits is only 1% (Elner and Shmida, 1981). In relation to wind or ant dispersal, epizoochory is an efficient mechanism for LDD but in relation to other means of dispersal, there is no guarantee that the dispersing animal will reach the appropriate place (Shmida and Elner, 1983).

3. Endozoochory

Endozoochory is generally a coevolved mutualistic relationship in which a plant surrounds seeds with an edible, nutritious fruit as a good food for animals that consume it. Birds and mammals are the most important seed dispersers, but a wide variety of other animals, including turtles and fish, can transport viable seeds (Corlett, 1998). The exact percentage of tree species dispersed by endozoochory varies between habitats, but can range to over 90% in some tropical rainforests (Smallwood, 1984). Seed dispersal by animals in tropical rainforests has received much attention, and this interaction is considered an important force shaping the ecology and evolution of vertebrate and tree populations (Terborgh, 1986). In the tropics, large animal seed dispersers (such as tapirs, chimpanzees, and hornbills) may disperse large seeds with few other seed dispersal agents. The extinction of these large frugivores (fruit-eaten) from poaching and habitat loss may have negative effects on the tree populations that depend on them for seed dispersal (Chapman and Onderdonk, 1998).

From the plant point of view, its “interest” is to attract the frugivores to eat its fruit but not damage the seeds. The attraction of the frugivores (mainly vertebrata, especially birds and mammals) to fruit dispersal is similar to flower pollination: reward to the dispersers is by display and advertisement. Rewards are found in fruit flesh as food materials – carbohydrates, proteins, oils, other materials, and water. Advertisement is expressed by fruit color, color contrast, smell, fruit size, and fruit density on plant (van der Pijl, 1982). Observations show that mutuality adaptation between fruits and distributors is expressed by fruit color or smell, for example, black or red fruits were eaten by birds while green, white, and yellow fruits were eaten mainly by mammals (van der Pijl, 1982). Debussche and Isenmann (1989) found high overlapping between birds and mammals in fruit-eating. Mammals like hedgehogs, foxes, and honey badgers eat fruits that are typical for birds and seem to be a factor for evolutionary selection. Also, Herrera (1989, 2002) made similar observations in Mediterranean chaparal vegetation in Spain.

The attracting force of the fruit for frugivores comes from fruit nutritional value and how easy it is to extract the nutrients from the juicy fruit flesh. The widespread nutrients in juicy fruit flesh are mainly carbohydrates and/or oils. Carbohydrate amount in different fruits is 5–90% dry weight, and oil amount in general is 1–50%. Protein content is low in general, 2–6% (Izhaki and Safriel, 1989).

The fact that fruits do not have enough proteins prevents frugivores from using juicy fruits as an exclusive food source, so they supplement their diet by eating insects, whose bodies have 70% protein content. Nutritious value is also affected by materials that reduce the amount of palatability, such as tannins or toxins. These materials are found in unripe and immature fruit. They prevent the attack of harmful fungi and insects and deter typical frugivores – birds and mammals – from eating the fruits before the seeds mature (Izhaki and Safriel, 1989).

The influence of seed dispersers on the evolution of fruit traits remains controversial, largely because most studies failed to account for phylogeny and/or focused on conservative taxonomic levels. Under the hypothesis that fruit traits have evolved in response to different sets of selective pressures by disparate types of seed dispersers (the dispersal syndrome hypothesis), Lomascolo et al. (2008) tested two dispersal syndromes, defined as groups of fruit traits that appear together more often than would be expected by chance: (1) bird syndrome fruits are brightly colored and small, because birds have acute color vision, and commonly swallow fruits whole; and (2) mammal syndrome fruits are dull-colored and larger on the average than bird syndrome fruits, because mammals do not rely heavily on visual cues for finding fruits, and can eat fruits piecemeal. If, instead, phylogenetic inertia determines the co-occurrence of fruit size and color, Lomascolo et al. (2008) observed that specific combinations of size and color evolved in a small number of ancestral species. They performed a comparative analysis of fruit traits for 64 species of Ficus (Moraceae) based on a phylogeny constructed using nuclear ribosomal DNA. Using a concentrated change test and assuming fruit color is an independent variable, Lomascolo et al. (2008) found that small-sized fruits evolve on branches with red and purple figs, as predicted by the dispersal syndrome hypothesis.

In most cases, the fruits are consumed by the animals and birds and deposited in their droppings or feces. In some cases, passage through the digestive system serves to remove impermeable waxes and coatings as well as germination retardants, and the feces provide moisture and nutrients to the seedlings. Sometimes, fruit-eating improves germination, sometimes it prevents it, and sometimes it has no influence (Howe, 1986). In the marine plant, *Najas marina*, it was found that the eating of seeds with hard peels by Mallard ducks (*Anas platyrhynchos*) improves the germination amount of seeds not digested by increasing the grinding.

Another classic and famous example that germination is entirely dependent on transition seeds through the stomach is the dodo birds, which were once inhabitants of Mauritius. Stanley Temple (1977) hypothesized that the extinction of the dodo was responsible for the near extinction of the tambalacoque or calvaria tree (*Sideroxylon grandiflorum*, formerly *Calvaria major*). He proposed that dodo and tambalacoque represented an obligate animal–plant mutualism in which the tambalacoque seeds had to pass through the dodo digestive system before they could germinate. Temple assumed that the tambalacoque's fruit, with its thick and hard endocarp, would mechanically prevent germination unless worn down by the dodo digestive system. He drafted a flock of turkeys to serve in a germination experiment.

Of the 17 tambalacoque pits he force-fed to the turkeys, his dodo-substitute, only three germinated. Temple also claimed that no tambalacoque trees were less than 300 years old, but had no data to support that other than second-hand estimates, since tambalacoque trees have no annual rings so their age is not easy to determine (Hershey, 2004; Temple, 1977).

Parasite plants such as *Viscum cruciatum* use the *Mistle Thrush bird* in order to reach another host plant (Fig. 2). Its fruit is a berry that is white, yellow, orange, or red when mature, and containing very sticky juice seeds. The seeds are dispersed when birds (notably the *Mistle Thrush*) eat the fruit, the seed is not digested, and when it comes out it does not fall because it is very sticky. To remove it, the bird uses its legs by wiping them on tree branches where they can germinate. This dispersal could be endozoochory but mainly the dispersal epizoochory is widespread.

Small frugivorous birds that feed largely on the fruits of stem-parasitic mistletoes have independently evolved in various parts of the world. Local populations of mistletoes may be dispersed almost exclusively by these birds. Four attributes of mistletoe dispersal systems may have enhanced the evolution of

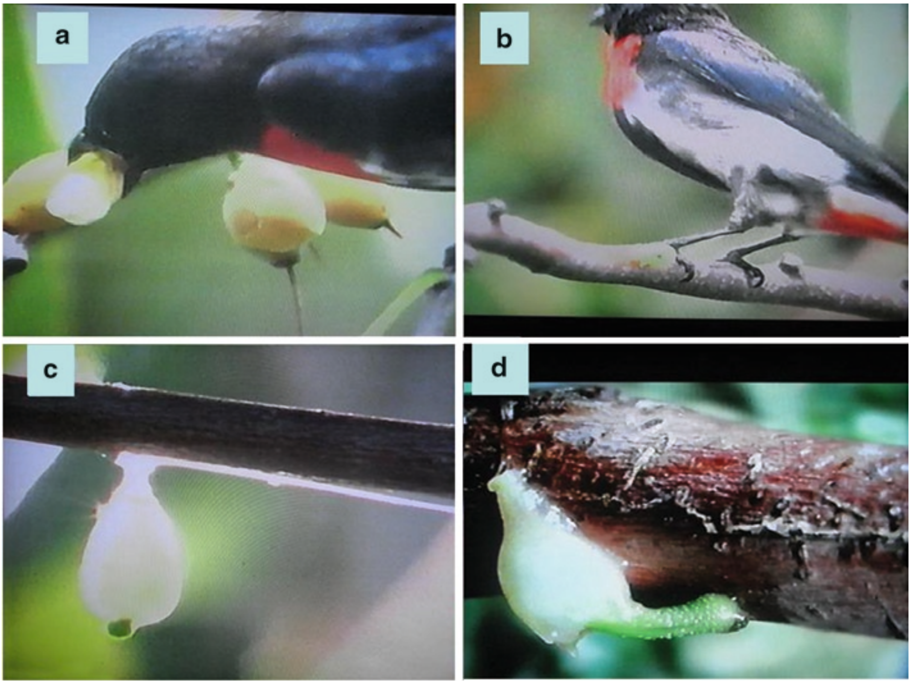

Figure 2. Dispersal of juicy fruit by birds that ensure seed germination on another host tree: (**a**) the bird eats the juicy fruit; (**b**) the bird sticks the seed by her legs on a branch; (**c**) the seed is stuck on a branch; (**d**) the seed starts to germinate into the tree branch.

reciprocal dependence between mistletoes and specialized dispersers: (1) safe sites for mistletoe seeds (i.e., the young branches of a compatible host) are precisely defined in space and time; (2) the viscidity of mistletoe seeds induces smaller dispersers to deposit seeds at safe sites; (3) frugivores differ markedly in the efficiency with which they disperse mistletoe seeds to safe sites; and (4) relatively large viscid fruits and adaptive fruiting displays exclude or deter most members of the potential disperser guild (Levin et al., 2003; Reid, 2006).

Some birds have anatomical adaptations as a result of a specialized diet of mistletoe fruit, and some mistletoe has fruiting displays that target specialized birds or a narrow disperser spectrum. Coevolution between guilds of mistletoes and specialized dispersers is, therefore, probable. The uncoupled selective pressures that might have driven this coevolution are the mistletoes' provision of fruit crops that are unavailable to more generalized frugivores, in return for seed dispersal to the small stems most suitable for infection. As in other mutualistic seed dispersal systems, phylogenetic, ecological, and life history factors constrain the evolution of monophyletic interdependence, resulting in varying degrees and patterns of reciprocal specificity between mistletoes and dispersers (Reid, 2006).

Most frugivores are vertebrate mammals or birds, and there are also known Osteichthyes and Reptilia that eat fruits. Some fish, especially *Tilapia zillii*, eat and secrete seeds of the *Najas delilei* marine plant (Agami and Vaisel, 1986). Birds are the most important dispersal factor of seeds. The mammals are mainly: bats, primates, rodents, and big mammals (megafauna) from different systematic groups (Ungulata, elephants, etc.). Big terrestrial mammals such as elephants (Kitamura et al., 2007), cows, horses, and pigs are used to eating a lot of fruits containing seeds. Part of the seeds is digested, but remaining many essential seeds not damaged remains in their droppings, and these seeds appear at a density assembly point that exposes them to stiff competition at germination time. Rodents eat these seeds from droppings, decrease the density of the seeds, and contribute the remaining seeds in order to survive. In Africa, many seeds of acacia species germinate from elephant droppings, and it seems that the acacia depends on the elephant eating for helping its germination. Also, in the southern Negev (Israel), there is a positive influence on the germination of acacia species. Seeds passing through the digestive system of *Dorcas Gazelle* are essential and germination increases. Another benefit of acacia is that beetles (*Bruchidoe*) harm acacia seeds and the *Gazelle* eats them also. Digestive juices destroy larvae and prevent damage to embryos. The improvement and acceleration in germination are very essential to germination promise in conditions of not regular soil wetting in desert area (Halevy, 1974). In the eastern North America, it was found that white-tailed deer represent a significant and previously unappreciated vector of seed dispersal across the North American landscape, probably contributing an important long-distance component to the seed shadows of hundreds of plant species, and providing a mechanism to help explain rapid rates of plant migration (Myers et al., 2004).

4. Synzoochory

Seed predators, which include many rodents (such as squirrels) and some birds (such as jays), might also disperse seeds by hoarding them in hidden caches (Forget and Milleron, 1991). The seeds in caches are usually well protected from other seed predators and, if left uneaten, will grow into new plants. Finally, seeds may be secondarily dispersed from seeds deposited by primary animal dispersers. For example, dung beetles are known to disperse seeds from clumps of feces while in the process of collecting dung to feed their larvae (Andresen and Levey, 2004).

Best known in this respect are the nutcrackers (*Nucifraga*), which feed largely on the "nuts" of beech, oak, walnut, chestnut, and hazel trees; the jays (*Garrulus*), which hide hazelnuts and acorns; the nuthatches; and the California woodpecker (*Balanosphyra*), which may embed literally thousands of acorns, almonds, and pecan nuts in bark fissures or holes of trees. Secondarily, rodents may aid in dispersal by stealing the embedded diaspores and burying them. In Germany, an average jay may transport about 4,600 acorns per season over distances of up to 4 km (2.5 miles). Woodpeckers, nutcrackers, and squirrels are responsible for similar dispersal of *Pinus cembra* in the Alps near the tree line.

5. Myrmecochory

Myrmecochory is the dispersal of seeds by ants. Foraging ants disperse seeds that have appendages called elaiosomes (Terborgh, 1986) (e.g., bloodroot, trilliums, acacias, and many species of Proteaceae). Although myrmecochory is a type of synzoochory, it will be discussed as a separate animal-dispersal mechanism. Myrmecochory is an example of true mutualism, which has arisen independently in so many groups of plants that there must be very strong pressures, which favor this type of dispersal mechanism (Handel and Beattie, 1990).

Elaiosomes are soft, fleshy structures that contain nutrients for the animals to eat (Fig. 3). The ants carry such seeds back to their nest, where the elaiosomes are eaten. The remainder of the seed, which is hard and inedible to the ants, then germinates either within the nest or at a removal site where the seed has been discarded by the ants (Chapman and Onderdonk, 1998).

There is also a well-documented body of evidence showing the advantages of seeds remaining in the nest, where they are protected against fire and/or predators (e.g., Christian and Stanton, 2004). The presence of an elaiosome may be important for the transport of the seed to the nest, but its absence after seed manipulation implies that the shape of the seed, its size, or the presence of surfaces that can provide a new handle for the ants, may be key factors in the outcome of the interaction, i.e., the final location of the seed. The final location of seeds with their elaiosomes removed was evaluated to assess the importance of possible handles in transporting ants during redispersal experiments of seeds from nests of six species of ants. The results indicate that seeds remained within

Figure 3. *Left*: Seeds of *Chelidonium majus* are small and black, and possess an elaiosome that attracts ants to disperse the seeds. (Image from: tt.ibt.lt/.../Papaveraceae/Chelidonium-majus/.) *Right*: Ant (*Messor sp*.) tow-boat seed of the plant *Ricinus communis.* (Photographed by Simon Danny.)

the nest because the ants were not able to transport them out of the nest. As a consequence of the elaiosome being removed, small ant species could not take *Euphorbia characias* seeds out of their nests. Only large ant species could remove *E. characias* seeds from their nests. Attaching an artificial handle to *E. characias* seeds allowed the small ant species to redistribute the seeds from their nests. On the other hand, *Rhamnus alaternus* seeds that have a natural handle after elaiosome removal were removed from the nests by both groups of ant species. If a seed has an element that acts as a handle, it will eventually be removed from the nest. The ant size and their mandible gap can determine the outcome of the interaction (i.e., the pattern of the final seed shadow) and, as a consequence, could influence the events that take place after the dispersal process (Gómez et al., 2005).

This dispersal relationship is an example of mutualism, since the plants depend on the ants to disperse seeds, while the ants depend on the plant seeds for food. As a result, a drop in numbers of one partner can reduce the success of the other. In South Africa, the Argentine ant (*Linepithema humile*) invaded and displaced the native species of ants. Unlike the native ant species, Argentine ants do not collect the seeds of *Mimetes cucullatus* or eat the elaiosomes. In areas where these ants have invaded, the numbers of *Mimetes* seedlings have dropped (Forget and Milleron, 1991).

Myrmecochory is widespread both taxonomically and geographically and is a phenomenon found in at least 3,000 species in 60 families of plants in a wide range of climates and habitats around the world (Beattie, 1983; Horvitz and Schemske, 1986). A major hypothesis concerning the benefits of myrmecochory, seed dispersal by ants, to plants is that ant nests are nutrient-enriched microsites that are beneficial to seedling growth. Horvitz and Schemske (1986) tested this hypothesis experimentally on a neotropical myrmecochore, *Calathea ovandensis*, and found that although ant-nest soil was significantly enriched in nitrate-nitrogen, magnesium, iron, manganese, cadmium, and percent of organic matter compared to randomly collected soil, seedling growth was not significantly improved by ant-nest soil. In the Australian environment, it was also reported that, compared to control soil, soil from nest mounds of *Aphaenogaster longiceps* enhances the growth of seedling roots and shoots by about 50% in glasshouse trials. However, this benefit of nutrient-enrichment probably only occurs when seeds are dispersed by ants that construct large, long-lived, nest mounds. This is very often not the case, and there is now increasing evidence that distance dispersal is often the major benefit of myrmecochory in Australia (Andersen 2006a, b).

Mediterranean and North American harvester ants (*Messor*, *Atta*, *Tetramorium*, and *Pheidole*) are essentially destructive, storing and fermenting many seeds and eating them completely. Other ants (*Lasius*, *Myrmica,* and *Formica* species) eat the fleshy, edible appendage (the fat body or elaiosome) of certain specialized seeds, which they disperse. Most myrmecochorous plants (species of *violet*, *primrose*, *hepatica*, *cyclamen*, *anemone*, *corydalis*, *trillium*, and *bloodroot*) belong to the herbaceous spring flora of northern forests. Tree poppy (*Dendromecon*), however, is found in the dry California chaparral; *Melica* and *Centaurea* species in arid Mediterranean regions. The so-called ant epiphytes of the tropics (i.e., species of *Hoya*, *Dischidia***,** *Aeschynanthus***,** and *Myrmecodia* – plants that live in "ant gardens" on trees or offer the ants shelter in their own body cavities) constitute a special group of myrmecochores, providing oil in seed hairs, which in ancestral forms must have served in wind dispersal. The primary ant attractant of myrmecochorous seeds is not necessarily oil, instead, an unsaturated, somewhat volatile fatty acid is suspected in some cases. The myrmecochorous plant as a whole can also have specific adaptations, for example, Cyclamen brings fruits and seeds within the reach of ants by conspicuous coiling (shortening) of the flower stalk as soon as flowering is over.

6. Ornithochores – Dispersal by Birds

Birds, being preening animals, rarely carry burr-like diaspores on their bodies. They do, however, transport the very sticky (viscid) fruits of *Pisonia*, a tropical tree of the four-o'clock family, to distant Pacific islands in this way. Small diaspores, such as those of sedges and certain grasses, may also be carried in the mud sticking to waterfowl and terrestrial birds.

Most ornithochores have conspicuous diaspores attractive to such fruit-eating birds as thrushes, pigeons, barbets (members of the bird family Capitonidae), toucans, and hornbills (family Bucerotidae), all of which either excrete or regurgitate the hard part undamaged. Such diaspores have a fleshy, sweet, or oil-containing edible part; a striking color (often red or orange); no pronounced smell; protection against being eaten prematurely in the form of acids and tannic compounds that are present only in the green fruit; protection of the seed against digestion – bitterness, hardness, or the presence of poisonous compounds; permanent attachment;

and, finally, the absence of a hard outer cover. In contrast to bat-dispersed diaspores, they occupy no special position on the plant. Examples are rose hips, plums, dogwood fruits, barberry, red currant, mulberry, nutmeg fruits, figs, blackberries, and others. The natural and abundant occurrence of *Euonymus* (cardinal's hat), essentially a tropical genus, in temperate Europe and Asia, can be understood only in connection with the activities of birds. Birds also contributed substantially to the repopulation with plants of the island Krakatoa after the catastrophic eruption of 1883. Birds have made *Lantana* (originally American) a pest in Indonesia; the same is true of wild plums (Prunus serotina) in parts of Europe and *Rubus* species in Brazil and New Zealand.

The fruits and seeds of plants are an important part of the diet of birds in forest ecosystems (McCarty et al., 2002). In Japan, which is mostly characterized by a temperate climate, bird-dispersed plants comprise a large proportion (40–80%) of the woody plant species in forest communities (Nakanishi, 1996), and their fruits are consumed by wide arrays of birds (Kominami et al., 2003). The interactions between fruits and birds have great importance for the maintenance of both the bird and plant populations. For the birds, the fruits are essential sources of energy, especially in the autumn, during bird migrations (Noma and Yumoto, 1997; Takanose and Kamitani, 2003), and in the winter, in temperate regions, when other available diets are scarce (Kwit et al., 2004; McCarty et al., 2002). For bird-dispersed plants, fruit consumption by the birds is a prerequisite for successful seed dispersal, although their seeds can be lost to seed predation by nonseed dispersing birds (Englund, 1993; Traveset, 1994a, b). The compositions of plant species consumed by the birds determine the structure of bird–plant interactions, and they are highly implicated in the stability and sustainability of both the bird and plant communities (Carlo et al., 2003; Kitamura et al., 2002; Moran et al., 2004). Yoshikawa et al. (2009) examined the interactions between bird-dispersed plants and fruit-consuming birds with various feeding strategies, by reviewing the plant species consumed by 14 bird species in Japan with four feeding types: gulpers (5 species), grinders (4 species), crushers (4 species), and peckers (1 species). Logistic regression analysis revealed that some crushers and the pecker preferred plants with dry or arillate pulp around the seeds. Yoshikawa et al. (2009) suggests that a frugivorous bird's feeding strategies, and particularly its fruit-handling behaviors and the fruit parts it ingests, influence the diversity of the plants it consumes. The crushers and the pecker, which feed exclusively on seeds, require more effort and time to consume this type of food, and this might cause a strong preference for specific fruit traits, and thus, consumption of a lower diversity of plant species.

In the Negev area (Israel), the bird *Pycnonotus xanthopygos* (Spectacled Bulbul) acts as a distributor of the parasite plant *Loranthus acaciatae*. The fruit of this plant is a berry (red when mature) containing seeds embedded in very sticky juice. The seeds are dispersed when these birds eat the fruit and remove the sticky seeds from the bill by wiping them on tree branches, where they can germinate (Fig. 4).

Figure 4. *Loranthus acaciatae* (wide leaves) is a parasite on the *acacia* tree visited by birds.

Vertebrate dispersal of fruits and seeds is a common feature of many modern angiosperms and gymnosperms, yet the evolution and frequency of this feature in the fossil record remain unclear. Increasingly complex information suggests that: (a) plants had the necessary morphological features for vertebrate dispersal by the Pennsylvanian, but possibly in the absence of clear vertebrate dispersal agents; (b) vertebrate herbivores first diversified in the Permian, and consistent dispersal relationships became possible; (c) the Mesozoic was dominated by large herbivorous dinosaurs, possible sources of diffuse, whole-plant dispersal; (d) simultaneously, several groups of small vertebrates, including lizards and, in the later Mesozoic, birds and mammals, could have established more specific vertebrate--plant associations, but supporting evidence is rudimentary; and (e) the diversification of small mammals and birds in the Tertiary established a consistent basis for organ-level interactions, allowing for the widespread occurrence of biotic dispersal in gymnosperms and angiosperms (Butler et al., 2009; Tiffney, 2004).

7. Human and Dispersal Plants

Human transportation is also an immense zoochorous agent, spreading invasive marine and terrestrial plant species that displace and replace native floras.

Dispersal of seeds by humans is also responsible for the spread of plants around the world, and may lead to nonnative species invasions. Matthias et al. (2008) provide quantitative evidence that humans may be more important than natural agents such as wind power for dispersal attachment to hikers' boots.

They found that while wind dispersal takes seeds a few meters, adhesion to boots dispersed seeds over 5 km. Only a few seeds may go very far, but these are important seeds – pioneers – since they colonize new sites. Salisbury (1942) directed attention to the accidental carriage and distribution of seeds by human activity, and pointed out that clothes can be one of a number of means of distribution.

Many vertebrate animals in tropical regions are seriously threatened by human development and the consequent deforestation and fragmentation that result. Hunting and illegal pet trade also seriously threaten many medium- and large-sized vertebrates in tropical areas. The end result will be forests that contain fewer individuals and fewer species. How the elimination of large vertebrates may affect tropical forest dynamics has only recently been explored. Evidence suggests that the absence of large- and medium-sized vertebrate dispersers will result in reduced seed movement for animal-dispersed species with very large seeds, reduced seed predation by granivorous vertebrates for large-seeded species, and a change in the composition of the seedling and sapling cohorts. Most avenues of research suggest that the elimination of vertebrate seed dispersers will result in changing tropical forest composition and structure. The long-term consequences of this are unknown.

Long-distance seed dispersal is currently of intense interest because of its critical importance for invasion dynamics, population structure, and vegetation response to climate and land-use changes (Bullock et al., 2002; Cain et al., 2000; Pitelka, 1997). Environmental change, whether natural or anthropogenic, is constantly altering local habitat conditions that influence demographic processes in plants. When previously unsuitable habitat conditions become suitable for establishment, successful colonization depends on dispersal of seeds into the altered habitat. If there are no nearby seed sources, "long-distance" dispersal is essential (i.e., >100 m; Cain et al., 2000). The dynamics of metapopulations and genetic structure, especially in fragmented habitats, and rates of plant migration (post-glacial and otherwise), are also strongly influenced by long-distance movements (Myers et al., 2004).

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PART 4:
ANIMALS AND HUMANS INVOLVEMENT

Landau
Molle
Whitford
Steinberger
Kociolek

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GRAZING LIVESTOCK, OUR CONNECTION TO GRASS: A MEDITERRANEAN INSIGHT

Why They Eat What They Eat, and How It Affects Us

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The presence of Man in Mediterranean harsh environments, where the conversion of sun energy to grass – including herbaceous and ligneous vegetation – is the only asset, would not be possible without domestic livestock. Grazing livestock have developed foraging behaviors – largely affected by their interaction with humans throughout history – that allow them minimizing the intake of toxic plant secondary metabolites (PSMs) and mitigating their negative effects. They have evolved to cope with extreme nutritional situations and to fit their reproductive cycles to seasonal cycles of nutrient availability. They can probably learn how to minimize the negative effects of parasites by ingesting plants containing PSMs with pharmaceutical properties where available. Most of this knowledge is taught mainly by mothers, but peer influence and individual learning by trial-and-error are also instrumental in surviving in an ever-changing world. Grazing livestock possess foraging cultures transmitted from generation to generation. With an emphasis on Mediterranean conditions, we review the scientific basis of foraging cultures, and how they may impact on our own body. In particular, we address the flow and modifications of fatty acids from grass to man, through the composition of milk or meat, which we consume, with emphasis on the roles of PSMs on the sensory properties and the health value of grazing livestock products. Interestingly, new technologies, such as stable element isotope analysis, are successful in identifying regional fingerprints in meat, probably related to foraging cultures that bridge between local human cultures and their environment.

1. Domesticating Grazing Animal as Means to Collect Grass

In this chapter, grass, the most ubiquitous form of transformed sun energy on continental Earth, stands for “photosynthetic vegetation,” encompassing a wide array of herbaceous and ligneous species subjected to grazing, as typical pastoral systems in the Mediterranean area rely on the exploitation of heterogeneous mosaics of grasslands and brushlands mainly by small ruminants. If agriculture can be considered as a way of harnessing sun energy by exploiting plants photosynthesis, then exploiting grass can be looked upon as the alternative in pre-agricultural societies or wherever ecological conditions limit agriculture (extreme temperatures, extreme winds, conditions that limit completion of domestic crop cycles such as too much or too little water, shallow or salty soils).

Being devoid of cellulolytic digestive enzymes, humans have a very limited ability to digest nonreproductive plant material, at the exception of leaves and stems at very early phenological stage, so optimal utilization of grass by humans is not straightforward.

As many ungulates harbor ruminal or caecal floras with cellulolytic abilities that can transform grass at all phenological stages into nutritionally usable meat, their domestication was pivotal toward more efficient grass utilization. Indeed, the synchrony in time (10,000 years ago) and contiguity in space (in the Mid-Eastern Fertile Crescent) of the domestication processes of grazing ruminants (Zeder and Hesse, 2000), and small grain and pulse-crops (Lev-Yadun et al., 2000), strongly backs this way of thinking.

From the variety of herbivore ungulates available in nature, only a few have been domesticated. Price (2002) lists some pre-adaptations as requisites for domestication. Strong territoriality is probably the most important attribute that prevents domestication of grazing ungulates. To exploit grass efficiently, one has to allocate a large number of animals to a given area for a number of days, a process called "stocking" in the grazing management jargon. Animals are frequently shepherded from grass-depleted to grass-rich areas. These practices are obviously not compatible with territoriality. Only nonterritorial herbivores, such as cattle, sheep, and goats, that had transhumance habits, but in which males competed for seasonal dominance over the whole flock, and not over a territory, were likely candidates to be successfully domesticated. In the first stage of domestication, animals were selected for smaller body size, a way to reduce nutritional requirement (see Zeder and Hesse, 2000; in goats), but domestication also required willingness to accept a wide variety of feeds – an attribute shared by cattle, sheep, and goats – in order to cope with periods of impaired nutritional availability.

2. Browsers, Grazers and What Is In Between

2.1. A HISTORICAL SCOPE ON FORAGING BEHAVIOR

Cattle, sheep, and goats differ in foraging selectivity. Authors traditionally consider cattle to be grass-eaters, or "grazers." As goats prefer foraging on ligneous vegetation, they are considered "browsers." Sheep are "grazers" that also like forbs. By using these categories, one implies that bovines, ovines, and caprines have strongly specialized dietary preferences, an attribute that seemingly contradicts domestication requirements. Indeed, the popular imagery typically depicts cattle and sheep grazing green lush grass in rolling landscapes, while goats in bipedal stance strive to reach up the most delicious twigs in a tree. In fact, goats will voluntarily ingest more browse than sheep (Rogosic et al., 2006) and cattle (Henkin, 2000), but oak foliage can represent 50% of the diet of beef cattle in an oak-rich environment in Galilee (Brosh et al., 2006), sheep can thrive in a shrubland in Northern Spain (Jáuregui et al., 2007), and goats graze all-grass pastures in the French Indies (Alexandre et al., 1997).

There is ample evidence that many feeding selectivity patterns have been acquired largely after domestication. A survey carried out in the Cazorla Natural Park of Spain (Garcia-Gonzalez and Cuartas, 1989) showed that wild goats (*Capra pyrenaica*) consumed a diet consisting of 41% browse and 59% herbaceous where domestic goats (*Capra hircus*) selected a diet with 81% browse and 19% herbaceous. The diets of wild sheep (*Ovis musimon*) contained 80% of herbaceous species, compared with 48% for domestic sheep (*Ovis aries*), which also included 25% of dwarf shrubs in their diets. In other words, while sharing the same heterogeneous environment, domesticated ruminants grazed less and browsed more than their wild counterparts, i.e., the browsing propensity of goats, and the fondness of sheep for forbs have been strengthened by domestication.

Why and when did that evolution happen? We hypothesize that the location and quality of pasture allowed to grazing animals in ancient times was a function of their relative economic importance. Early domesticated livestock were plainly eaten, a custom referred to as “primary use.” “Secondary use,” i.e., exploiting domesticated animals without killing them, i.e., by harvesting traction, wool and milk, came later. Extensive processing of dairy products – from cattle, and not small stock – was found in ceramic potsherds dating from 6,500 to 5,000 BC in Northwestern Anatolia (Evershed et al., 2008), i.e., relatively soon after domestication. The first wooden plow was developed in Mesopotamia about 4,000–6,000 BC (Hanson and Reicosky, 2008). As cattle were milked, and used for draft and plowing, they were probably well cared for, i.e., the best grass was probably reserved to them. Small ruminants yielded only meat until the fourth millennium BC, when the wool mutation occurred in sheep and dramatically upgraded their economical importance (Sherratt, 1983). In his book on the Awassi breed of sheep, Epstein (1985) presents carvings from Ur (2,400 BC) that feature curly coats strongly suggestive of wool. He documents Canaanite textual reports on wool quality from 1,500 BC. In contrast, goat wool – mohair – appeared much later and, outside Turkey, significant flocks of Mohair-yielding Angora goats were rare until the end of the nineteenth century. In other words, cattle and sheep had much higher economic value than goats for most of the time since domestication.

Sheep digest starch much better than cattle (Colucci et al., 1989; Fig. 1), and they feature a particularly long small intestine (18–35 m, 25 m, on average; Epstein, 1985). Indeed, wool was so valuable that wool-producing sheep grazed on unsuccessful drought-stricken barley crops, they exploited wheat aftermath,

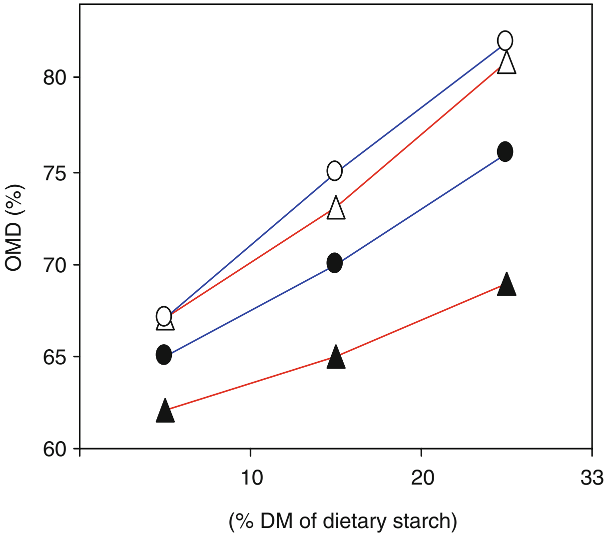

Figure 1. Organic matter digestibility (OMD) in sheep (*circles*) and cattle (*triangles*) given diets with different percentages of starchy concentrates. Open symbols stand for restricted rations and closed symbols stay for rations given ad libitum (after Colucci et al., 1989).

and were even fed with barley grain during droughts (Epstein, 1985), as practiced today by Bedouin herders.

The biblical commandment "Thou shalt not boil a kid in the milk of his dam" (Exodus 23, 14) suggests that goat were already milked at some time in the first half of the first millennium BC. The Babylon Talmud, compiled before the second and fifth century AC, clarifies the relative status of cattle and small ruminants: "Thou shalt not keep small stock in the Land of Israel, but instead, in the woodlands of Israel and Syria. …in the deserts of Judea and close to Acre… but you can keep large stock because one should not mandate laws that most people will not observe." (Baba Kama, Nezikin, 79b). In other words, in the Ancient Fertile Crescent, crops had to be protected against grazing incursions and goats were kept off limits. Cattle were too important in domestic economy for people to graze them far from home. If goats produced milk, they were kept in confinement – and probably hand-fed with browse – and if not, they were grazed in woodlands. Such legislation, restricting goats, and sheep to a lesser extent, to woodlands and shrublands deeply contributed to the evolution of foraging preferences in small stock as reported by Garcia-Gonzalez and Cuartas (1989).

2.2. FORAGING BEHAVIOR: WHY SIZE MATTERS

Plant fiber is digested by the ruminal flora in the rumen and the rate of fiber digestion is a major factor in determining rumen-fill, and subsequently, grass intake.

Cattle, sheep, and goats differ in their body size, but the rumen represents the same proportion of their body weight (BW). As ruminal passage rate is similar in sheep and cows, their intake of fibrous material is also similar, on body weight basis. However, nutritional requirements are proportional to body surface that is a linear function of metabolic BW ($BW^{0.75}$). Small animals have a higher maintenance cost per unit of BW (Van Soest, 1982). For a sheep weighing 40 kg, $BW^{0.75}$ equals 15.9, and for a cow weighing 400 kg, $BW^{0.75}$ equals 89.4. If the sheep and cow ingest 1 and 10 kg of dry matter, respectively, they have achieved the same intake on BW basis, but, relative to $BW^{0.75}$, their respective intake values are 63 and 111g. In other words, the sheep needs to forage on feed higher in nutritional value, compared with the cow, in order to compensate for lower ingestive capacity relative to $BW^{0.75}$. Sheep and goats do that by adopting a more selective foraging behavior than cows, a trade-off between quality and quantity (Demment and Greenwood, 1988). The theory claiming that the smaller the body size, the more selective the ruminants, has some exceptions such as the moose, but globally, cattle can be considered as “grazers,” and small stock as “concentrate selectors.” An implication of this theory is that males and females, which differ in body size, will consume diets that differ in energy density, and may occupy different ecological niches. Another implication has to do with being predated: selective grazing is more time-consuming than bulk-grazing and selective ruminants are more exposed to predation (Shrader et al., 2007).

The historic differences in the management of cattle, sheep, and goats by humans have affected their different behaviors as grazers or concentrate selectors. Cattle will twist their tongues around grass before biting and chewing. Sheep will select for the highest parts of grass, i.e., the more nutritious parts of leaves, avoid coarse stems, and exhibit high preference for starch-rich seeds, a behavior enabled by their impressively prehensile and mobile lips. Goats will favor browsing, even if other nutritious alternatives are available (Landau et al., 2002), a behavior favored by bipedal stance, and lip and maxillary structure.

2.3. THE DAILY DILEMMA OF WHAT TO EAT

The co-evolution between grass species and herbivorous livestock is not without problems. At some phenological stages, plants take advantage of being predated, as seeds can be dispersed in the feces of grazing animals (endo-zoochory). Some seeds also have adaptations that allow them to attach to wool (exo-zoochory; Shmida and Ellner, 1983) and benefit from the presence of livestock without being predated. But many plants have developed aids to deter herbivory, mainly physical (size, height, and thorns) and chemical adaptations (toxins globally termed “plant secondary metabolites” or PSMs). The major PSMs are tannins, terpenes, and alkaloids, but nitrates, saponins, oxalates, and phytates can also harm or even kill livestock. PSMs are ubiquitous in rangelands. Not an expert of PSM chemistry, but known for his common sense and observation skills, Rabbi Yehezkiel Landau

(1776) wrote: "Look at the beast of the land, ….they understand how to save their souls from things that kill them, and the shepherd of sheep and cattle, he will testify, when they graze in the paddock, they avoid and prevent themselves from all grass that is a deadly drug for them".

2.3.1. Learning the Hard Way: Individual Food Aversions

At first, it seems extremely difficult to learn what to eat and what to avoid, as the relative availability, the location, and the toxin contents of plants are ever-changing. The reader will find a formal mathematical approach to this problem, as tackled by goats in the Judean Hills of Israel (Perevolotsky et al., 1998) – where, on a typical day, goats will encounter 24 plant species, 37% of which will eventually be grazed. As stated by Provenza (2003), "life for herbivores exists at the boundary between order and chaos." Out of a chaos of spatially, temporally, and chemically ever-changing conditions, grazing livestock have to create order and predictability.

They learn to detect nutrients and consume them in sufficient amount while minimizing ingestion of toxins in the form of PSMs. The basis of that learning is the postingestive malaise felt by animals following the ingestion of a toxin (Provenza, 1995; Fig. 2). Two levels of hierarchy seem to determine foraging decisions. Soon after ingestion – a matter of minutes in heifers, after the ingestion of quebracho tannin (Landau et al., 2000) – a chain of affective processes, following the postingestive malaise, decreases intake rate. The higher level is a cognitive process that integrates odor, taste, and texture with postingestive feedback from cells and organs in response to concentrations of biochemicals in foods, which results in attributing "flavor" to each food. The senses of smell, taste, and sight enable animals to discriminate among foods and provide pleasant sensations – liking for a food's flavor – associated with eating. Postingestive feedback calibrates sensory experiences – like or dislike – in accord with a food's utility to the body (Fig. 2).

The neurological basis of these processes is theorized by Provenza (1995). Sheep given repeated doses of the toxicant LiCl synchronously with the ingestion of a plant will associate the malaise with the plant, a process termed "conditioned

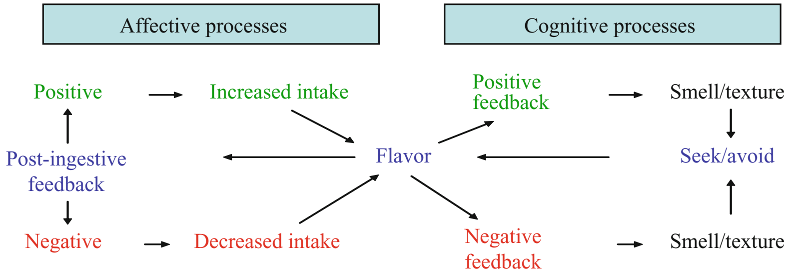

Figure 2. Post-ingestive feedback as the basis of food preference. (After Provenza, 1995).

feed aversion." Although ruminants do not vomit, the postingestive malaise involves interactions between the solitary nucleus, a major coordinator of autonomic nervous system regulation of gustatory, gastrointestinal, and chemoreceptive aspects of homeostasis, and the area postrema, one of the few loci in the brain where the blood–brain barrier is lacking, which serves as a chemoreceptor area associated with vomiting. Postingesting malaise can be mimicked by dosing animals with LiCl, and anti-emetic drugs attenuate food aversion in sheep (Provenza et al., 1994). Aversion with LiCl resembles the natural process of learning to avoid harmful plants. For instance, using LiCl, lambs have been averted to the Mediterranean lethal forb *Ferula communis*, which contains anticoagulants (Landau et al., 1999) but triggers a very mild postingestive effect, if any.

All PSMs decrease feed intake at pasture. Animals learn to attenuate this effect by consuming more small meals (Landau et al., 2000) instead of a big one. Globally, tannins bind to proteins – dietary, microbial, and enzymatic. Condensed tannins are active along the gut, and defecated, but hydrolyzable tannins, terpenes, and alkaloids all need liver detoxification.

The negative effects of PSMs are merely a question of dose. In the 1980s and 1990s of the previous century, scientists from Australia and New Zealand, working with forage species of medium tannin content such as birdsfoot trefoil (*Lotus corniculatus*) and sulla (*Hedysarum coronarium*) consistently reported that tannins had the positive effect of protecting dietary proteins from being altered in the rumen (see review by Barry and McNabb, 1999), whereas Mediterranean studies agreed that tannins impaired protein digestion and nitrogen balance (Silanikove et al., 1997; Decandia et al., 2000). It appeared that the discrepancy between them was mainly a question of dose. Indeed, well-adapted goats are able to select diets with condensed tannin between 4% and 5% of dry matter ingested, a concentration that is optimal for protein metabolism (Kababya et al., 1998).

Evidence accumulates that supplemental dietary protein mitigates the effects of tannins (Silanikove et al., 1997) and terpenes (Campbell et al., 2007) and that PSMs can have positive effects one on another: alkaloids from endophyte-infested tall fescue had less effect on feed intake by sheep when associated with tannins or saponins. The sequence of ingesting PSMs also affects feed intake: lambs ate more total foods with terpenes + tannins when fed tannins → terpenes → alfalfa/barley than when fed alfalfa/barley → tannins → terpenes (Lyman et al., 2008; Mote et al., 2008). This strongly suggests that botanical variety, associated with a variety of PSMs, is an asset for livestock in their daily combat against poisonous plants. When turned to new feeding stations, goats will typically show phases of feed sampling, featuring a variety of plant species, followed by phases of intense foraging, as soon as feeds are regarded as safe, followed by a phase of diversification, a process that is modulated by shepherds (Meuret, 1997) to stimulate appetite or for targeted grazing.

The persistence of aversions is challenged by social facilitations, which introduces the role of learning from mother and group-mates in acquiring foraging habits.

2.3.2. Foraging Skills: Mothers as Vectors of Foraging Cultures

The odor of milk is very much affected by the neutral volatile compounds ingested at pasture (Fernandez et al., 2003). This infers that volatile molecules absorbed by animals are not totally detoxified in the liver, and reach placental circulation. Therefore, animal are exposed to food flavors pre- and post-partum. Lambs exposed to garlic and onion as embryos will eat them readily as adults (Provenza, 2003). Heifers that suckled mothers fed ammoniated wheat straw throughout wintering performed better when later exposed to straw as adults than heifers naïve to straw (Wiedemeier et al., 2002).

Offspring adopt preferences for foods that are eaten by their mothers and these behaviors endure for years. When founder mothers coming from different environments were transferred to a new farm in the French Indies, their original foraging behaviors acquired far away from the farm still affected their offsprings for more than two generations (Biquand and Biquand-Guyot, 1992). In Israel, when kids from the Damascus breed (with high natural propensity to consume the tannin-rich small tree *Pistacia lentiscus*) were fostered at birth to Mamber mothers (with low propensity to consume *P. lentiscus*) and followed them at pasture, they acquired their fostering mothers' behaviors (Glasser et al., 2009; Fig. 3).

After weaning, the influence of mother declines and that of group-mate peers of same age increases. Being part of such a group has advantages and drawbacks. If an animal grazes all by itself, the impact of competition with mates is decreased, but it needs to carry out all the time-consuming flavor verification by itself, and be more alert to predators, which decreases grazing duration. In a group, animals can follow a group-mate, rely on what he eats, and be less alert to threatening predators, but then competition for feeding is more intense (Shrader et al., 2007). This is an

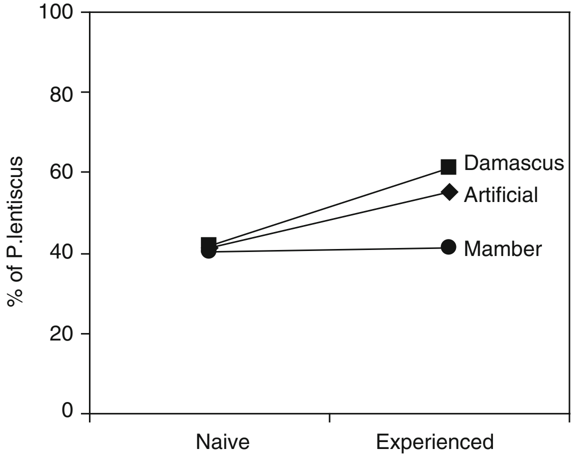

Figure 3. Effect of educating mother (Damascus, Mamber, or artificial milk machine) on the percentage of *Pistacia lentiscus* offered with *Phillyrea latifolia* in kids' diets. (After Glasser et al., 2009.)

example of a complex trade-off involved in foraging decisions that grazing livestock has to take daily.

2.3.3. More Than a Hint That Grazing Livestock Can Self-Medicate Against Parasites

Given the negative impacts of PSMs and the positive effects of nutrients on a herbivore's physiology, a superficial analysis of the rules that guide foraging behavior may lead to the conclusion that animals will strongly avoid PSMs and select nutrient-rich forages. However, not only do herbivores manifest partial preferences (Westoby, 1978; Landau et al., 2002), they also ingest substantial amounts of PSMs (Provenza, 1995).

Internal parasites are one of the greatest disease problems in grazing livestock worldwide (Min and Hart, 2003; Waller, 2006). Considerable attention has been given recently to bioactive plants that have some effect on internal parasite populations (Jackson and Miller, 2006). Plant-derived alkaloids and terpenes have antiparasitic properties (Kayser et al., 2003), and some tannins have strong anthelmintic properties (Molan et al., 2003). As sheep learn to avoid pasture patches infested with fecal helminthes (Hutchings et al., 2001) and, in the most elaborate studies to date, to selectively ingest three medicines – sodium bentonite, polyethylene glycol, dicalcium phosphate – that lead to recovery from illness due to eating too high amounts of grain, tannins, and oxalic acid, respectively (Villalba et al., 2006), they might be able to self-medicate against internal parasites (Villalba and Provenza, 2007). Research is still needed, with far-reaching implications on veterinary practice and on the safety of the meat that grazing livestock produce and we eat.

3. What They Graze Is What They Are, and What They Are Is What We Eat

During intake, digestion, and metabolism, most (say >60%) of the grass biochemical components such as carbohydrate polymers and proteins are broken into smaller molecules and re-arranged to become part of the herbivore's body itself or the microflora and protozoa populations hosted in the gastrointestinal tract. Further, potential nutrients are transferred via blood stream to the mammary gland of lactating animals where they can be incorporated into milk. Usually, less than 40% is not retained either in the body or milk and is wasted as *excreta* and eructation gases.

It is therefore quite obvious that meat and milk composition tend to mirror the dietary composition of ruminants; hence, the linkage between their diets and ours is overall straightforward. For instance, in small ruminants, recent results of experiments have clearly shown that the fatty acid (FA) composition of milk and cheese in sheep (Addis et al., 2005) or milk and suckling kid meat (Nudda et al., 2008) can be effectively tracked back to their diets.

3.1. MILK AND CHEESE FROM GRASS, HEALTHIER?

Apart from the hygienic and sensory values, dairy products nowadays are considered not only as protein and fat providers, but also as functional foods, because they contain components that can directly affect the consumers' health such as beneficial polyunsaturated fatty acids (PUFA), rumenic acid (RA, C18:2 9 *cis* 11 *trans*) – representing >70% conjugated linoleic acid (CLA) isomers, vaccenic acid (VA, C 18:1 11*trans*), and the so-called n-3 or ω3 FA. All these FAs have shown, at least in laboratory animals, anticarcinogenic or antiatherogenic effects (Banni and Martin, 1998). Some of them (RA and VA) are present in nature only in ruminants' products because they are by-products of microbial metabolism in the rumen. Therefore, nutritionists' look on cheese produced from grazing livestock is changing from an item that should be kept under control due to its high content of fat to a source of several beneficial FAs (so-called "nutraceuticals"). For instance, cheese from sheep grazing vegetative pastures based on mixtures of grass and legumes can often contain a concentration of RA ≥ 20 mg/g of fat which, on the basis of studies carried out on rodents (Baer et al., 2001), is thought to be optimal for consumers.

In contrast with protein and lactose concentrations, milk fat content and fatty acid composition are very sensitive to dietary conditions in ruminants. In grazing livestock, milk fat and its FA composition change during lactation as a consequence of several factors such as genotype, parity, lactation stage, management, and nutrition. As to nutrition, energy balance and the intake of dietary fat precursors are the main driving forces that affect FA profiles in milk. A negative energy balance tends to increase fat content and the proportion of oleic acid (C18:1) in the fat, because under these conditions the fat mobilized from body reserves is not fully utilized as fuel for body functioning due to the shortage of glucose. Hence, it can be easily used by the mammary gland and secreted in milk (Bocquier and Caja, 2001; Pulina et al., 2006). The dietary precursors or sources of milk fat can be parted into two classes: (i) cellulose, a precursor for the de novo synthesis of short- to medium-chain FAs (C < 16); and (ii) long-chain FAs (C > 16), which originate in the food and are unmodified or partially hydrogenated in the rumen by rumen microflora (Fig. 4).

Vaccenic acid sourcing from the hydrogenation of dietary C18:2 and C18:3 can be converted either in the rumen or in the herbivore's tissues, into RA by the action of Δ^9 desaturase. Elongase and desaturase are involved in the synthesis of long-chain PUFA ω6 (n-6) or ω3 (n-3) such as eicosapentenoic acid (EPA, C20:5-n3) and docosahexanoic acid (DHA, C22:6-n3.), of which the level in ruminants' products is usually very low.

The implications of small ruminant farming systems on milk production and its quality have been recently summarized by Morand-Fehr et al. (2007), with emphasis on milk yield and composition, in particular fatty acid composition (CLA, PUFA), α-tocopherol, terpenes, and general value for health, globally showing a trade-off between milk yield and nutritional quality.

Feeding regimens based on fresh grass usually result in dairy products with high RA, VA, and PUFA contents, i.e., of high "nutraceutical" value. (e.g. Pulina

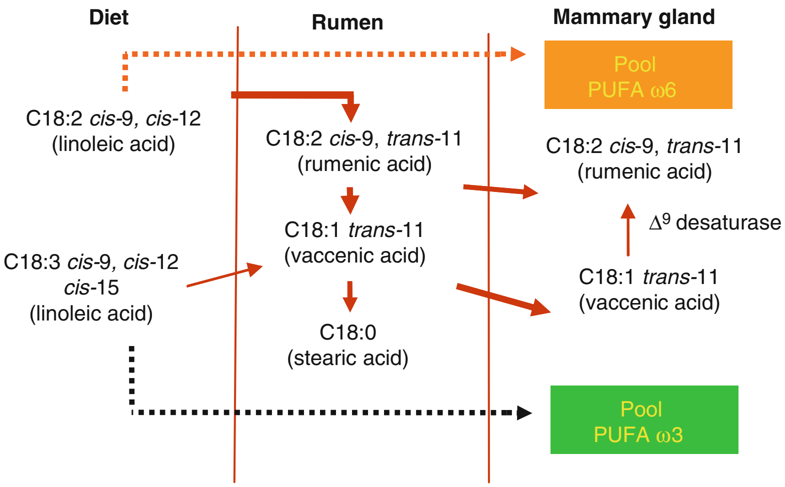

Figure 4. Simplified pathway of CLA, n-3 and n-6 PUFA synthesis.

et al., 2006, for sheep and Chilliard et al., 2003 for goats). Indeed, grass has a relatively high content of lipids associated to thylacoid membranes of chloroplasts. These lipids are in turn rich in long-chain FA such as α-linolenic acid (C18:3 *cis* 9, *cis* 12, *cis* 15), and to a smaller extent, linoleic acid (C18:2, *cis* 9, *cis* 12). However, their concentrations widely vary with forage species, cultivars, and phenological stage (Dewhurst et al., 2003; Cabiddu et al., 2005). The content of the above FA decreases abruptly after cutting due to plant lipoxygenases activity, which converts them into volatile organic compounds such as alcohols and aldehydes (Dewhurst et al., 2003). Therefore, conserved forages have a markedly lower content of RA and VA precursors than the fresh undisturbed forage they come from. In pastures dominated by plants of the Poacecae family, the younger and leafier is the sward the higher the content of PUFA in the grazed herbage (i.e. the greener the better). In general, the content of precursors in the grazed herbage is reflected into the contents of PUFA, RA, and VA in the dairy products. While this is expected for PUFA, it is not straightforward for RA and VA since the extent of biohydrogenation in the rumen and the intensity of postruminal desaturation of VA to yield CLA can vary quite widely due to several factors. The extent of FA postruminal desaturation increases during pasture maturation, along with the decrease of precursors concentration in the grazed herbage (Cabiddu et al., 2005).

We contend that milk from pastures is healthier than milk from confined animals. But can pasture-milk be imitated by feeding supplements? Not so easily. The use of supplements enriched with CLA or ω3 precursors can be used to compensate for the gaps of C18:3 and C18:2 concentrations in ruminants'diet during winter, due to herbage shortage, and in spring, due to its quality decay, but the use of vegetable oils or oilseeds as supplements may dramatically increase the total level of *trans* FA monoenes that are deleterious to animal and human health, at the exception of VA. Indeed, the increase in the content of biohydrogenation products such as C18:2, 10 *trans* 12 *cis* or C18:1 10 *trans* is associated, among others, to milk fat depression syndrome in ruminants (Bauman and Grinari, 2000). This shift of rumen metabolism often associated with diets high in nonprotected lipids or in starch, is negative twice, as it reduces the fat content and, at the same time, worsens FA composition.

In addition to fatty acids, other lipid components such as carotenoids, and tocopherols, precursors of vitamins A and E, respectively, have a much higher concentration in milk and cheese from grazing than stall-fed ruminants. Furthermore, apart from vitamin E, the total anti-oxidant potential of these products is enhanced while cholesterol is reduced, allowing for a higher ratio between anti-oxidants and oxidants in the consumer's diet as reviewed by Morand-Fehr et al. (2007) .

The N content of forage (Gerson et al., 1986), and the content of PSMs can modulate the level of beneficial FAs in dairy products as well as its trend during grazing season. In forage legumes containing condensed tannins (CT) (e.g. sulla, *Hedysarum coronarium*), these PSMs interact with rumen microbes reducing PUFAs biohydrogenation and hence enhancing their incorporation of PUFAs in dairy products. For this reason, sheep grazing sulla during flowering with 4% of condensed tannins in their diet can still have a high proportion of PUFAs in their milk. And for the same reason, neutralizing the effects of tannins with polyethylene glycol will decrease milk CLA (Cabiddu et al., 2009). In vitro research results have recently highlighted the role of polyphenol oxidase (PPO) in curbing biohydrogenation efficacy in forage species such as red clover (*Trifolium pratense*), which have a low concentration of phenols and condensed tannins. In particular, in an in vitro study, a line of red clover cultivar selected for high PPO content when incubated in rumen liqueur for 24 h was able to decrease the biohydrogenation of C18:2 and C18:3 by 8% and 14%, respectively, when compared with a low-PPO line of the same cultivar (Lee et al., 2007).

Forbs such as garland (*Chrysanthemum coronarium*) or chicory (*Chichorium intybus*) may also contribute to further enhance the nutraceutical value of ruminants' dairy products (Landau and Molle, 2005), thanks to a moderate content of terpenes that can impact on rumen microflora and depress biohydrogenation activities. Garland can accumulate high contents of fat in flowers and seeds, hence counteracting the usual decay of FA, which is found in spring in annual grass-based pastures. However, terpenes may affect – sometimes negatively – the sensory properties of cheese as found by Addis et al. (2006).

3.2. FLESH FROM GRASS

The most natural diet for a ruminant is grass, and the most logical way of spending a ruminant's time is grazing. However, economical conditions from the 1970s

Table 1. Grain price ($/ton) increase in 2007, adapted from "Daily National Grain Market Summary" (USDA, 2007; http://www.ams.usda.gov/mnreports/SJ_GR850.txt).

	10 Jan. 2007	**11 Jan. 2008**
Wheat		
Kansas City	179	321
Minneapolis	185	402
Corn, US No 2 Yellow		
Kansas City	150	188
Minneapolis	139	182
Soybeans, US No 1 Yellow		
Kansas City	251	439
So Iowa	247	443

of the last century, and in particular, dumping prices of corn in the United States, have promoted the practice of fattening weaned offspring with grain. What happened to grain prices in 2007 (Table 1), together with ethical considerations, emphasize the need to revisit the issue of lamb/kid fattening.

Would substituting grain with pasture for fattening lambs and kids – where possible – result in the production of low-fat, low-calorie, and healthier meat types? As expected, a trade-off between quantity and quality of meat was identified when Boer goat kids kept at pasture were compared with confined counterparts fed concentrate and hay. Confined kids had superior carcass traits, but exhibited higher cooking loss and a three- to sixfold higher C18:2 n-6/C18:3 n-3 ratio (Ryan et al., 2007), i.e., less favorable to human health, compared with grazing kids. Under temperate conditions, the meat of lambs following their Italian Merino mothers at pasture and slaughtered at 100 days of age had higher intramuscular polyunsaturated fatty acids (PUFA) and higher ratio of PUFA to saturated FA than counterparts with hay- and concentrate-fed mothers (Scerra et al., 2007). In France, lambs gained as much weight when feeding on lush green grass than counterparts feeding on concentrate and hay in confinement; grazing lowered carcass n-6 PUFA and increased n-3 PUFA and RA (Aurousseau et al., 2004). The ratios of C18:2 n-6 to C18:3 n-3 were favorably low in grazing lambs.

Meat from grass is healthier, but not necessarily tastier to all palates. Two unpleasant compounds, skatole and indole, are higher in the fat of lambs fed with herbage than concentrates. Again, PSMs have a role to play: skatole, and the sheep meat odor are lower in the fat of animals supplemented with tannins (Priolo et al., 2008). Meat from grass-fed lambs is darker in color than meat from stall-fed animals up to 24 h of display, and less tender and juicy than meat from confined animals, as judged by a trained panel of assessors. Typical "lamb" and "fatty" flavors are higher in stall-fed lambs, whereas meat from grass-fed animals had more pronounced liver flavor (Priolo et al., 2002). As oxidation processes are responsible for the flavor, color, and nutritive value of meat, turning lambs to pasture can have implications on meat shelf-life, with legumes promoting higher GSH-Px, an anti-oxidant enzyme, in meat, than other pasture species (Petron et al., 2007).

So, odor and taste are different in grass-fed animals. But how would lamb meat consumers respond? In a survey carried out in six European countries (Greece, Italy, Spain, France, United Kingdom, and Iceland), following a taste panel with two lamb types differing in production system, a clear difference appeared between families originating in Mediterranean and Northern Europe: the former preferred concentrate-fattened lamb, and the latter, grass-finished lamb (Sanudo et al., 2007). This is not surprising, as Mediterranean lambs are traditionally fattened with concentrates, whereas Northern lambs are finished at pasture. Also, Northern European cuisine includes more browning and baking of meat, which mitigates the effect of meat odors on the dish flavor, whereas more grilling or roasting is practiced around the Mediterranean.

Finally, if a demonstration of the intricate relationship between agricultural and cultural – and in particular, culinary – practices is needed, an up-to-date chemical analysis can provide it, showing that grass does not affect fat only. In a study of lamb meat traceability carried out in the frame in a cooperative European program, 193 samples of defatted lyophilized lamb meat from the United Kingdom, Ireland, France, Germany, Italy, and Greece, encompassing pasture and confinement systems, were subjected to multielement (H, C, N, S) stable isotope ratio analysis (Camin et al., 2007). C and N isotopic ratios were affected by feeding practices and climate, S by geographic location and surface geology, and H by hydrology. Multivariate analysis of ratios between them resulted in 78% correct classification of meat samples. In other words, a piece of defatted meat is much more than water and protein. The climate, history, and geography of a region, the subsequent grazing practices, and the adaptations of animals to the grass and those of the grass to animals, all are concealed in a tiny piece of defatted meat.

4. Conclusions

Grass and domestic livestock, the main components of the most important ecosystem driven by man from as early as 10,000 years BC have co-evolved through millennia. By domesticating ruminants, shepherds pioneered the exploitation of cellulose, the most common depot of sun energy on Earth and shepherding is still the main activity in regions where agriculture is not sustainable. To harvest grass, shepherds rely on livestock foraging cultures transmitted by animals from generation to generation. These foraging cultures largely affect livestock products. Science has advanced in understanding the mechanisms underlying the connection between grass and livestock products and between these products and human life. In particular, dairy products and meat are usually better for human's health when livestock is grazing a variety of plant species than when it is kept confined in intensive systems.

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HERBIVORE–PLANT INTERACTIONS AND DESERTIFICATION IN ARID LANDS

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1. Introduction

In a seminal paper on animals in ecosystems, Chew (1974) stated that, although only a small fraction of plant biomass is consumed by animals, the importance of animals as agents affecting the structure, properties, and processes of ecosystems must be evaluated. Chew based this evaluation of the function of animals in ecosystems on his research in the Chihuahuan Desert of eastern Arizona. The focus on animal activities that affect the structure and function of arid ecosystems provided the conceptual framework for animal studies for the past three decades. The most important animal activities are those which have direct or indirect effects on plants: establishment, growth and mortality, and/or spatial distribution. Arid lands around the world have experienced or are currently experiencing degradation that is known as desertification (Reynolds et al., 2007). Animal–plant interactions that have an effect on desertification are among the most important functions of animals in arid ecosystems (Whitford, 1993).

Desertification has been defined as land degradation in arid and semiarid areas that is the result of various factors, including climate variation and human activities (Williams and Balling, 1996). The interactions between animals and plants affect desertification in arid environments in a number of ways. These interactions may exacerbate desertification processes or contribute to the stability of desertified ecosystems. Some animal–plant interactions are direct, e.g., herbivory, while others are indirect, e.g., seed dispersal and soil modification. Animal–plant interactions that contribute to changes in the structure of plant communities or the spread of alien plant species may enhance some degradation processes such as soil erosion or contribute to soil stability and resistance to degradation.

Most studies that provide data useful for examining the effects of animal–plant interactions on desertification or the contribution of animal–plant interactions to the resilience of desertified ecosystems were conducted in arid and semi-arid regions, where desertification began approximately 1.5–2 centuries ago, coincident with the introduction of the livestock industry. In these landscapes, the structure of some of the component ecosystems is in a state of flux while other component ecosystems appear structurally stable. In these environments, animal–plant interactions may be direct, e. g., herbivory, or indirect, e.g., seed dispersal and soil modification. In degrading or relatively stable degraded ecosystems, herbivory can contribute to changing plant species composition or modifying the morphology of dominant plant species. Animals are frequently agents of seed dispersal, which may be critical for the expansion of native shrubs into areas where they were absent earlier. Animals that modify soil may affect the distribution of critical resources (water and nutrients), which has been identified as one of the most important consequences of desertification (Whitford, 2002). Here, we review studies that have addressed animal–plant interactions and desertification.

2. Herbivory – Plant Architecture

The behavior of some insects can have a marked effect on the architecture of woody perennials. For example, insects that kill part of live stems in the process of creating a safe environment for oviposition, can have an effect on the physiological response of the plant to the loss of apical dominance. One such insect is *Oncideres rhodostica* (Coleoptera, Cerambycidae). *Oncideres rhodosticta* is a twig-girdling beetle that utilizes mesquite (*Prosopis glandulosa*) stems for oviposition and larval development sites. Female beetles chew girdles around mesquite stems, severing the phloem and xylem, disrupting water and nutrient transport and killing the stem above the girdle (Polk and Ueckert, 1973). The beetle then oviposits distally from the girdle, allowing the egg to avoid being coated in sap flowing down the stem in response to the girdle (Whitford et al., 1978; Dussourd and Eisner 1987). Mesquite responds to *O. rhodosticta* girdling behavior by producing new stems from nodes below the girdle, compensating for photosynthetic area loss on the dead girdled stem, and potentially increasing the stem density of individual plants (Whitford et al., 1978). Increase in stem density when multiple stems are produced below the girdle changes the morphology of the shrub and, more importantly, how the shrub functions in the landscape. In a study designed to compare the effects of *O. rhodostica* girdling on mesquite in grasslands with mesquite plants in coppice dunes, there were no differences in the number of old or new girdles on mesquite plants among the dune and grassland plots (Fig. 1). Mesquite in grassland produced more new stems per girdle after 1 year than the coppiced shrubs of the dunes. The new stems below the girdles were longer in the grassland than in the dunes. The volume of plant-captured soil was correlated with shrub stem density (Duval and Whitford, 2008). Positive correlations of mesquite volume with both old and new girdles suggest a feedback loop where girdling increases stem density, stem dense shrubs provide greater resources to emerging female *O. rhodosticta* the following year, and the plant responds by growing more new stems. Beetles contribute to the increase of resources (oviposition sites and food) in the form of compensatory growth stems for the next generation of beetles that will emerge from the girdled stems. This interaction of the beetle and *P. glandulosa* shrubs supports the resource regulation hypothesis of Craig et al. (1986).

Figure 1. Mesquite dune landscape.

Figure 2. Small mesquite seeding with long stems browsed off by rabbits.

There is a correlation between soil capture and stem density, which provides evidence that increases in plant-stem density increases soil capture by individual *P. glandulosa* shrubs (Van de ven et al., 1989). As girdling by *O. rhodosticta* increases stem density, mesquite plants capture more aeolian sand, which is deposited around the base of the shrub. This is the mechanism of dune formation around mesquite plants that eventually results in coppice dune landscapes. Since coppice dune landscapes represent the end point in degradation of desert grasslands, the girdling of mesquite by this beetle increases the probability that the landscape will eventually convert to the most desertified state. Continued girdling of emergent stems from developing dunes provides a positive feedback that maintains the dunes in the landscape.

Browsing by jackrabbits (*Lepus californicus*) has been found to modify the morphology of small honey mesquites (*Prosopis glandulosa*) (Fig. 2). Jackrabbit browsing on small mesquites results in a net loss of biomass. However, the most important effect of browsing was the change in architecture of the *P. glandulosa* shrubs. Unbrowsed mesquite had long branches and extended crown cover. Browsed mesquite had many stems packed into a compact matrix armed with spines. The compact, spiny, stem matrices produced one or more shoots that were sufficiently tall to escape browsing by the jackrabbits (Martinez and Lopez-Portillo, 2003). The high stem density of the browsed, small mesquite produces a trap for aeolian sand. Compact mesquite will serve as focal plants for the formation of coppice dunes in areas with low grass cover. The effects of stem girdling cerambycid beetles and browsing by native lagamorphs that change stem density and architecture of mesquite, were documented in studies on the expansion of *P. glandulosa* into black grama grassland (Hennessy et al., 1983). The study included areas that had been grazed by livestock and areas from which livestock had been excluded. Vegetation changes in the 45 years since the initial measurements documented complete dominance of mesquite, loss of black grama grass, and the formation of many new mesquite coppice dunes in both grazed and ungrazed areas.

Herbivory by rabbits was found to have an effect on the morphology and distribution of the Mediterranean woody shrub, *Retama sphaerocarpa*. Herbivores produced an open shrubland with fewer, thicker-branched, large, *R. sphaerocarpa* compared to areas subjected to mechanical cutting of the shrubs. Mechanical cutting is a management procedure used to maintain the *Retama* savanna shrublands. Intense herbivory during exceptionally dry periods was reported to produce some effects similar to mechanical cutting, i.e., denser aerial biomass (Sal et al., 1999). The distribution patterns and morphology of *R. sphaerocarpa* and the maintenance of the structure of the Mediterranean savanna-like shrublands are largely attributed to herbivory on the dominant shrub.

3. Herbivory – Plant Vigor and Mortality

Desertification in the southwestern United States and northern Mexico is generally described in terms of expansion of invasive native shrubs (mesquite, *P. glandulosa* and creosotebush, *Larrea tridentata*) into desert grasslands. Creosotebush expansion has been down slope from bands of *L. tridentata* on rocky, well-drained soils at the top of hill slopes or piedmont slopes to the finer textured sandy to sandy-loam soils on the low-slope angle basin slopes (Whitford, 2002). Jackrabbits (*Lepus californicus*) sever most of the stems of some of the multistem *L. tridentata* shrubs during the usually dry winter months in the Chihuahuan Desert. Jackrabbits selectively browse those shrubs with the highest stem water content (Steinberger and Whitford, 1983). Most of the stem with leaves attached is dropped adjacent to the browsed shrub. The stem and leaf wastage decomposes at a different rate than the naturally senesced material. However, the differences in decomposition appear to have little, if any, effect on the physiological status of the shrubs or on soil processes.

Following stem browsing by jackrabbits, *L. tridentata* shrubs exhibit compensatory growth, producing multiple, new shoots from nodes below the cut (Steinberger and Whitford, 1983). In many cases, heavy browsing changes the plant morphology, creating a shrub volume smaller than expected, with much denser foliage in comparison to unbrowsed shrubs. It has been suggested that heavy browsing enhances plant vigor and survivorship due to compensatory growth. In creosotebush-dominated shrubland, the shrubs with the highest stem density cause deposition of wind-transported soil fines and plant litter that form small mounds below the shrub canopy (Whitford et al., 1996). The development of finer textured soil mixed with decomposing litter under the shrub canopies produces a soil patch that supports higher density of soil biota than surrounding soils (Santos et al., 1978). Subcanopy soil with fine texture and organic matter contributes to the nitrogen-rich soils under shrubs (Schlesinger et al. 1996). The change in morphology of heavily browsed *L. tridentata* also affects water redistribution by the shrubs. There is significant stem flow of rainfall intercepted by the canopy of creosotebush that is funneled to deep soil storage by transmission along roots (Martinez-Meza and Whitford, 1996).

Herbivory that involves severing of stems of shrubs can have significant effects on the plant species composition of areas that are still subject to degradation processes. Tarbush, *Flourensia cernua*, is a native woody shrub species that was distributed along ephemeral water courses prior to the initiation of desertification processes. This species moved from the margins of ephemeral streams into areas receiving overland flow from the streams. Thus, on the lower slopes of the piedmonts, tarbush and creosotebush established in areas where grass cover was reduced. On the upper piedmont, creosotebush is dominant with some tarbush on the interfluve ridges. On the ridges, approximately 35% of the tarbush shrubs were heavily browsed compared to only 4% of the creosotebush. Along the drainage channels on the upper piedmont, jackrabbits heavily pruned 21% of the tarbush but less than 1% of the creosotebush. Jackrabbits preferentially pruned tarbush shrubs in both locations but the differences in proportion of *F. cernua* shrubs pruned compared to *L. tridentata* shrubs was greatest in the drainage channels (Roth et al., 2007).

Creosotebush was slightly more abundant on lower piedmont slopes, with tarbush accounting for 41% of the shrubs per unit area. Nearly half of the tarbush shrubs were dead compared to less than 5% of the creosotebush. Most of the dead tarbush exhibited evidence of moderate to heavy browsing before the shrubs died. The dead tarbush shrubs were only 50% of the predicted size based on basal stem diameters. There was no evidence of compensatory stem growth in *F. cernua* shrubs that were moderately or heavily browsed by jackrabbits in any of the locations. Even in monotypic stands of *F. cernua*, the plants with severed stems exhibited no evidence of compensatory growth by new stems from lateral nodes below the severed end of the stem. Both *F. cernua* and *L. tridentata* are browsed by jackrabbits during the dry winter months. Pruning of stems of one-shrub species (*L. tridentata*) by jackrabbits resulted in increased vigor as a result of compensatory growth and, in the other species (*F. cernua*), pruning of stems by jackrabbits resulted in reduced vigor and mortality (Roth et al., 2007).

The long-term implication of these differences in response to browsing by rabbits is change in community dominants in degraded grasslands that underwent transition to tarbush in the past and are now shifting to creosotebush dominance. The process of vegetation change in desertification may change drivers, e.g., from domestic livestock overgrazing to selective browsing by a native herbivore. This change in drivers can change plant-species composition and spatial distribution of essential resources before temporally stable plant communities are established.

The resilience by compensatory growth of arid region shrubs to herbivory varies not only with the amount of biomass lost to herbivory but with season (wet or dry season) and rainfall (Oba et al., 2000). Long-term studies of herbivory effects on the dwarf shrub *Indigofera spinosa* showed that overcompensation occurred under some conditions but not others. *I. spinosa* exhibited overcompensatory growth for three consecutive years, with 30% biomass removal by clipping during the wet season. In 2 of the 5 years of the study, there was overcompensation when shrubs were defoliated in the dry season but not in the other three yeas of the study. Moderate to severe (50–90%) defoliation resulted in undercompensation, i.e., failure to return to the preclipping biomass. Full year defoliation resulted in undercompensation even at the light (30%) clipping regime (Oba et al., 2000). The results of this study suggest caution in making generalizations based on single-year or single-season studies of herbivory and compensatory growth in arid-region shrubs.

Growth and survivorship of mesquite in vegetation arcs were found to be a function of stem browsing by jackrabbits and packrats (*Neotoma* sp.) (Fig. 3). Herbivory by these animals had a greater effect on growth and survival of young *P. glandulosa* than water availability (Lopez-Portillo et al., 1996). In landscapes characterized by vegetation arcs separated by gentle erosion slopes, herbivory affected the survivorship of young plants, thereby affecting the plant-species composition of the vegetation arcs.

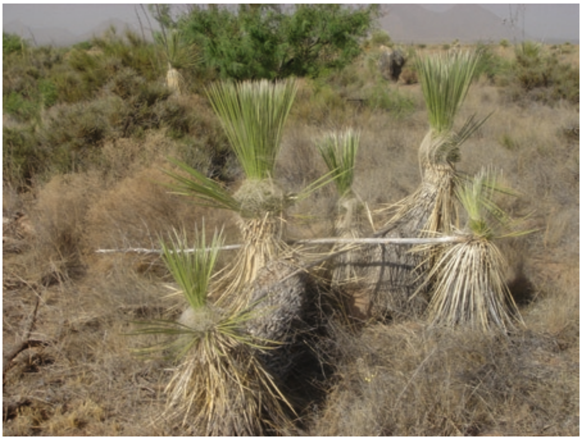

Figure 3. *Yucca elata* with foliage browsed by woodrats (Neotoma spp.).

The spread of mesquite (*Prosopis* spp.) into historical grasslands has been enhanced by the management decision to extirpate black-tail prairie dogs (*Cynomys ludovicianus*) from arid rangeland grasslands. Eradication programs in the western United States reduced the area occupied by prairie dogs to less than 1% of their former distribution and have resulted in an effort by conservation groups to have the species listed as an endangered species (Ceballos et al., 1993). An experimental study found that prairie dogs and the herbivores and granivores associated with their colonies probably maintained grassland and savanna by preventing the establishment of mesquite and other woody species. Mesquite seeds and pods disappeared three to 99 times greater on prairie-dog colonies than off-colony areas (Weltzin et al., 1997). Survival of *Prosopis* spp. seedlings that were protected from vertebrate herbivory was the same on and off colonies. On prairie dog colonies, prairie dogs and associated herbivores girdled and destroyed mesquite seedlings within 2 days after seedlings were planted on colonies. One-year-old mesquite seedlings were reduced by 50% on prairie-dog colonies. Repeat aerial photography showed that *Prosopis* spp. canopy cover on a colony eradicated in 1950 (27%) increased to a level of off-colony mesquite stands (approximately 65% canopy cover) within 23 years (Weltzin et al., 1997). This is an example of how the failure to appreciate the importance of a herbivore in the maintenance of a grassland-savanna has probably contributed to desertification in the southwestern United States. Prior to European settlement, the fossorial mesomarsupial, the burrowing bettong (*Bettongia lesueur*) had the widest distribution of any native mammal in Australia. Following European settlement of interior Australia, the burrowing bettong was extirpated by hunting and poisoning for the same reasons that prairie dogs were largely extirpated in North America (Noble et al. 2007b). The burrowing bettong is now considered rare and endangered. "There is now mounting evidence that *B. lesueur*, together with other mesomarsupials such as the ubiquitous *B. penicillata* (brush-tailed bettong) and *Onychogalea fraenata* (bridled nailtail wallaby), collectively contributed to several other fundamental ecosystem processes including promoting the dispersal of mycorrhizal spores and seeds" (Claridge and May, 1994; Noble, 2001) and by selective browsing, together with episodic fire, regulating populations of native shrub species now regarded as "woody weeds" (Noble, 1996; Noble and Grice, 2002). In pre-European Australia, frequent fire and burrowing bettongs kept shrub and small tree populations low. The results of a modeling study are particularly revealing. With low-shrub density and high-fire frequency, the final densities of plants remained low both in the presence of *B. lesueur* (100 shrubs/ha) and in their absence (200 ha^{-1}). At low-fire frequencies, burrowing bettongs were predicted to keep shrub densities low (250 ha^{-1}). However, in the absence of bettongs and low-fire frequency, shrubs were predicted to increase to high levels (>1,000 ha^{-1}) (Noble et al., 2007a). With more than a half-century of fire suppression and elimination of bettongs, reintroduction of fire for shrub control was insufficient to change the dense shrub ecosystem to a more open savanna-like ecosystem.

Frequent fire resulted in post-fire coppicing of many shrub species that provided green biomass at or near ground level during drought. Some native mammals probably depended on such vegetation for forage. Intense browsing on resprouting shrubs would impose sufficient physiological stress on the plants to kill a large percentage of the population. These studies provide evidence that herbivory by mesomarsupials, especially burrowing bettongs, were essential for maintaining the open savanna-like ecosystems before the arrival of Europeans. This has led to attempts to reintroduce burrowing bettongs into areas that are dense shrubland in order to use frequent fire and bettong browsing to return the system to a savanna parkland (Noble et al., 2007a).

Noble et al. (2007a) reviewed anecdotal evidence that other mesomarsupials contributed to the regulation of shrub populations by browsing shrubs seedlings and coppices that regenerated after fire. Another rare and endangered mesomarsupial, the nail-tail wallaby, *O. fraenata*, consumed large quantities of shrub foliage. The rufous bettong (*Aepyprymnus rufescens*) browsing of seedlings of the white cypress pine (*Callitris glaucophylla*) in combination with fire maintained the region in northern New South Wales as an open savanna woodland.

Exclosure studies in Western Australia clearly demonstrated that fire and browsing by the mesomarsupial, the quokka (*Settonix brachyurus*), prevented tree-seedling recruitment. Experiments in mallee regions of New South Wales provided evidence that macromarsupials, especially the western gray kangaroo, could eliminate seedlings of mallee pine (*Callitris verrucosa*) by selective browsing (Noble, 2001).

Rodent and insect herbivory is the main factor eliminating the establishment of trees in the northern Mongolian steppe. In experiments where larch, *Larix sibirica*, was planted in plots from which livestock were excluded by fences, grasshoppers and rodents damaged the seedlings during the entire growing season (Dulamsuren et al., 2008). This study documented the importance of grasshopper and rodent herbivory in maintaining the steppe grassland free of trees.

In the Monte Desert of Argentina, the fossorial herbivorous rodent, the tuco-tuco (*Ctenomys mendocinus*), is reported to damage 39% of the total shrubs. *C. mendocinus* was recorded as browse damaging 65% of the *Larrea divaricata*, 41% of the *Lycium chilensis*, 38% of the *Junellia seriphiodes*, and 33% of the *Mendora decemfida* shrubs (Tort et al., 2004). There were no data provided on the long-term growth responses of these shrubs to herbivory by the tuco-tuco in this study. However, since *Larrea tridentata* is closely related to *L. divaricata*, it is likely that this species would exhibit compensatory growth of stems from below the severed ends of the stems. However, other shrub species browsed by the tuco-tuco may parallel tarbush (*F. cernua*), with cumulative damage causing death of the shrub. We suggest that herbivory by the tuco-tuco is an important factor affecting the species composition of the shrub communities in the Monte Desert.

Herbivory is also an important animal–plant interaction that affects the physiological status, survivorship, and abundance of perennial herbaceous plants, which are important components of desert ecosystems. In the Chihuahuan Desert, several species of prickly-pear cacti are relatively abundant and important plants contributing to faunal diversity. These plants are also subjected to intense herbivory by lagomorphs (*L. californicus* and the desert cottontail, *Sylvilagus auduboni*) during the dry winter months. Plant size and not spinescence appears to be the key determinant of presence or absence of browsing. Lagamorph herbivory is not directed at seedlings or small individuals with less than ten cladodes (pads). *Opuntia* with more than 30 cladodes were little affected by herbivory. Because of the large volume and architecture of large *Opuntia* sp., browsing is predominately on the peripheral, older cladodes. The large size insures that there is sufficient photosynthetic area and primordia for new cladodes, flowers, and production of fruit provided by interior cladodes. Thus, heavy browsing on the mid-size cacti reduces the number of cladodes, reduces flower and fruit production, and may even kill the plant if browsing is intense (Hoffman et al., 1993). Since browsing by rabbits does not have adverse effects on seedlings and small individuals or on large prickly-pear, herbivory on these plants is relatively benign.

A South American rodent (*Microcavia australis*) gnaws the bark of the Monte Desert shrub *Geoffroea decorticans* and plants that are heavily gnawed do not survive the loss of bark. *G. decorticans* plants with large diameter trunks have greater survival after bark loss than shrubs with small diameter trunks. Also, shrubs that are a long distance from *M. australis* burrows are subject to less damage by the rodents. The size–distance relationships of the *G. decorticans* shrubs from *M. australis* burrows has long-term effects on the abundance and spatial distribution of the shrub (Tognelli et al., 1999).

In the arid regions of Israel, the dorcas gazelle (*Gazella dorcas*) feeds on different parts of the lily *Pancratium sickenbergeri*. In the summer months, gazelles dig in the sand to remove all or part of the bulb. Partly eaten bulbs have a lower probability of leaf production the following winter (the rainy season) and probably have lower lifetime flower production (Saltz and Ward, 2000). In lily populations subjected to gazelle herbivory, the plants respond by greater depth of bulb growth than recorded in populations with no herbivory. In winter, gazelles browse only on the tips of leaves because of the calcium oxalate concentrations in the leaves, resulting in little effect on the physiological status of the lily. However, in experiments where the lily was subjected to various levels of simulated browsing of leaves (0%, 25%, and 50% of leaves clipped), plants in the intermediate clipping treatments overcompensated in leaf area produced after clipping. Even the highest level of clipping had no significant effect on the number of fruits per plant (Ruiz et al., 2008). Compensatory growth that exceeds photosynthetic area lost is important for the maintenance of *P. sickenbergeri* and is an important adaptation for plant species subjected to herbivory in arid ecosystems.

In the autumn, gazelles eat most of the flowers. Some of the plants produce a second flower that may or may not be eaten by gazelles (Saltz and Ward, 2000). Gazelle-lily interactions appear to be either benign (summer feeding on leaf tips) or negative during the other seasons. Dorcas gazelles are probably more important than edaphic features of the landscape in determining the abundance and distribution of the lily.

Herbivory may have negative effects on certain plants by affecting reproduction by destroying infloresences and indirectly eliminating specialist pollinators. *Yucca elata* (Agavaceae) is a common evergreen perennial in the arid regions of the southwestern United States and northern Mexico. Young *Y. elata* plants grow as a rosette of narrow, sharp-tipped leaves. In mature plants, the leaf rosette is supported by a fibrous caudex. The inflorescence stalk grows rapidly and bears several hundred flowers. The young inflorescence stalks and flowers are highly desired by cattle, and ranchers manage their herds for maximum utilization of the *Yucca* inflorescences. This management strategy has the effect of reducing populations or eliminating *Y. elata* from the ecosystem. Cattle also browse *Yucca* leaves, especially during the dry winter and spring months (Herbel and Nelson, 1966).

Cattle browse young vegetative growth in the center of the rosette, causing a loss of apical dominance of the browsed rosettes. Browsing rosettes leads to an increase in the number of caudices per plant. The most important effect of browsing on central leaves in the rosette is the reduction in floral infloresences in the browsed *Y. elata*. This has long-term implications for maintenance of viable populations of this important plant (Kerley et al., 1993).

Yucca elata is pollinated exclusively by the mutualistic yucca moth *Tegeticula yuccasella*, for which *Y. elata* seeds are the obligate habitat and food source for the larvae. If cattle eat all or almost all of the flowers, the year's cohort of *T. yuccasella* will fail to reproduce. If intense browsing of the flower crop continues for several years, the yucca moth may become locally extinct. Yucca moths are weak fliers, suggesting poor dispersal to unaffected *Y. elata* populations. Thus, local *Y. elata* populations may become infertile and incapable of sexual reproduction (Kerley et al., 1993). In Mexico, human harvest of *Yucca fillifera* flowers for food has extirpated the specialist yucca moth, *Tegeticula mexicana*. As a consequence, there are large regions in northern Mexico where *Y. fillifera* flowers are not pollinated and there is no fruit production (Ridaura-Sanz, 1979). Since the dried inflorescence stalks are nest and/or perch sites for several species of birds, loss of infloresence stalks and/or reduction in abundance of *Yucca* sp. may have serious consequences for biodiversity in areas where this plant is a subdominant.

Differential mortality of shrubs results not only from vertebrate herbivory but also has been reported to result from activities of invertebrates. Seed harvester ants, *Pogonomyrmex rugosus*, were reported to alter the vegetation in the *Larrea tridentata*-*Ambrosia dumosa* shrubland of southern Nevada. Thirteen of seventeen species were absent from nest discs and immediate vicinity of the nests. Woody vegetation was sparse and less variable in composition at the nest discs and edges of nests (Lei, 1999). *P. rugosus* frequently defoliates or severs the stems of woody plant germinants on the nest discs and in close proximity to the nest (Whitford, W. G., 1972–1975, unpublished observations). This behavior of harvester ants contributes to the spatial structure of shrub-dominated ecosystems and to the maintenance of zones of low shrub densities in the vicinity of the nests.

4. Herbivory and Graminivory

While herbivory on some shrubs has effects on ecosystem structure and function as well as on the trajectory and end points of desertification, herbivory on other shrubs, grasses, or herbaceous plants may be benign or contribute to desertification or resilience of desertified ecosystems as a function of the abundance of the target plants in the landscape. Overgrazing of desert grasslands frequently results in increased abundance of an evergreen, subshrub, *Gutierrezia sarothrae* (snakeweed), which is poisonous to livestock (Ralphs, 2002). Snakeweed is multistemmed, with a crown of dense inflorescence stems bearing seeds. *G. sarothrae* stems are severed close to the soil surface during dry winter months by jackrabbits (*L. californicus*). The incidence of herbivory on the stems of snakeweed is virtually the same in mesquite coppice dunes, overgrazed grassland, and ungrazed grassland (Roth et al., 2008). There is no evidence that herbivory by jackrabbits on *G. sarothrae* has an effect on size or mortality of this species. However, rodents sever the infloresences containing seeds. Because of the low mass relative to the large volume of the severed infloresences, the cut material is easily moved by wind. It is very likely that wind-blown seed heads disperse the seeds of this undesirable plant species. Thus, herbivory on snakeweed by rodents may contribute to higher abundance of *G. sarothrae* in overgrazed grassland.

While jackrabbit browsing had no effect on mortality and population size of *G. sarothrae*, these parameters were affected by the specialist, snakeweed grasshopper, *Hesperotettix viridis* (Insecta, Orthoptera). In an experiment using caged grasshoppers on individual *G. sarothrae*, mortality was positively related to the number of grasshoppers per plant. One grasshopper per plant killed 53% of the plants, three grasshoppers per plant killed about 69% of the plants, and five grasshoppers per plant killed 91% of the plants (Thompson et al., 1996). A natural population explosion of *H. viridis* resulted in the death of most of the snakeweed in the area and an increase in grass biomass. However, there were no increases in grass biomass during years with low to medium density grasshopper populations (Thompson et al., 1996). While a specialist grasshopper may cause a short-term reversal of a desertification trajectory (increasing abundance snakeweed and reducing grass production), it only occurs during population outbreaks. During most years, *H. viridis* herbivory on *G. sarothrae* is benign.

Most research on desert rodents has focused on granivory and the effects of granivory on soil seed banks. However, most desert rodents feed on plants or plant parts during some or all of the year and may, thereby, have an effect on desertification (Kerley and Whitford, 1994). In the Chihuahuan Desert, several species of kangaroo rats, *Dipodomys* spp., are known to cut large numbers of tillers from a variety of grasses (Kerley et al., 1997; Sipos et al., 2002). Fourteen species of perennial grasses, three species of annual grasses, and one invasive alien species (*Eragrostis lehmannania*) were reported to be subject to removal of flowering and vegetative tillers by rodents. Rodents also severed stems of three of the most abundant perennial herbs and three of the most abundant annual herbs (Kerley and Whitford, 2009). This indicates that rodent herbivory is not limited to a few grass species or just monocotyledons. The herbivorous behavior of rodents has largely been unrecognized but recent experimental evidence documents the extent and contribution of this behavior to the maintenance of ecosystems in a degraded state.

In an experiment comparing the effects of tiller cutting of the bunch grass *Sporobolus flexuosus* by rodents, caged grass tussocks that were protected from rodents were compared to plants subject to browsing. Plants subjected to rodent-pruning exhibited very little regrowth of tillers even when pruned tussocks were protected from further herbivory. The lack of recovery (growth) of severed tillers is probably due to the loss of the apical meristem (Briske, 1991). Loss of vegetative tillers markedly reduced the vigor of the grass tussocks. Most of the tillers were severed before seed set. Although medium-size kangaroo rats consume relatively few grass seeds (Reichman, 1975), the effect of rodent graminivory was to reduce the average seed production by a factor of 65. This supports the hypothesis (Kerley et al., 1997) that kangaroo rat graminivory may have a large effect on seed dynamics of desert grasses.

In another experiment where graminivory was examined in relation to tussock size and distance to neighboring tussocks, graminivory was highest on the smallest plants and considerably higher on small plants that were surrounded by bare soil. Graminivory did not occur on tussocks that were in contact with other tussocks. These relationships suggested that graminivory would be highest in overgrazed areas where small and germinant grasses are surrounded by bare soil (Kerley and Whitford, 2009).

The contribution of graminivory by rodents to the prevention of recovery of overgrazed grassland was recently documented in a study of paddocks that were intensively grazed for 1 or 2 days a year between 1995 and 1997 (Roth et al., 2009). Grazed plots had very little grass cover when compared with the ungrazed controls although grazing had been excluded from all plots for more than 5 years. Rodent graminivory reduced the canopy cover of the grasses in the overgrazed plots between 73% and 95% (Table 1) in comparison with little reduction in canopy

Table 1. Proportion of tillers cut by rodents on ungrazed plots and plots intensively grazed by cattle relative to the total percentage of cover of each grass species measured at the end of the growing season. Proportions of total cover clipped by rodents that are different ($p < 0.05$) are indicated by different letters. (Modified from Roth et al., 2008.)

	Ungrazed plots	**Grazed plots**
Grass species	**Proportion of total clipped**	**Proportion of total clipped**
Bouteloua eripoda	5.1% a	73.3% b
Aristida purpurea	3.3% a	73.3% b
Aristida ternipes	24.7% a	77.5% b
Sporobolus spp.	16.2% a	97.0% b

area of the grasses in the ungrazed control plots. The level of rodent graminivory in the overgrazed plots has undoubtedly contributed to the lack of recovery of grass cover in the absence of grazing.

These data also demonstrate that in grasslands that are only moderately degraded by expansion of undesirable shrubs such as mesquite, graminivory by rodents is benign, with little effect on the structure of the grassland.

Graminivory on seedlings contributes to the virtual absence of grass in shrublands. In a study of survival of transplanted black-grama grass (*Bouteloua eriopoda*) seedlings in grassland, shrub-grass matrix ecotone, and shrubland, there was higher loss of seedlings to herbivory by small mammals in the shrubland than in the grassland or ecotone areas.

5. Indirect Effects of Animals on Plants

While graminivory and herbivory are important processes that contribute to desertification or to the maintenance of desertified ecosystems as alternate stable states, there are less direct but similar effects of animals on plants in arid regions. For example, animals may serve as the primary dispersal agents of seeds of woody plants that are undesirable in rangelands. Phytophagous insects may contribute to the growth and vigor of shrubs in desertified shrublands by affecting soil processes. Several types of soil disturbance are known to affect the patch dynamics of arid ecosystems and may contribute to the resilience of desertified ecosystems.

Prior to the introduction of the livestock industry, honey mesquite (Prosopis glandulosa) distribution was limited to the margins of ephemeral streams and ephemeral lakes (Johnston, 1963). The dispersal of mesquite from riparian habitats into upland grasslands has been attributed to domestic livestock. Livestock consume the seed-pods and seeds of mesquite, which are reported to have enhanced germinability after passing through a herbivore-digestive tract. Livestock deposit the seeds in nutrient-rich dung in areas that are frequently distant from the parent plants (Brown and Archer, 1989). While livestock are agents for long-distance dispersal of mesquite, heteromyid rodents (Dipodomys spp., Chaetodipus spp., Perognathus spp.) are thought to be agents of short distance dispersal and to enhance germination by seed scarification and by burying seeds in shallow, scatter hoard, caches (Duval et al., 2005). The germination and establishment of mesquite depends on weather conditions prior to the dispersal of the seeds and the abundance of rodents. In an experiment examining the germination and establishment of mesquite in three microsites in a desert grassland, all of the seeds and seedlings were removed from cattle dung pats in all locations. During the growing season of this experiment, no grasses or herbaceous annuals flowered and the only plants that set fruit were mesquite. The dry conditions and lack of other foods probably account for the destruction of dung pats and excavation of all of the caches.

Experiments in the following 2 years examined germination and establishment of mesquite in simulated rodent caches with rodent exclusion cages over one-half of the caches. Despite relatively high rates of germination in both caged and open caches, over-winter survival was zero in all microsites except for the rodent exclusion caches in burned grassland. In the repeat experiment the following year, high rates of germination were followed by relatively high over-winter survival of seedlings in the burned-site caged caches (28.6%) and unburned grassland open sites (50%). The most frequently documented cause of mortality was rodent or rabbit browsing. Thus, rodent dispersal of mesquite seeds and successful establishment is dependent on weather patterns, rodent population size, and cache microsite.

In Australia, one of the invasive, undesirable shrubs in salt-bush (*Atriplex vesicaria*) rangeland is nitrebush *Nitraria billardieri* (Zygophyllaceae). Nitrebush is a conspicuous component of many overgrazed salt-bush areas. Emus (*Dromaius novehollandiae*) congregate in areas with abundant nitrebush during the late summer fruiting season. The fruits are small drupes that are consumed in large quantities by Emus. During the peak fruiting period, nitrebush seeds comprise more than 90% of the crop and proventriculus (Noble, 1975). Germination studies showed much higher germination of nitrebush seeds after passing through Emus (67% after 24 days) compared to 17% germination of hand-collected seeds. Germination of Emu-digested seed was much faster (50% within 4 days vs. 3% in 4 days for hand-collected seeds (Noble, 1975)). The relationship between Emus and nitrebush is similar to that of cattle and mesquite seeds with respect to dispersal and enhanced germination. Although woody weeds that produce large fruits and seeds are primarily dependent on animals for dispersal, most plants in relatively intact and desertified arid ecosystems are wind-dispersed. In desertified systems with large patches of barren soil, most seeds are either deposited with litter under shrubs that create eddy currents in winds or in animal produced soil pits (Whitford, 2002). One example of digging and diversity and productivity of plants in the soil disturbed patch, was documented in studies of vegetation on residual burrowing bettong warrens. Species diversity and biomass of forbs and grasses were higher on warren sites compared with off-warren sites (Noble et al., 2007b). Similar results have been reported for banner-tail kangaroo rat (*Dipodomys spectabilis*) mounds in the Chihuahuan Desert of North America (Mun and Whitford, 1990), for crested porcupine (*Hystrix indica*) excavations in the Negev Desert, Israel (Boeken et al., 1995), and for the Cape porcupine (*Hystrix africaeaustralis*) in the semi-arid regions of South Africa (Bragg et al., 2005). Warrens of bettongs, mounds of kangaroo rats, and excavations of porcupines and other large, relatively persistent soil disturbances, frequently represent fertile patches within a relatively infertile matrix and patches with higher infiltration and water storage. These features may therefore serve as refugia for plant species that cannot establish and/or survive in the low-resource areas between patches. Nests of some ants (harvester ants: *Messor* and *Pogonomyrmex*) are well documented as representing nutrient-rich patches and as supporting higher biomass and species richness of annual plants on the periphery of the nest discs (Whitford et al., 2008).

Even small, short-lived excavations can serve as seed traps and contribute to the stability of ecosystems. In banded mulga (*Acacia aneura*), the excavations produced by the large varanid lizard (*Varanus gouldii*) in the process of digging out ground-nesting spiders, serve as traps for seeds of grasses, forbs, and mulga (Whitford, 1998). The varanid pits are concentrated at the interception zones of the vegetation bands. The seed trapping pits produced by *V. gouldii* contribute to the dynamics of the banded vegetation by concentrating seedlings and young plants at the upslope edges of the bands where run-off water accumulates. In North American deserts, heteromyid rodents collect and store seeds in burrows and scatter hoard seeds in spatially dispersed, shallow excavations (Price et al., 2000). The scatter-hoard caches are important germination and establishment sites for plant species that produce seeds selected by the rodents. Establishment of plants from seed initially scatter-hoard-cached by kangaroo rats (*Dipodomys merriami*) was an order of magnitude higher than those from seed that escaped harvest by rodents or ants (Longland et al., 2001). Even scatter-hoard caches that are retrieved by the rodents serve as traps for litter and other seeds. These are examples of indirect effects of animals that affect the structure and function of both desertified and relatively undisturbed ecosystems.

Herbivory by insects can have an effect on the vigor of invasive shrubs. Growth of an invasive shrub, creosotebush (*L. tridentata*), is both water- and nitrogen-limited (Fisher et al., 1988). In a study designed to examine the contribution of herbivorous insects to short-term nitrogen cycling in creosotebush (*Larrea tridentata*) shrublands, these insects contributed approximately 20% of the canopy to soil nitrogen flux. Sap-sucking phytophagous insects were the most abundant arthropods but leaf-chewing phytophagous insects accounted for the highest biomass of the foliage arthropods (Lightfoot and Whitford, 1990). Leaf-chewing insects produced more frass (fecal material) of partially or wholly digested leaf material. Sap-sucking insects excrete a variable volume of liquid that is dependent in-part on the amino acid content of the plant fluid. The frass of sap-sucking insects is also a dilute solution of simple carbohydrates. The frass not only supplies nitrogen to the soil, and eventually the plant, but the simple carbohydrates provide a readily useable energy source for the microbes in the subcanopy litter and soil. Microbial biomass in arid ecosystems is dependent on the supply of labile carbon, and rates of nutrient cycling are dependent on the microbial biomass (Parker et al., 1984). Phytophagous insects on desert shrubs can indirectly contribute to the growth and reproduction of the shrubs via their contribution to nutrient cycling and, thus, to the resilience of shrub-dominated desertified ecosystems.

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Biodata of **Dr. J. Patrick Kociolek**, author of ***"Microscopic in Size: Macroscopic in Impact. Diatom–Human Interactions"***

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MICROSCOPIC IN SIZE: MACROSCOPIC IN IMPACT. DIATOM–HUMAN INTERACTIONS

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1. Introduction

1.1. OVERVIEW

Diatoms are single-celled, photosynthetic organisms that have cell walls made of silica dioxide or glass. They have chlorophylls a and c, and large amounts of the accessory pigments xanthophylls and carotenoids, giving living diatoms a golden brown hue. Unlike green plants and their allies, the storage product of the photosynthetic activity of diatoms is a lipid or oil (Opute, 1974). Diatoms are a part of the tree of life, currently referred to as stramenopiles, a lineage that includes other golden-brown algae, as well as water molds and a variety of small flagellates (Patterson, 1989; Leipe et al., 1994).

Diatom cell walls, in the form of a pill-box or Petri dish (i.e. in two pieces; the word diatom comes from the Greek and means "to split into two"), are perforated with a number of types of pores and structures that function to allow an exchange of nutrients, secretion of mucilaginous stalks, and even movement. A whole cell is referred to as a frustule, each with a large and smaller theca (epitheca and hypotheca, respectively), as well as few to numerous girdle bands that help to join the valves together. While the systematics of the group is undergoing a revolution due to the application of molecular techniques in determining its evolutionary history (e.g. Medlin et al., 2000; Sims et al., 2006), most of the structure of the taxonomy for the groups is still based on cell wall morphology (e.g. Round et al., 1990).

1.2. LIFE HISTORY

As individual cells, diatoms undergo vegetative cell division, one to several times per day. This asexual reproduction requires dealing with complexities imposed by the rigid glass cell walls. Thus, for each of the two thecae of the "mother" cell, a new, smaller theca is created internally. Over many cell divisions, an implication of this method of developing new cells is that the overall population decreases in size (MacDonald, 1869; Pfitzer, 1871). The resulting size diminution has concomitant implications for valve shape (Geitler, 1932), making the diagnosis and identification of species along the size range of a taxon more difficult.

At some point in the size series, and many times related to environmental cues, diatoms undergo sexual reproduction. These diploid organisms undergo meiosis, and the products of reduction division in terms of gametes may be as few as one gamete, and as many as 128 gametes (Drebes, 1977). One group of diatoms produce isogametes that are amoeboid, while another forms eggs and sperm. The flagellated sperm have a most unique arrangement of microtubules in their flagella, with a 9 + 0 arrangement, lacking the central doublet (Manton and von Stosch, 1966; Heath and Darley, 1972). Almost every other eukaryotic flagellum or cilium has a 9 + 2 arrangement of microtubules (Mitchell, 2007). After syngamy, the resulting non-siliceous zygote expands in volume and, in some groups, length, and reaches a maximum size. This auxospore may be covered in a variety of siliceous scales or strips, characteristic of the group and species. Following this elongation of the auxospore, the next division creates what is termed as the "initial valve" or "Erstlingzelle." Of the maximum size, this valve may express some deformities or abnormalities not present in the typical vegetative cells. The auxospore and initial valve are most susceptible to environmental influences. The next division usually produces the typical vegetative cell morphology. Thus, sexual reproduction not only affords diatoms the exchange of genetic information among individuals in the population, but also allows the cell lineage to regain maximum size. Lewis (1984) has described the interplay (or lack thereof) and timing between diatom sex and environment. In certain situations, auxospores can be produced asexually (Round et al., 1990).

1.3. STATIC CELL WALLS AND POLYMORPHISM

Common dogma among diatomists is that diatom variation is negligible. "…most diatoms divide to produce valves that are virtually identical to the parent valves…" (Round et al., 1990, p. 84) represents a popular explanation of the diatom cell walls. This has included the idea that reproduction of the cell walls from one generation to the next is done so faithfully, that diatoms were used as test objects for light and electron microscopes. The general feeling is that some records of polymorphisms do exist; however, in his review of species concepts in diatoms, Mann (1999) gave little space discussing variation generally, and polymorphism specifically. This phenomenon has usually been considered as limited in number, the range of the polymorphism is not so extreme as to budge the foundations of our systematic schemes, and the more disparate cases have been called into question (Mann, 1999).

Yet, the interplay between development, genetics, and environment (the "triple helix" of Lewontin, 2000) is only now being understood, as data continue to be compiled detailing the ways the glass cell walls of diatoms may be transformed. During cell division and subsequent cell wall morphogenesis, environmental conditions can influence how the cell wall is laid down, to the point of the diatom producing valve features that are different from the valves produced under different environmental conditions. A wide range of environmental variables, some natural and others induced or accentuated by humans, have been identified to affect cell wall morphology. They include salt (e.g. Stoermer, 1967; Schultz, 1971; Paasche et al., 1975; Tuchman et al., 1984; McMillan and Johansen, 1988), silica (e.g. Theriot, 1987; Theriot and Stoermer, 1981, 1984), temperature (e.g. Syvertsen, 1977), seasonality (Kociolek and Stoermer, 1988), pH (Hustedt, 1937–1938), metals (Doucette et al., 1987; Gensemer, 1990), and nutrient levels (Theriot et al., 1988; Hürlimann and Straub, 1991).

The entire genome of a single diatom, *Thalassioria pseudonana*, has been sequenced (Armbrust et al., 2004). While a wide variety of studies have used molecular and biochemical data to generate hypotheses about diatom relationships (e.g. Medlin et al., 2000; Sims et al., 2006), and thereby document variability across the taxa (see also Medlin et al., 1991 on *Skeletonema*), very few studies have documented intraspecific variation. Gallagher (1982) demonstrated variation among clones and within-populations of *Skeletonema costatum* across time (seasons) in Narragansett Bay, Rhode Island. Bourne et al. (1992) showed tremendous variability in molecular sequence data for cell nominally identified as *Cyclotella meneghiniana*. Soudek and Robinson (1983) and Lewis et al. (1997) documented variation across geographic space for *Fragilaria capucina* and *Asterionella formosa*, respectively, although the morphologies of the populations were identical. Wood et al. (1987), working with *Thalassiosira tumida*, were able to identify a genetic component to variation associated with morphology. They noted that the amount of variability is high, and argued that in the context of directed evolution (intense, directional selection), significant morphological change could occur across relatively short time intervals.

1.4. DISTRIBUTION

As a group, diatoms are widely distributed. Species occur in almost every place where there is or has been water. Their ecological breadth spans many environmental spectra. For example, with regard to temperature, certain species of diatoms occur at the mouths of hot springs, being among the first eukaryotes present at near-boiling water (Jones et al., 2000), while other species are known to occur in sea-ice (Quillfeldt et al., 2003). There are species found in the most nutrient-dilute freshwaters (Lange-Bertalot and Metzeltin, 1996) and others that live at the outfalls of sewage-treatment plants. The group spans nearly eight orders of magnitude in terms of hydrogen-ion concentration, with some species living in acid mine drainage (DeNicola, 2000) and others living at a pH of about 10.5, the level where silica begins to dissolve (Ryves et al., 2001). And the group may be found across a wide salinity gradient, with some species found in inland saline lakes, within the marine environment, estuaries, and very dilute freshwaters. Some authors have termed them “the ubiquitous diatoms” (Lohman, 1960).

1.5. DIVERSITY AND CLASSIFICATION

The group has many described species. A recent database of diatom names indicates that over 60,000 species of diatoms have been described (Fourtanier and Kociolek, 2008).

Diatoms have been traditionally split into two major groups, the centric and pennate diatoms. This split recognizes some basic differences between the two groups in terms of general valve organization, sexual reproduction, and chromatophore number and arrangement. The centric diatoms have radial symmetry, are oogamous, and usually have numerous, small discoid chromatophores. Pennate diatoms have bilateral symmetry, are nearly all isogamous, and usually have a few, large chromatophores. Round et al. (1990) revised the higher level classification, distinguishing the pennate raphid diatoms (those diatoms having a raphe are capable of relatively rapid and prolonged movement) and araphid pennates, which lack a raphe system. The recognition of three classes of diatoms, centric, araphid, and raphid, echoes the system developed by H.L. Smith nearly 100 years before Round et al.'s system.

A molecular approach to diatom classification (e.g. Medlin et al., 2000; Sims et al., 2006) more or less supports a system developed by Simonsen (1979), where centric and araphid diatoms are non-monophyletic. That is, these groupings include some but not all of the descendants of a common ancestor. Only the raphid pennates are monophyletic. While these relationships are based on a relatively few number of taxa, it seem inevitable that the classification of the past will give way to a new set of higher level groupings. Williams and Kociolek (2007) indicated that this will result in a sea of change of information for the discipline.

2. Diatoms and the Aquatic Food Chain

Diatoms are important players in the food chains of almost all aquatic ecosystems. Owing to their biochemical makeup, including oil, they are the preferred food source for a wide array of primary consumers in almost all of these systems (EPA, 1995; Ross et al., 2000; Power and Dietrich, 2002; Schnetzer and Steinberg, 2002). In studies where primary consumers are offered a choice between diatoms and other food types (other primary producers, but also bacteria), invariably diatoms are selected (Ahlgren et al., 1990). Some suggest the omega-3 fatty acids produced by diatoms is the reason for their selection; thus, resources are not just numbers driven, i.e. quantity, but involve quality as well (Brett and Goldman, 1996, 1997). Because of their important role in aquatic food webs, a significant amount of work has been done to understand the implications of a variety of perturbations on aquatic ecosystems, and their effects on diatoms. The implication is that due to declines in diatom populations, or significant changes in metabolism of storage products due to the perturbations (Sicko-Goad et al., 1989; Lazinsky and Sicko-Goad, 1990; Schelske and Sicko-Goad, 1990; Sicko-Goad and Andresen, 1993), such effects could affect other ("higher") parts of the food chain, including many fish species.

3. Diatoms and Global Carbon, Oxygen, and Silica Cycles

Through their large numbers as well as high rates of carbon fixation and oxygen evolution, diatoms are important players in connecting a variety of nutrient cycles within the aquatic systems, and with the terrestrial ecosystem and atmosphere, thus being major players in global biogeochemical cycling. The ubiquity of diatoms, and their large numbers in marine ecosystems, makes them significant agents for primary production. They play important roles in carbon cycling in the oceans (Francois et al., 1993; Hayes, 1993). It has been estimated that diatoms are responsible for more than 25% of all the primary production on earth (Falkowski and Raven, 1997; Falkowski et al., 1998), and may evolve more oxygen than all the rainforests combined (Falkowski et al., 2000). Diatoms are the major player in the production of biogenic silica, where they take dissolved silica out of the water and create their cell walls (Brzezinski, 1985; Dugdale et al., 1995; De la Rocha et al., 1998; Dugdale and Wilkerson, 1998; Ragueneau et al., 2000; De la Rocha, 2006). The isotopes of oxygen and silica in biogenic silica can be used to examine nutrient cycling and climate changes over time, especially in systems such as the southern oceans where carbonates are not well preserved (Takeda, 1998; White, 2001; Leng and Barker, 2006). In freshwaters, declines in diatom productivity due to the reduced availability of silica, have been attributed to climate change (Verschuren et al., 2002; Verburg et al., 2003).

4. Poisonous Diatoms

For a long time, diatoms had been considered as the "good guys" of the plankton, serving as the base of the food chain and taking up carbon dioxide and evolving oxygen. In fact, in books published in the 1960s and 1970s dealing with algae and humans, including toxic algae, there is no mention of toxic diatoms (e.g. Jackson, 1964). However, about 20 years ago, toxic blooms of domoic acid-producing diatoms were discovered along the SE shore of Canada (Bates, 1997). The taxa responsible for those blooms were many species of the genus *Pseudo-nitzschia* (Trainer et al., 2008) and *Amphora coffeaeformis*. *Pseudo-nitzschia* blooms have now been widely reported, including off the coasts of Japan, Korea, China, Chile, Argentina, Costa Rica, Morocco, Tunisia, New Zealand, Adriatic Sea, Mediterrean Sea, Turkish coast, Mexico, and east and west coasts of the United States (e.g. Qi et al., 1996; Fryxell et al., 1997; Ochoa et al., 1997; Kotaki et al., 1999, 2000; Akallal et al., 2002; Bargu et al., 2002; Vargas-Montero and Freer, 2004; Besiktepe et al., 2008; Orlova et al., 2008; Quijano-Scheggia et al., 2008).

Domoic acid is a neurotoxin, first isolated from a red alga *Chondria armata*, with the common name "doumoi" in Japan (hence the name of the toxin). Domoic acid may bioaccumulate in planktivores, such as shellfish and certain fish species. In 2003, domoic acid poisoning killed over 700 marine mammals off the coast of California (Goldstein et al., 2008). When contaminated shellfish are ingested by humans, domoic acid causes amnesic shellfish poisoning (ASP, Todd, 1993), the effects of which may include memory loss, brain damage, and even death (Pulido, 2008). Once a bloom of toxic diatoms is discovered, shellfisheries are closed. Tens to hundreds of millions of dollars are lost each year due to the closure of shellfisheries around the world.

5. Fossil Diatoms

Owing to their glass cell walls, diatoms have an excellent fossil record. Oldest known deposits are of marine origin, and extend back to the Jurassic, about 140 mya (Rothpletz, 1896; Harwood and Gersonde, 1988, 1990). The earliest known freshwater deposits are from the Eocene (Lohman and Andrews, 1968). Molecular analyses, based on a "molecular clock" of relationships, places the origin of the diatoms in the Permian, about 250 mya (Kooistra and Medlin, 1996). While current evidence suggests that a small group of pigmented flagellates are the sister group to diatoms, diatom origins are still debated, with even hypotheses suggesting an extra-earth origin. Fossil diatoms have many applications, from practical products to purely academic pursuits.

5.1. DIATOMACEOUS EARTH

Diatomaceous earth (or diatomite) is usually defined as sediments that are composed of 85% or greater silicon dioxide derived from diatoms. The origin may be freshwater, estuarine, or marine, though the most extensive deposits are of freshwater or marine origin. They occur all over the world, with some of the largest and best-known deposits being located in Europe, Asia, North and South America, Australia, and Africa (Dolley, 2008). It is estimated that in a cubic centimeter of diatomite, one billion cells may be present. The fact that some deposits are many hundreds of meters in area and depth, and the productivity of these systems, many of which are considered to be upwelling zones in the marine environment, is impressive.

Diatomaceous earth has been used by humans in a wide variety of ways. One of the most interesting applications was as a stabilizing agent for nitroglycerin, and the development and distribution of dynamite and munitions by the Nobel family (Nobel, 1868.) The highly unstable nitroglycerin was mixed with diatomite, which, due to its great surface area (due to all the little holes and surfaces of the complex cell walls, many of which are fractured under the pressure during sedimentation), was able to stabilize the product. This allowed dynamite to be mass produced and shipped (without blowing up along the way), resulting in a significant fortune accruing to the Nobels. That fortune was the basis for establishing the widely acclaimed and known Nobel Peace Prize.

Other applications of diatomaceous earth include filtration of a wide range of products, from beverages (beer, wine, soft drinks, and teas) to swimming pools.

It is also used as an abrasive, coating, in dry cleaning, and in pesticides, among many others (Cummins, 1975). It has also been used by primitive people as a food additive, termed "mountain flour" (Bermehl) by some cultures. Production worldwide exceeds 2.1 million metric tons (Crangle, 2008), with a value in excess of US $440 million.

5.2. EVALUATING ENVIRONMENTAL CHANGE

An attribute of diatoms, long understood and appreciated by ecologists, is their fidelity in terms of tolerances or preferences related to environment, and they have been used to evaluate a wide range of environmental conditions, both near-term marine and freshwater diatom records (Holocene and Pleistocene) as well as on longer time scales. In the freshwater realm, these have included salinity, pH, and a wide array of nutrients, some serving as proxies for climate change (Gasse, 1988; Birks et al., 1990; Fritz et al., 1991; Stine, 1994; Wilson et al., 1994; Blinn, 1995; Gasse et al., 1996; Laird et al., 1996; Bradbury, 1999). Large-scale projects for these broad research program have included Lake Baikal, CASPIA (for salt lakes, Juggins et al., 1994), and PIRLA (acid precipitation, Charles and Whitehead, 1986). An archive of paleo lake data is housed in the World Data Center for Paleoclimatology (see http://www.ncdc.noaa.gov/paleo/paleolim/diatom.html).

Fossil marine diatoms have been applied widely to assess climate, sea-surface temperatures, physical characteristics of the oceans, extent of sea-ice, and productivity (e.g. Alexander and Niebauer, 1981; Sancetta and Silvestri, 1986; Abrantes, 1988; Alvarez-Borrego and Lara-Lara, 1991; Barron, 1992a; Koc Karpuz and Jansen, 1992; Sancetta et al., 1992; Schrader, 1992). Large, multinational projects that have generated many samples, papers, and insights into physical, chemical, and biological characteristics and changes in marine ecosystems, which included recovery and analysis of diatoms, are the Integrated Ocean Drilling Program (and related programs, the Deep-Sea and Ocean Drilling Programs; see http://www.oceandrilling.org/; http://www-odp.tamu.edu/; these websites give an excellent overview of the programs and access to past publications, data, and ways to access core material previously taken) and ANDRILL, which is focused on Antarctica (see http://www.andrill.org/ for the research program and ways to participate).

5.3. COMMUNITY STRUCTURE AND ECOLOGICAL REPLACEMENT

Ecological replacement can be discussed as the origin, establishment, and exclusion of one species by one or more other species. Such a pattern may be interpreted from a variety of perspectives, ecological and/or evolutionary. Based on the wealth of material from large ocean coring programs that have captured material across broad lenses of space and time, the turnover of diatom floras have been documented (Strelnikova, 1990; Barron, 1992a, b; Barron and Baldauf, 1995). Barron (2003) lists many species that either went extinct or migrated (and became more regional or local in their distributions), in response to cooling events through the Cenozoic.

While floristic surveys of freshwater fossil deposits have helped to paint a picture of ecological conditions of the habitat represented by the fossil deposits, little has been made of the community structure of the diatom communities themselves. Many times, the compilation of the individual taxa is made and discussed (e.g. VanLandingham, 1964), but an overview of the community, with fossil and/or modern analogs, levels of diversity, or other measures of community structure are rarely given. An exception to this is a rarely cited article by Van Landingham (1970), in which he describes the pH, trophic status, salt concentration, and flow of a variety of ancient lakes, from Pleistocene to Oligocene in age, based on the diatoms present. Interestingly, for some physical and chemical features, Van Landingham's analysis (his Plate 7) shows more uncertainty in terms of species preferences in the younger deposits than in the older ones.

In Lake Baikal, for example, Khursevich et al. (2002, 2003a, b, c, 2004) described the changes in the centric diatom flora there over 10 million years, from *Concentrodiscus* to *Mesodictyon*, *Mesodictyopsis*, *Stephanopsis*, *Stephanodiscus*, *Cyclotella*, and *Cyclostephanos*, where overlap between the species of the different genera is quite small. In western North America, Bradbury and Krebs (1995) described, from approximately the same time frame as in Lake Baikal (i.e. Miocene to Pleistocene), the sequence of genera *Aulacoseira* to *Actinocyclus*, cyclotelloid genera, *Cyclostephanos,* and *Stephanodiscus*. A pattern of taxa similar to western North America has been described for deposits on the Balkan peninsula (Ognjanova-Rumenova, 2006). Discussion of these seemingly similar patterns across wide geographic space over 10 million years is necessary.

5.4. PALEOECOLOGY

Interpretation of fossil freshwater diatom data has been made through a number of lenses. Many of the discoveries of the deposits were made initially by geologists, and the lenses they looked through were focused on structural causes or explanations. Hence, interpretations of environmental context was made relative to the landscape structure, shifts, mountain orogeny, etc. (see many interpretations in the summaries provided in Feth, 1964).

Intense investigations have occurred regarding the degradation and eutrophication of recent freshwater bodies over the last 40 years. Eutrophication has been viewed as a natural process (Hutchinson, 1969, 1973), speeded up by natural or human-induced processes (Wetzel, 1975.). While recent Holocene sediments have been used extensively to document changes over time (see review by Hall and Smol, 1999), surprisingly little has been studied regarding the natural eutrophication process in pre-human systems, especially some of the lake systems that have been or were in existence for (relatively) long periods of time. Van Landingham (1970) suggested that certain Oligo-Miocene lakes reflect oligotrophic conditions, while other Miocene lakes may have been eutrophic. These systems should allow us to pose questions, such as "Do the changes seen in diatom community structure (autecological and synecological) in human-induced eutrophicated systems mirror the natural process of eutrophication?" If there are differences, what are they? This is applicable to understanding whether the "natural" course of water body evolution differs from systems that are anthropogenically impacted.

6. Evolution

6.1. EXTINCTION

Strelnikova (1990) gave an overall picture of the origin and extinction of major groups of marine diatom diatoms. At the level of genus, Fourtanier and Kociolek (1999, 2003) indicated that many genera, nearly the majority of those described, are known only as fossils.

While in recent sediments and collections, we can talk about local extinctions (e.g. *Gomphoneis* from the southern Great Lakes; Kociolek and Stoermer, 1988), the fossil record demonstrates the phenomenon that extinction is forever. Extinction events are well-documented in the freshwater fossil diatom record. Khursevich and Cherniaeva (1989) documented a new family Ectodictyonaceae, exclusively from fossil material around Lake Baikal, which occurred and subsequently went extinct in the Miocene. A number of diatom genera are also known to be extinct, including the non-pennate genera *Tertiariopsis*, *Tertiarius*, *Alveolophora Mesodictyon*, *Mesodictyopsis*, *Stoermeria*, the pennate genera *Ambistria*, and *Gomphopleura*. Some of these were restricted to one area (e.g. Lake Baikal), whereas others had broader geographical distributions (e.g. *Stoermeria*). There are literally thousands of extinct species of marine and freshwater diatoms. The timing of some of these freshwater extinctions appears synchronous, within a system or across large geographic space. For example, extinction of several species of *Gomphoneis* occurred within two subgroups of the genus right at the Miocene–Pliocene boundary, in Europe and western North America (Kociolek and Stoermer, 1989).

6.2. GIGANTISM

In many freshwater lineages, the evolution of forms that are significantly greater in size and volume than sister taxa has been demonstrated. We have seen giant species of *Gomphoneis*, *Reimeria*, and *Gomphonema* in western North America from the Miocene (Kociolek and Stoermer, 1987, 1990). This phenomenon may be explained by the abundance of silica and other nutrients after extensive mountain building and volcanism (Taliaferro, 1933). A similar situation is seen in *Gomphoneis* from approximately the same time frame in Europe (Kociolek and Stoermer, 1988). Alternatively, Finkel et al. (2005) described a decrease in the mean valve area in marine diatoms across the Cenozoic, driven by climate-related factors.

6.3. ADAPTIVE RADIATION

This term can be defined as when a lineage "flowers" in terms of morphology and taxa (Futuyma, 1979). Round et al. (1990, p. 124) indicated that through the Miocene, there was a "flowering of marine diatom diversity." This situation is hinted at for the genus *Azpeitia* (Fryxell et al., 1986), and documented for lineages comprising the genus *Crucidenticula* and the genera *Denticulopsis* and *Neodenticula* (Yanagasawa and Akiba, 1990). The Miocene also saw the diversification of species of *Delphineis* (Andrews, 1988; Yanagasawa, 1993), and a lineage of forms within the genera *Rossiella*, *Bogorovia*, and *Koizumia* (Yanagasawa, 1996). Gombos (1980) described the radiation of genera in the Asterolampraceae across the Eocene and into the Early Oligocene. In freshwater systems, the late Miocene and Pliocene were the times of the origin of many new species and closely related genera, including *Gomphoneis*, *Gomphonema* (Kociolek and Stoermer, 1990), and *Actinocyclus* (Bradbury and Krebs, 1995). Many of these radiations occurred at approximately the Miocene–Pliocene boundary, when a large variety of new habitats seems to have opened up with mountain orogeny and creation of large lakes systems (e.g. Feth, 1964) and abundant nutrients (Taliaferro, 1933).

6.4. TEMPO OF EVOLUTION

In a series of papers published between 1945 and 1950, James Small, a mathematician interested in questions regarding evolutionary pattern and process and quantitative data to examine those questions, looked to the diatoms for a critical data set to explore questions regarding tempo and mode of evolution. Small (1946, 1948a, b, c) anticipated by several decades what today would be called "punctuated equilibrium" (Eldredge and Gould, 1972; Gould and Eldredge, 1977) model for explaining the pattern of diatoms through time. He showed that evolution in the diatoms (in terms of the origin of new taxa) happened in a step-wise fashion, with periods of stasis followed by bursts of evolutionary activity, followed by additional periods of stasis. However, he did not express support for the Darwinian explanation of evolutionary change in his analysis, that is, long, slow, and gradual change. Though dated, Small's work offers a variety of interesting, testable hypotheses, ready-made for diatomists interested in evolutionary biology and fossil record.

Khursevich et al. (2002) indicated the stratigraphic sequence of species within the genus *Mesodictyopsis* Khursevich and Kociolek from Lake Baikal, the only place where the genus has been identified. Most species do not overlap temporally, and the duration of the species varies greatly. However, it seems likely that the divergence, tempo, speciation, and factors affecting extinction of the seven species of the genus, can be determined. Kociolek and Stoermer (1989) described the evolutionary history of the genus *Gomphoneis*, including the burst of morphological diversity as the genus appeared on the scene in the late Miocene. Additionally, Theriot et al. (2006) examined the morphological evolution of *Stephanodiscus yellowstonensis* from *S. niagarae*. In contrast to Small's sweeping approach of previously published data, Theriot and colleagues painstakingly quantitatively evaluated the morphological changes in *Stephanodiscus* species with SEM, through a finely sectioned core from Yellowstone Lake. They demonstrated the relatively rapid (in terms of decades) changes in the valve's fine structure, though the changes were small from one core section to the next. Other studies examining evolutionary rates include those of Sorhannus (Sorhannus, 1990; Sorhannus et al., 1998) for *Rhizosolenia* species.

7. Archeology

Diatom remains in a variety of materials have been used to discuss a wide range of issues relating to past human settlements and cultures. Diatoms associated with archeological sites have been used to help date the remains (Mannion, 1987; Straub, 1990), with some fantastic claims about an arrival of humans in the new world much earlier than previously estimated (Hardaker, 2007). Diatoms associated with cultural sites have also helped to describe the environmental setting of the area and time, allowing scientists to infer modes of life and conditions of the local population (e.g. Newman, 1977; Alhonen and Matiskainen, 1980; Gibson, 1986). Few studies have used diatoms, however, to examine changing habitats and climates, and to investigate adaptations of locals to the dynamic world around them. Association of diatoms with clay, and their incorporation into pottery and other artifacts, have allowed archeologists to examine the sources of materials used by cultures (local or distant), and to describe whether cultures were involved in commerce to gain materials and/or objects due to a lack of needed materials in their region. Association of diatom taxa from particular environments has shown, for example, the harvesting of shellfish from particular marine environments (Denys, 1992). Presence of diatoms on the teeth of human remains has shown consumption of shellfish by humans in certain locales (Boyadjian et al., 2007). Furthermore, the presence of diatom silica in the material from the pyramids suggests that Egyptians used a concrete-like material thousands of years before the Romans (who are usually given credit for the origin of concrete).

8. Assessment of Modern Environmental Conditions

Relating diatoms to the ecological situation they occupy has a long history. Kolkwitz and Marsson (1908) were some of the first to relate algal species, including diatoms, to water quality. A wide variety of "systems" were developed indicating the relationship between diatom species present and particular physical or chemical conditions of the water (e.g. Kolbe, 1932; Hustedt, 1937–1938, 1953, 1957; Krasske, 1939; Petersen, 1943; Jørgensen, 1948, 1950; Moller, 1950; Cholnoky, 1958, 1968).

For over 50 years in the United States, diatoms have been applied to understanding the quality of freshwaters. Much of the pioneering work in this field was done by Dr. Ruth Patrick of the Academy of Natural Sciences of Philadelphia, who just turned 100 years old. While as a group, diatoms are found across a wide spectrum of ecological conditions, individual species have more narrowly defined niches.

Diatoms occupy the primary interface between the physical–chemical realms and the biological realms (converting carbon to sugars, taking up carbon dioxide and releasing oxygen cycling silica), and due to their short generation times, they are the front edge of reacting to a wide range of changes. Diatoms have been used to study changes in pH, temperature, salinity, nitrogen, phosphorus, and metals, whether those inputs have been from the atmosphere, terrestrial ecosystems, or directly into aquatic systems. They are excellent indicators of past climates and climate change, as well as the many direct perturbations impacting the aquatic ecosystems, including more general land-use patterns to point-source pollutants.

Another feature that makes diatoms a preferred tool for understanding the ecological conditions and change is their generation times. Unlike some aquatic organisms that live from months to years, diatoms divide from one to several times a day (meaning that a single cell can turn into a billion cells in the course of a month). Thus, they react almost instantaneously to the environmental conditions that they face. And like other biomonitors, diatoms face the environmental music 24/7. Intermittent monitoring of, say, chemistry, by humans takes only a narrow snapshot of ecological time and conditions. Studies have suggested that diatoms offer a better understanding of water quality than water chemistry (Stevenson, 1998).

Understanding the autoecology of species, their individual tolerances, and preferences allows us to make certain inferences about the relative conditions of aquatic ecosystems. Furthermore, we can examine the structure and pattern of the entire diatom communities. This synecological approach uses a wide range of diversity and species richness measures to understand water quality. In addition to the numerous species and large number of individuals (making this group useful in terms of developing robust statistical analyses), there are a number of other features of diatoms that make them a group that can convey much ecological information about the status and trends of aquatic systems all over the world, including rivers and streams (e.g., Archibald, 1972; Descy, 1979; Lange-Bertalot, 1979; Chessman, 1986; Watanabe et al., 1986; Biggs, 1990; Bahls, 1993; Rosen, 1995; Whitton and Kelly, 1995; Lowe and Pan, 1996; Pan et al., 1996; Stevenson, 1998; Bate et al., 2004) lakes (e.g. Coté, 1983; Charles, 1985; Kingston and Birks, 1990; Hall and Smol, 1992), brackish waters (Simonsen, 1962; Snoeijs, 1989, 1994; Leskinen and Hällfors, 1990; Wendker, 1990; Sündback and Snoeijs, 1991; Juggins, 1992; Grönland, 1993; Witkowski, 1994) and coastal marine zones (e.g. Hohn, 1959; Hein and Koppen, 1979; Admiraal and Peletier, 1980; Baillie and Welsh, 1980; Varela and Penas, 1985; Lukatelich and McComb, 1986; Sullivan, 1986; Riaux-Gobin, 1987; Coleman and Burkholder, 1995). Tens of millions of dollars are spent annually utilizing diatoms to assess water quality. In the United States, this occurs across continental-scale monitoring programs to mandated programs within individual municipalities.

9. Fuels of the Future

Though extensive discussions and debates about fossil fuel reserves are at the top of the mind today, impacting high-level domestic and foreign policy of many nations, the limits of those reserves and possible options for fuels have been discussed for some time (Meier, 1955). Furthermore, while the current mode has been to use domestic food crops to augment oil with ethanol (corn mostly, but others as well), this is seen as a stop-gap measure, not a long-term strategy. Diatoms are thought by some to have been the source of some fossil-fuel reserves, and there is intense interest in biofuels generally and specifically focused on diatoms. The case for diatoms as a potential source of biofuels is several fold. First, they naturally produce oil. Some species of diatoms are prodigious oil producers, achieving up to 60% of their body weight in oil (Sheehan et al., 1998). They are not only able to produce a lot of oil relative to their body size and weight, but the rate of production is high, given enough nutrients, carbon dioxide, and growth conditions that provide optimal physical and chemical conditions (Sheehan et al., 1998). Nitrogen and phosphorus sources could be effluent from sewage-treatment plants. Extraction processes of oil from diatoms have been worked out. In fact, calculations based on current rates of production and yields have suggested that a culture facility of the size of the Mojave Desert would be enough to supplant current levels of imported oil into the United States (Sheehan et al., 1998). Furthermore, paradoxically, these aquatic organisms actually would require less water to produce a barrel of oil than any terrestrial organism (Sheehan et al., 1998). Efficient photosynthetic activity of diatoms would also help serve as a carbon dioxide sink.

Challenges to realizing the potential of diatoms as biofuels include the fact that only a small fraction of species has been assayed. Currently, finding species that may have even higher levels of oil production is basically a “hunt and peck” approach, with few roadmaps to success. Collaboration between biologists and engineers might be a much more successful enterprise for finding species with optimal oil production characteristics versus many individual, discipline-specific approaches, which more or less describes the current situation. Development of techniques to maximize production by species currently investigated includes the right combination of physical and chemical conditions, including the development of bioreactors from the traditional large, open, swimming pool-like arrangement to structures that are oriented vertically and which not only maximize oil production, but lend themselves to mass scales. Extraction techniques are also an intense area of investigation and development. Almost all work is focused on finding the “best” species, and focusing on monocultures, similar to large-scale terrestrial crop production.

Though the amount of money being invested in the possible development of diatoms as a large-scale biofuel source is large relative to the past, it is almost impossible to understand the scale of development at this point. Unlike past efforts, where the investigations were mostly government-sponsored, today much of the investment is by private investors. And with this approach, many companies have kept the results of their R and D internally, outside the regular scientific literature. Electronic blogs and new websites are appearing fast, as amateurs to new investors are trying to gather and share information on this exciting prospect.

10. Nanotechnology

The limits of human-produced engineering solutions are being encountered at the nanoscale (10^{-9} m; 1 mm + 1,000,000 nm). The idea of microfabrication and nano-sized widgets accomplishing a wide range of tasks in the fields of engineering, medicine, and biology (to name a few) has led to how these ultra-small objects will be designed. Diatoms produce an amazing variety of features and networks in their superstructures and substructures. Owing to the silica nature of their cell walls, diatom structures might lend themselves to industries that also use silicon, such as semiconductors. The three-dimensional and intricate structure of diatom cell walls may have antibodies associated with them that retain their biological activities, and this complex may serve as a relatively inexpensive, testing tool for immunology (Townley et al., 2008). In addition, defining and elaborating the physical and transmittance properties of diatom cell walls may be critical for establishing whether nanostructures produced by diatoms are useful in the development of electro-optical devices via biologically-produced structures (De Stefano et al. 2007), reflecting and anti-reflecting surfaces and coatings (Parker and Townley, 2007), and nanoelectrode arrays in electrochemical or optical sensors (Losic et al., 2006). Other applications might include dispensing medicines through nanocapsules and as a wide range of nanofilters for everything from dust to Anthrax cells. An entire issue of the Journal of Nanotechnology (Gordon et al., 2005) was devoted to the topic of the intersection between diatoms and this emerging field.

11. Forensic Sciences

The use of diatoms by forensic experts to determine the timing and place of potential drowning victims has a long history (Pollanen, 1998; Rohn and Frade, 2006). The basis of this application is when a living person has drowned, diatoms enter the lungs, and can make their way through the body, such that they also end up in other organs (liver, kidney, brain) and systems (circulatory). Examination of human organs and tissues can reveal, due to the types and species of diatoms present in the body, not only if the person was drowned in a freshwater or marine ecosystem, but many times, the exact water body. Also, the pattern of colonization of the exterior of a dead body by diatoms can indicate the time between drowning and recovery of the body (Cameron, 2004; Zimmerman and Wallace, 2008).

Diatoms are an amazing group of organisms, whose interface with humans extends from the very basic (providing oxygen for breathing), to the very practical (the many uses of diatom fossil for a wide array of products) to the academic. Though small, their impacts affect the entire planet and many of the species that inhabit it, including *Homo sapiens*.

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PART 5:
PLANT DEFENSES

Smith
Seckbach

Biodata of **C. Michael Smith**, author of ***"Biochemical Plant Defenses Against Herbivores – From Poisons To Spices"***

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BIOCHEMICAL PLANT DEFENSES AGAINST HERBIVORES

From Poisons to Spices

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1. Introduction

Fossil evidences from some of the earliest terrestrial communities indicate that insect herbivory began near the close of the Silurian, around 420 million years ago (MYA), by early insects with primitive piercing and sucking mouthparts (Labandeira, 1998). During the Early Permian (~280 MYA), plant foliar feeding and skeletonization evolved on some seed-bearing plants. Approximately 200 MY later, specialized associations between plants and their insect herbivores, such as feeding by hispine Chrysomelid beetles on ginger, occurred in the late Cretaceous (Wilf et al., 2000). Many arthropods living prior to the Jurassic (~200 MYA) were polyphagous, but shifts in arthropod feeding from polyphagy to specialized oligophagous feeding and subsequently monophagy occurred during this period. The occurrence of plant alkaloid and terpenoid metabolites at a similar point in the fossil record suggests that plants began to actively evolve these compounds for defensive purposes. These compounds, along with the phenolics, are collectively known as defensive allelochemicals or antiherbivory compounds (Whittaker, 1970).

Changes in insect host plant selection have been dependent on the dynamic equilibrium between the genetic potentials of plants and insect herbivores at various points in co-evolutionary time, their rates of change relative to one another, and the environment in which these interactions have occurred. Each organism has struggled for adaptive advantage. Plants have survived due to the development of physical and chemical defenses to survive insect herbivory. In response, insects have deployed physiological or morphological "countermeasures" to overcome plant defenses. This chapter provides an overview of the discovery of the numerous allelochemical defenses employed by plants to maintain their adaptive advantage against insect herbivory. For additional commentaries, readers are referred to the in-depth reviews of Kessler and Baldwin (2002) and Chen (2008).

2. Current Status

In the early stages of insect plant selection, plant defensive allelochemicals regulate insect behavior, through the production of volatiles that disrupt insect olfactory orientation to the plant, and through the production of feeding deterrents and feeding inhibitors that may force an insect to reject the foliage of a prospective host plant (Tuomi, 1992; Smith, 2005). Once some degree of acceptance of plant foliage occurs by an insect herbivore, any of a number of defensive allelochemicals may also reduce the growth, development, survival, and fecundity of an insect. The adverse effects of allelochemicals ingested by insects range from mild growth inhibition to death, and lethal effects may be chronic or acute. Acute mortality affects early immature stages, while chronic effects are often delayed until the later larval instars, and the pupal and adult stages. Here, chronic allelochemical effects may be exhibited as reduced body size and weight, prolonged periods of development in the larval and pupal stages, and reduced adult fecundity.

2.1. REPELLENTS

Volatile hydrocarbons emitted by plant foliage comprise a great variety of arthropod repellents. Monoterpenes from the vapors in foliar resin of grand fir, *Abies grandis* (Dougl. ex D.Don) Lindl., repel several species of beetles (Perttunen, 1957; Bordasch and Berryman, 1977). Repellents in foliage of rice *Oryza sativa* (L.), adversely affect the feeding behavior of several species of leafhoppers and planthoppers (Khan and Saxena, 1985, 1986; Saxena and Okech, 1985). Volatiles from strawberry species with high essential oil content repel feeding by the two-spotted spider mite, *Tetranychus urticae*, and the strawberry spider mite, *Tetranychus turkestani* Ugarov and Nikolsik (Dabrowski and Rodriguez, 1971). Foliar volatiles produced in leaves of wild tomato, *Lycopersicon* species, repel feeding by *T. urticae,* a leafminer, *Liriomyza trifolii,* and the potato aphid, *Macrosiphum euphorbiae* (Thomas) (Hawthorne et al., 1992; Guo et al., 1993).

2.2. FEEDING DETERRENTS

Allelochemicals in many plants deter insect feeding. Phenolic, alkaloid, and terpenoid deterrents are frequently produced and stored in leaf cell walls, vacuoles, or specialized structures such as leaf trichomes and surface waxes. Metabolically, deterrents can exist constitutively or may be expressed by de novo synthesis following tissue damage incurred after insect feeding. Several species of beetles are strongly deterred from feeding on willow, *Salix alba* L., cultivars that produce high concentrations of salicin – *n*-derived phenolic glucosides (Kelly and Curry, 1991; Kendall et al., 1996). Feeding by *Epicauta* spp. beetles and the vegetable weevil, *Lisstrodes*

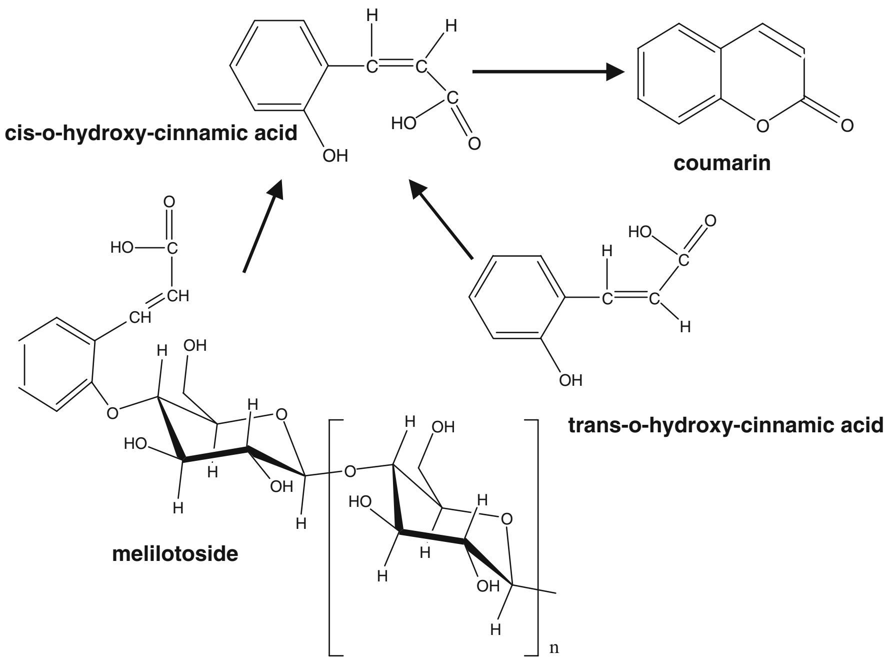


Figure 1. Metabolic pathway toward production of coumarin, an allelochemical in *Melilotus officinalis* that deters feeding of *Listroderes costirostris obliqus*, *Epicauta fabricii*, and *Epicauta vittata*.

costirostris obliqus (Klug), is deterred by the phenolic compound coumarin in the leaves of yellow sweetclover, *Melilotus officinalis* Pursh (Matsumoto, 1962; Gorz et al., 1972). Coumarin is the deterring allelochemical, it occurs after the hydrolysis of the phenolic glycoside melilotocide and its precursor trans-*o*-hydroxy-cinnamic acid (Fig. 1). Dihydroxyphenols and methoxyphenols, i.e., ferulic acid and synapic acid, in some wheat cultivars function as feeding deterrents to several species of aphids (Dreyer and Jones, 1981; Leszczynski et al., 1995).

In graminaceous plants, phenolics (rutin, chlorogenic acid, flavonoids) act as deterrents. The aphid *Schizaphis graminum* is deterred from feeding by procyanidin, *p*-hydroxybenzaldehyde, and the cyanogenic glycoside dhurrin (Fig. 2) in aphid-resistant sorghum cultivars (Dreyer et al., 1981). *p*-Hydroxybenzaldehyde also deters feeding by locusts on sorghum foliage (Woodhead and Cooper-Driver, 1979; Woodhead, 1982).

Insect feeding deterrents occur in several leguminous plants, and the isoflavone vestistol (Fig. 3) in subterranean clover, *Trifolium subterraneum,* and *Lotus pedunculatus* Cav. have been demonstrated to impart deterrence to feeding by a mite and the beetle *Costelytra zealandica* (White) (Russell et al., 1978; Wang et al., 1998). Related isoflavones from soybean foliage reduce growth of the beetle

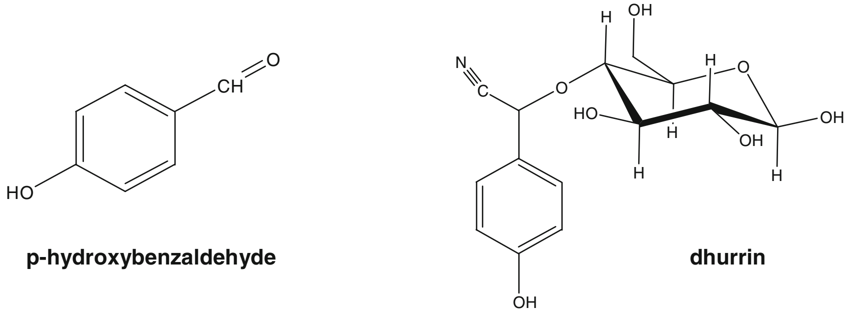


Figure 2. Chemical structures of dhurrin and p-hydroxybenzaldehyde, recognized as insect-feeding deterrents.

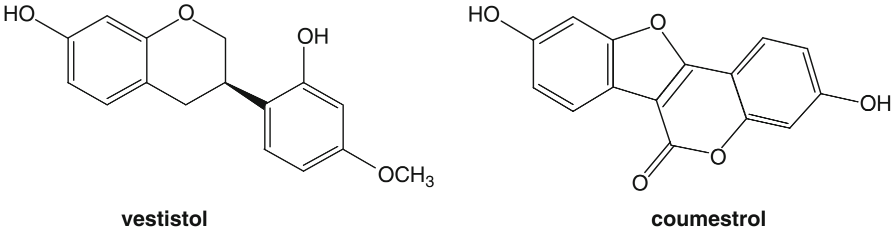


Figure 3. Isoflavone allelochemicals from leguminous crop plants. Vestitol from *Medicago sativa* and *Lotus pedunculatus*, a feeding deterrent to *Costelytra zealandica*. Coumestrol from *Glycine max* foliage, reduces growth of *Pseudoplusia includens* immatures.

Epilachna varivestis (Mulsant), and the lepidopterous larvae *Pseudoplusia includens* (Walker) and *Anticarsia gemmatalis* (Hübner) (Rose et al., 1988; Fischer et al., 1990).

The role of foliar glycoalkaloids as feeding deterrents produced in both the foliage and roots of solanaceous plants is well established. Alkaloids wild *Solanum* species deter above – ground feeding of the leafhopper, *Empoasca fabae*, and subterranean feeding by larvae of the beetle *Agriotes obscurus* (L.) (Sinden et al., 1986; Jonasson and Olsson, 1994). Sesquiterpene lactones and norditerpene dilactones occurring across both ranges of plant taxa (*Helianthus, Rhododendron, Podocarpus*) deter both foliar and root feeding of several species of Coleoptera, Orthoptera, and Lepidoptera (Doss et al., 1980; Kubo et al., 1984; Gershenzon et al., 1985). Finally, high levels of condensed tannin in the leaves of aspen, *Populus tremuloides* Michx., cotton, and sorghum deter larval feeding by several species of Lepidoptera and a midge, *Contarinia sorghicola* Coq., resulting in their reduced growth (Lane and Schuster, 1981; Manuwoto et al., 1985; Kumar and Singh, 1998).

2.3. TOXINS

Plant alkaloids, ketones, and organic acids have toxic effects to insects (Table 1). The review of the toxic effects of plant alkaloids on insects by Wink (1993) provides detailed discussions of the roles of these compounds in plant–insect interactions. The alkaloids leptine, solanocardenine, and α-tomatine that occur in foliage of certain genotypes of solanaceous plants (potato and tomato) are toxic to a broad range of insects, including the Homptera, Coleoptera, and Lepidoptera. Alkaloids produced in different *Lupinus* species are toxic to the aphid *Myzus persicae* (Sulzer) (Berlandier, 1996), and deter feeding by the weevil *Sitona lineatus* (L.) (Cantot and Papineau, 1983).

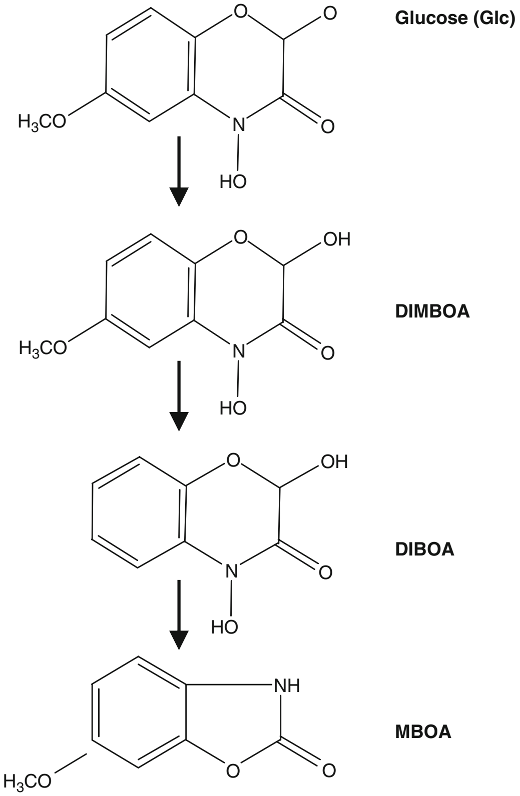
Accessions of the solanaceous plant *Lycopersicon hirsutum* f. *glabratum* Humb. & Bonpl. also produce methyl ketone and acylsugar toxins in leaf glandular trichomes. These compounds are toxic to the larval stages of several species of Lepidoptera and Diptera (Kennedy and Yamamoto, 1979; Williams et al., 1980; Dimock and Kennedy, 1983; Lin et al., 1987; Hawthorne et al., 1992; Eigenbrode et al., 1996).

A hydroxamic acid in the foliage of maize, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, (DIMBOA) is one of the most widely studied plant allelochemicals affecting insects (Bergvinson, 1997). When healthy foliage becomes damaged from insect feeding, DIMBOA present in plants as a 2-β-0-D-glucoside is enzymatically converted to the DIMBOA aglucone (Fig. 4). The benzoxazolin-2-one MBOA, a stable end product of DIMBOA degradation, occurs in maize

Table 1. Plant allelochemicals exhibiting toxic effects to insects. See text for references.

Plant genus	Allelochemical	Insect affected
Citrus	Linalool	*Anastrepha suspense*
Daucus	Chlorogenic acid	*Psilia rosae*
Geranium	Organic acids	*Tetranychus urticae*
Helianthus	Terpene lactones	*Homoeosoma electellum*
Lactuca	Isochlorogenic acid	*Pemphigus bursarius*
Lupinus	Lupinine, lupanine	*Myzus persicae*
Lycopersicon	α-tomatine	*Leptinotarsa decemlineata*
		Helicoverpa zea
	2-tridecanone	*H. zea, L. decemlineata*
	2-undecanone	*Manduca sexta*
		Spodoptera exigua
	Acylsugars	*Keiferia lycopersicalla, S. exigua*
Phaseolus	Arcelin protein	*Zabrotes subfasciatus*
	Phaseolin protein	*Callosobruchus maculatus*
Solanum	Glycoalkaloids	*Leptinotarsa decemlineata*
Vigna	Vicilin protein	*C. chinensis*
Zea mays	DIMBOA	*Diabrotica virgifera virgifera*
	6-MBOA	*Ostrinia nubilalis*
		Sesamia nonagriodes

Figure 4. Chemical structures of DIMBOA (2,4-dihydroxy-7-methoxy, 4-benzoxazin-one), DIBOA (2,4-dihydroxy-1,4-benoxazin-3-one), and MBOA (methoxybenzoxazolinone) present in maize foliage and participating in defense against insect herbivory.



foliage and roots and is toxic to larvae of several species of Coleoptera and Lepidoptera (Klun et al., 1967, 1970; Gutiérrez and Castañera, 1986; Xie et al., 1992; Assabgui et al., 1995).

DIMBOA acts as a digestive toxin in the insect midgut, while 6-MBOA exhibits more chronic effects, reducing the efficiency of conversion of digested food (Houseman et al., 1992; Yan et al., 1995; Ortego et al., 1998). DM_2BOA-DIMBOA is a unique component of insect-resistant maize plants (Hedin et al., 1993), and DIMBOA and DIBOA, the related 2,-α-glucoside, also deter the feeding of several of the same insects (Robinson et al., 1982; Yan et al., 1999).

Other allelochemicals are toxic to insect herbivores. The anacardic acids romanicardic acid and geranicardic acid (Fig. 5) exuded by leaf trichomes of geranium, *Pelargonium x hortorum* (L.), are toxic to *T. urticae* (Gerhold et al., 1984; Grazzini et al., 1995). In addition, chlorogenic acid and isochlorogenic acid in specific genotypes of lettuce, *Lactuca sativa* L., and carrot, *Daucus carota* L., are toxic to the fly, *Psilia rosae* (F.), and to the lettuce root aphid, *Pemphigus*

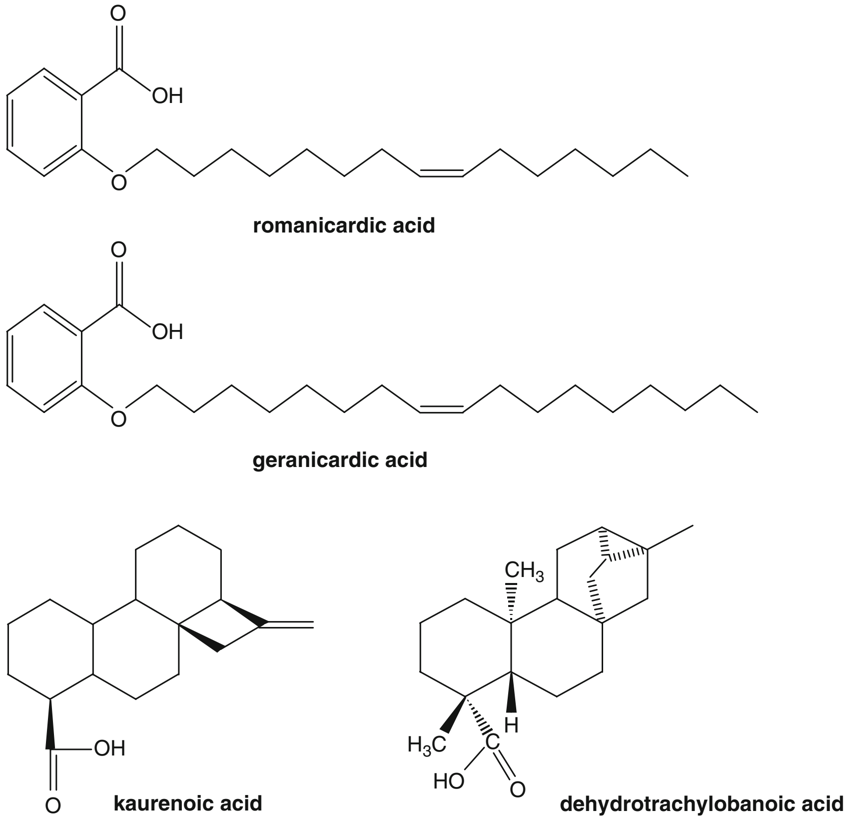


Figure 5. Organic acids affecting growth and survival of insect herbivores. Romanicardic acid and geranicardic acid from a geranium cultivar toxic to *Tetranychus urticae*; kaurenoic acid and trachylobanoic acid from *Helianthus* species that restrict growth of *Homeosoma electellum.*

bursarius (L.), respectively (Cole, 1984, 1985). Terpenoid metabolites have strong toxic effects toward insects. The sesquiterpene lactone 8, β-sarracinoyloxycumambranolide, produced by glandular leaf trichomes of *Helianthus* species, is toxic to larvae of the moth, *Homoeosoma electellum* (Hulst) (Rogers et al., 1987). The terpenoid saponin, produced in insect-resistant forages, is toxic to larvae of the beetle, *Costelytra zealandica* (White) (Sutherland et al., 1982).

2.4. PLANT PROTEINS INHIBITING INSECT GROWTH

Many plant proteins have been identified that adversely affect insect growth by inhibiting key digestive enzymes (Table 2). Often, these proteins exhibit acute

Table 2. Plant proteins identified as inhibitors of insect growth and development. See text for references.

Inhibitor type	**Gene**	**Plant source**	**Affected insects**
Trypsin	CpTi	*V. unguiculata* *Pisum sativum*	*Acyrthosiphon pisum* *Chilo suppressalis* *Helicoverpa zea* *Otiorhynchus sulcatus* *Sesamia inferens*
	WTI-1	*Psophocarpus tetragonolobus*	*C. suppressalis*
	SKTI	*Glycine max*	*H. zea* *Nilaparvata lugens* *Tenebrio molitor* *Spodoptera exigua* *Spodoptera litura*
	BTICMe	*Hordeum vulgare*	*Sitotroga cerealella*
	TI	*Ipomoea batatas*	*S. litura*
Cysteine Proteinase	OCI	*Oryza sativa*	*Chrysomela tremulae* *Diabrotica undecimpunctata howardi* *Diabrotica virgifera virgifera* *Leptinotarsa decemlineata* *Myzus persicae* *Sitophilus oryzae* *Tribolium castaneum*
	ATCYS	*Arabidopsis thaliana*	*Chrysomela populi*
α-Amylase	αAI-1	*Phaseolus vulgaris* *Vigna unguiculata*	*Bruchus pisorum* *Callosobruchus chinensis* *Callosobruchus maculatus* *Zabrotes subfasciatus*
Lectins	GNA	*Galanthus nivalis*	*Diatraea saccharalis* *Lacanobia oleracea* *Maruca vitrata* *M. persicae* *N. lugens* *Sitobion avenae*
	TEL	*Talisia esculenta*	*C. maculatus*
	WGA	*Triticum aestivum*	*Lipaphis erysimi*

inhibition that results in insect death. Prominent among these are serine proteinases that inhibit insect endopeptidases. Bowman–Birk proteinsae inhibitors (Bowman, 1944; Birk, 1985) from various legumes contain reactive sites for trypsin and chymotrypsin.

The cowpea trypsin inhibitor CpTi adversely affects growth in several insects (Gatehouse and Boulter, 1983), and was used to create some of the first insect-resistant transgenic plants. Kunitz trypsin inhibitors, which occur in legume and cereal crops, possess reactive sites for trypsin and chymotrypsin. Soybean trypsin inhibitor (SKTI) affects the growth of several insect pests (Applebaum et al., 1964; Broadway and Duffey, 1986; Rahbé et al., 2003b). SKTI and inhibitors from barley (BTI-CMe) and sweet potato (TI) have been used to develop transgenic insect-resistant plants (Xu et al., 1996; Graham et al., 1997; Yeh et al., 1997; Ding et al., 1998; Altpeter et al., 1999; Lee et al., 1999; Mochizuki et al., 1999). Plant cysteine proteinase inhibitors are 12–18 kDa proteins lacking disulfide bonds that inhibit the insect growth. Oryzacystatin (OCI), a cysteine proteinase inhibitor from rice, inhibits the growth of several species of Coleoptera (Liang et al., 1991; Chen et al., 1992; Edmonds et al., 1996) and *M. persicae* (Rahbé et al., 2003a) (Table 2). OCI and ATCYS inhibitors from *Arabidopsis thaliana* have been used to create transgenic plants that inhibit the midgut digestive proteinase activity of several Coleoptera (Leple et al., 1995; Lecardonnel et al., 1999; Delledonne et al., 2001).

Plant α-amylase inhibitors form complexes that block starch breakdown in the insect midgut. The α-amylase inhibitor αAI-1 from common bean, *Phaseolus vulgaris* L., inhibits the growth of larvae of the cowpea weevil, *Callosobruchus maculatus* (F.), and the adzuki bean weevil, *Callosobruchus chinensis* (L.) (Ishimoto and Kitamura, 1989; Ishimoto et al., 1996).

Schroeder et al. (1995) transformed plants of pea, *Pisum sativum* L., to express the αAI-1 gene for resistance to the pea weevil, *Bruchus pisorum* (L.). In a related manner, seeds from genotypes of cowpea, *Vigna unguiculata* L. Caupí., and common bean, *Phaseolus vulgaris* L., produce the storage proteins arcelin, phaseolin, and vicilin, that inhibit larval growth of *C. maculatus* and the weevil, *Zabrotes subfasciatus* (Minney et al., 1990; Macedo et al., 1995; Xavier-Filho et al., 1996; Moraes et al., 2000). These proteins function by tight binding to the chitin lining of the larval midgut endothelium (Sales et al., 1992).

Plant lectins, which bind specifically to cell surface carbohydrates (Van Damme et al., 1998), inhibit the growth of larval Coleoptera, Homoptera, and Lepidoptera (Powell et al., 1993; Fitches et al., 1997; Gatehouse et al., 1997; Powell et al., 1998; Machuka et al., 1999; Macedo et al., 2004) (Table 2). Genes encoding lectins from snowdrop, *Galanthus nivalis* L., (GNA-*Galanthus nivalis* agglutinin), and wheat germ (WGA), impart transgenic insect resistance (Gatehouse et al., 1996; Rao et al., 1998; Birch et al., 1999; Nutt et al., 1999; Stoger et al., 1999; Maqbool et al., 2001; Kanrar et al., 2002; Setamou et al., 2002).

2.5. PLANT NONPROTEIN ALLELOCHEMICALS INHIBITING INSECT GROWTH

In addition to plant protein inhibitors, many other plant allelochemicals also inhibit insect growth, and exhibit chronic effects such as reduced size and weight, or prolonged development (Table 3). Kaurenoic acid and trachylobanoic acid (Figure 5), diterpene acids produced in the florets of arthropod-resistant sunflower cultivars, retard the development of larvae of several species of Lepidoptera (Elliger et al., 1976). Similarly, oxalic acid exuded from leaf trichomes of chickpea plants and ultimately ingested by larvae of the moth *Heliothis armigera* (Hubner), reduces

Table 3. Plant allelochemicals that inhibit insect growth. See text for references.

Plant	Growth inhibitor	Insect affected
Arachis	Quercetin glycosides	*Spodoptera litura*
Azalea	Heptadecanoic, oleanic and ursolic acid	*Stephanitis pyrioides*
Cicer	Oxalic acid	*Helicoverpa armigera*
Glycine	Coumestrol	*Pseudoplusia includens*
Gossypium	Terpenes, sesquiterpene lactones, heliocides and related terpenoids (gossypol and hemigossypol)	*Heliothis virescens* *Spodoptera littoralis* *Helicoverpa zea* *Earias vittella* *Pectinophora gossypiella* *H. virescens*
Helianthus	Kaurenoic acid, dehydrorachylobanoic acid	*Lepidoptera*
Manihot	Rutin, kaemferol glycoside	*Phenacoccus manihoti*
Picea	Resin acids	*Pissodes strobi*
Vigna	Isorhamnetin, quercetin	*Aphis fabae*
Zea	Apimaysin, chlorogenic acid, 3′-methoxymaysin, maysin	*H. zea*

larval weight and lengthens the length of larval development (Yoshida et al., 1995). Fatty acids, i.e., heptadecanoic acid, *n*-hentriacontane, oleanic acid, and ursolic acid contained in epicuticular lipids of azalea, *Azalea indica* L., genotypes reduce the development of the azalea lace bug, *Stephanitis pyrioides* Scott *S. pyrioides* (Wang et al., 1999). Oleanic acid in the epicuticular lipids of olive fruits also inhibits egg development in the fly, *Bactrocera oleae* (Gmelin) (Kombargi et al., 1998).

The flavone glycosides maysin, apimaysin, 3′-methoxymaysin, and chlorogenic acid (Fig. 6) are produced silks of maize plants resistant to feeding of *H. zea* (Waiss et al., 1979). Larval weight gain is negatively correlated with concentrations of maysin glycosides (Wiseman and Snook, 1995; Guo et al., 1999). Centipedegrass, *Eremochloa ophiuroides* (Munro), clones with increased maysin and chlorogenic acid content also reduce larval growth in the moth *Spodoptera frugiperda* (J. E. Smith) (Wiseman et al., 1990). Flavonoid glycosides or aglycones in cowpea, *Vigna unguiculata* L. Caupí., cassava, *Manihot esculenta* Crantz, and groundnut, *Apios americana* Medik., also reduce the growth of insects (Stevenson et al., 1993; Calatayud et al., 1994; Lattanzio et al., 2000).

Terpenoids, e.g., gossypol produced in foliar glands of cotton plants, reduce the larval growth of several species of pest Lepidoptera (Lukefahr and Martin, 1966; Meisner et al., 1977; Mohan et al., 1994). Related terpenoids – hemigossypol and heliocides 1, 2, and 3 – play similar roles in larval growth reduction (Bell et al., 1975; Stipanovic et al., 1977; Hedin et al., 1992) (Fig. 7).

2.6. GLANDULAR TRICHOME ADHESIVES

Glandular trichomes on the leaves, stems, and reproductive structures of various species of *Solanum, Medicago,* and *Fragaria* produce adhesive allelochemicals

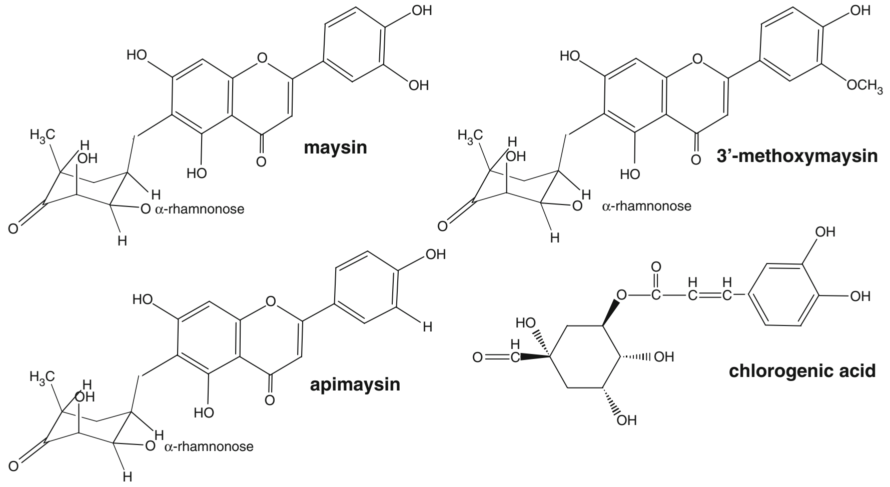


Figure 6. Flavone glycosides maysin, apimaysin, and 3′-methoxymaysin, and chlorogenic acid from foliage of insect resistant maize and centipedegrass cultivars inhibit growth of Lepidopteran larvae.

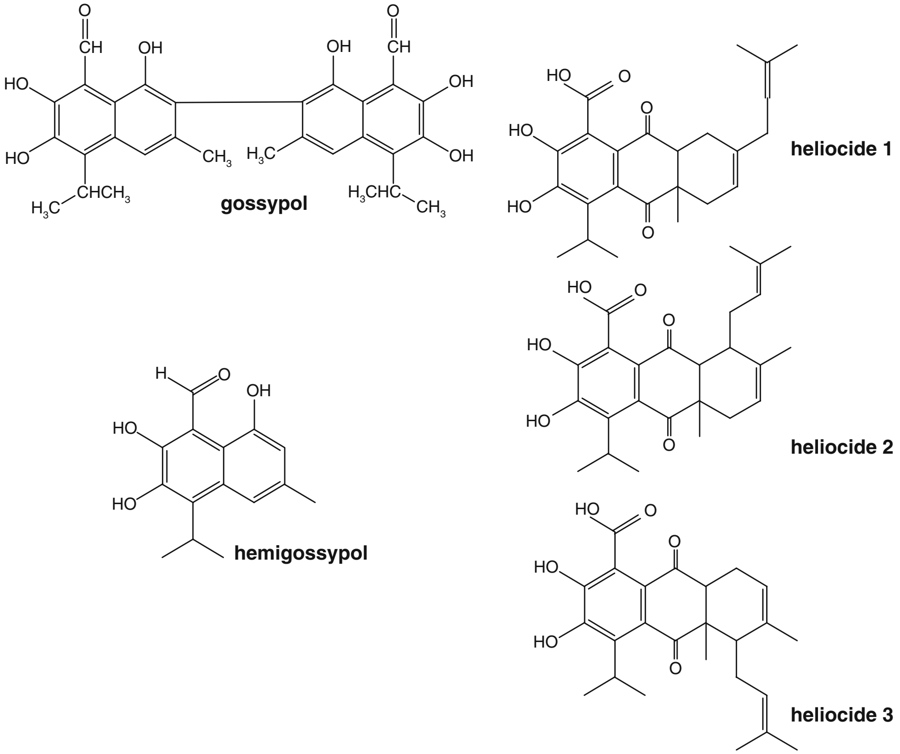


Figure 7. Chemical structures of terpenoids produced in insect-resistant cotton cultivars that inhibit the growth of foliar feeding Lepidoptera: gossypol; hemigossypol; heliocides 1, 2, and 3.

that lack contact toxicity, but entrap the insect, resulting in death by starvation. Several wild *Solanum* species produce fields of such short and tall glandular trichomes. Long trichomes exude an acyl sugar adhesive containing short-chain carboxylic acids that trap the insect tarsi. This in turn allows rupture of short trichome glands that produce a chlorogenic acid resin and a polyphenol oxidase catalyst that, as a consequence of oxidation, harden and kill the trapped insects. The review of Tingey (1991) and more recent contributions (Yencho et al., 1994; Hill et al., 1997; Malakar and Tingey, 2000) provide excellent accounts of the effects of this trichome system on several species of pest beetles, aphids, and Lepidopterous larvae.

Glandular *Medicago* trichomes exude an adhesive composed of aldehydes, alkanes, and esters (Triebe et al., 1981) that entrap and kill larvae of the weevil, *Hypera postica* Gyllenhal, (Shade et al., 1975; Kreitner and Sorensen, 1979) and nymphs of *Empoasca fabae* (Shade et al., 1979; Ranger and Hower, 2001). *Fragaria* trichome exudates kill *T. urticae* mites by similar means of entrapment (Luczynski et al., 1990).

2.7. ALLELOCHEMICALS INDUCED BY INSECT HERBIVORY

In addition to constitutive plant defenses, plants also exhibit inducible responses that lead to the production of allellochemical defenses. Owing to polyphenol oxidase and peroxidase activities and reactions, insect feeding induces the production of phenol oxidation products across a wide range of plant taxa, including chlorogenic acid and rutin (see Smith, 2005, Chapter 9). Peroxidase, a key enzyme in plant cell wall lignification, is also involved in the metabolism of reactive oxygen species (ROS), which cause direct oxidative tissue injury in insects (Bowels, 1990; Ni et al., 2001; Boyko et al., 2006). Foliar terpenoids are also induced in response to insect herbivory to cotton (McAuslane et al., 1977; McCall et al., 1994) and pine (Watt et al., 1991; Raffa and Smelley, 1995).

3. Major Recent Developments

Plant recognition of insect-inflicted tissue damage stimulates the production of signaling molecules that also direct the production of defensive allelochemicals (Kessler and Baldwin, 2002). In the past decade, oligonucleotide technologies have provided the means to sequence the genomes of Arabidopsis and rice. These developments, coupled with cDNA technologies (macro- and microarrays, subtractive substitution hybridization (SSH) cDNA libraries) have permitted studies of the expression of genes encoding both signaling proteins and defensive allelochemicals. Research to date indicates that in general, insects with chewing mouthparts inflict more extensive plant tissue damage that elicits different plant signaling responses than those induced in response to the "stealth" feeding by insects with piercing – sucking mouthparts. Signaling pathways driven by jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and abscisic acid (ABA) induce the production of plant defenses in response to insect attack (Turner et al., 2002; Kaloshian, 2004; Smith and Boyko, 2007). Lipoxygenases, which function in cell membrane lipid degradation, are important intermediates in the production of JA and related oxylipins. Transcripts encoding lipoxygenase (*LOX*) genes and related JA precursors are strongly induced by insect feeding on foliage of cotton, tobacco, sorghum, and wheat (Loughrin et al., 1995; Voelckel et al., 2004; Boyko et al., 2006; Park et al., 2006; Liu et al., 2007). Methyl JA-induced accumulation of ferulic acid and phenolic polymers leads to cell wall strengthening and increased insect resistance in barley and maize (Bergvinson et al., 1994; Lee et al., 1997). Mewis et al. (2005) demonstrated that JA and related oxylipins in Arabidopsis function in the production of glucosinolate defenses against aphid feeding. JA and ET synergistically induce a peptidase-like protein in squash, *Cucurbita moschata* Duchesne, involved in resistance to *B. argentifolii* (van de Ven et al., 2000), and JA-mediated genes encoding toxic storage proteins occur in transcriptomes of insect – challenged *Arabidopsis* and tobacco plants (Moran and Thompson, 2001; Stotz et al., 2002).

Aphid feeding on susceptible host plants elicits upregulation of SA pathway components (see review of Smith and Boyko, 2007), suggesting a role for SA in plant defense responses to aphid herbivory, and Li et al. (2008) determined that foliage of soybean plants resistant to the soybean aphid, *Aphis glycines* Matsumura, upregulate both SA and JA signaling pathway genes. Kempema et al. (2007) demonstrated that feeding by the silver leaf whitefly, *Bemisia argentifolii* Bellows and Perring, on Arabidopsis foliage greatly increases the production of SA – related transcripts, but antagonizes JA-induced defense responses.

Plant defense responses to insects induced by ET may also be antagonized by those induced by SA (Stotz et al., 2002). Transcriptional cross-talk between SA and JA pathways occurs in sorghum, in which both SA and methyl jasmonate (MeJA) induce JA synthesis (Salzman et al., 2005). As discussed in reviews of Kaloshian (2004) and Kazan and Manners (2008), cross-talk between signaling pathways may allow plants to choose an optimum defense strategy, depending on the type of insect attacking the plant, the signaling molecules involved, and the severity of plant tissue damage.

During feeding, insects also secrete salivary chemicals that induce plant defense responses and attract pest natural enemies. A fatty acid conjugate elictor (FAC) N-(17-hydroxylinolenyl)-L-glutamine (volicitin), from the larval regurgitant of *S. exigua*, is biosynthetically related to JA (Boland et al., 1998) and induces maize plants to emit volatiles that attract *S. exigua* natural enemies (Frey et al., 2000). FACs from tobacco hornworm, *Manduca sexta* (L.), larvae elicit the release of volatiles, JA production and defensive allelochemicals in *Arabidopsis* leaves (Halitschke et al., 2000, 2001). Schmeltz et al. (2003) demonstrated that *S. exigua* feeding stimulates similar reactions in maize plants.

4. Future Research Foci

Insects are only one of the selectional forces affecting the physical and chemical defenses that plants have developed to survive and evolve. Plants must also survive attack by phytopathogens and nematodes, as well as short- and long-term abiotic stresses resulting from changes in temperature and moisture. Such plant responses require the allocation of many plant metabolic resources, which may lower plant fitness and reduce biomass and seed yields. However, many plants produce some of the same allelochemicals in response to attack by phytopathogenic fungi (glycoalkaloids, glucosinolates, hydroxamic acids, isoflavonoids, saponins) as they do in response to insect feeding (Morrissey and Osbourn, 1999).

Past knowledge of the biochemical pathways responsible for producing the plant allelochemicals reviewed here, coupled with new and emerging knowledge about the signaling pathways plants use to recognize insect herbivory and subsequently initiate defense responses, are now being merged to provide a more thorough picture of plant defense responses to insects. cDNA arrays for Arabidopsis, barley, soybean, tomato, and wheat have provided initial genome-wide representations of plant signaling and defense genes involved in response to insect feeding. Additional genomic technologies are now providing new avenues of research in plant–insect interactions, and are providing in-depth information about the relationships between signaling molecules and their role in the production of allelochemical defenses.

Nevertheless, many questions remain to be answered about how both insect-based and plant-based elicitors are induced in plant responses to insects. Additional research is critical to better understand how different species of plants integrate elicitor signals generated in response against both insects and diseases, and the specificity of different signaling molecules in relation to the feeding of different insects. In spite of scores of studies conducted, it is difficult to make overall predictions about the specific elicitor signals involved in plant response, and as a result, much research about signal cross-talk is necessary. Research at the plant molecular, genetic, and biochemical levels will be critical for future progress to better understand how different plants generate and integrate signals to produce defenses against insects.

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THE *XANTHIUM* GENUS

Cocklebur Toxins Against Hostile Surroundings and Its Pharmacological Properties

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1. Introduction

Cockleburs (*Xanthium* spp.) are genera of flowering plants in the family of Asteraceae (Compositae), native to the Americas and eastern Asia. The number of species is debatable, ranging from a few to a dozen, and they are a model of plant–animal interaction. The name *Xanthium* originated from “xanthos” meaning yellow (the fruit turns from green to yellow and finally to brown). Many plants contain defense structures located as external projections (thorns, burning hairs, repulsive odors, etc.), while other plants accumulate internal toxic compounds against their attackers and external assaults. These toxic plants belong to several botanical families. Among the poison plants is the Nettle (Urtica) which contains burning acids in its hairs. Others possess toxic compounds in their leaves, fruits, seeds, or bulbs. Among them are the poison ivy, black locust (*Robinia pseudoacacia*), daffodils bulbs, oleander leaves, dumb cane (*Dieffernbachia* which possess them in all parts), castor bean seeds, wisteria (in their seeds, pods), oaks (within their foliage, acorns), mistletoe (in berries), nightshade (Datura), and *Xanthium* spp. the cocklebur weeds, family Asteraceae (Compositae). The leaves of noxious cocklebur weed contain xanthanolies, such as xanthinin and xanthatin, which serve as plant regulators – and growth inhibitors – and contain other toxins. Recently, this plant has been turned into a useful medical herb.

The flowers are of two types, the one with short terminal branches produces only pollen, and the other in clusters in the axils of the leaves produces seeds. Burrs are covered with hooked spines; these specialized structures are designed to use epizoochory (dispersion by means of tangled attachment to the surface of animals or human clothes), a kind of natural prototype of Velcro. In addition, burrs may also be distributed by water.

Xanthium is a model for photoperiod-responding plants. Plants and animals react physiologically to the photoperiod (length of day or night). There are long-day (LD), short-day (SD), and day-neutral (DN) plants. The cocklebur is a SD plant, and only one long night is sufficient to cause it to flower. In animals, the photoperiod causes color changes as well as behavioral changes (hibernation, sexual habits, and resizing of sex organs). The phytochrome (red–far-red absorbing pigments) system is involved in the circadian clock. A new blue light, ultraviolet–photoreceptor (in plants and animals) is also responsible for the normal development of plants and animals (including the circadian clock in organisms).

Asian species of cocklebur are also used in traditional Chinese medicine. In the Chinese literature, the cocklebur has a long list of pharmaceutical remedies, despite its somewhat toxic nature. Overdose of *Xanthium* fruit may yield many health problems, such as nausea, vomiting, diarrhea, and abdominal pain. The pills or tablets may insure the treatment of health hazards. In the Western literature, *Xanthium* appears less as a medical plant and is rather considered as a highly toxic herb for grazing animals.

2. The Cocklebur (*Xanthium* spp.)

Cockleburs are distributed worldwide and known as invasive noxious weeds. They grow in waste places, roadsides, around agricultural fields, and on beaches and water bodies. The flowers form pollen above the female flowers, which produce seeds in cluster in the axils of the leaves. The fruits are hard, spiny, globose burrs, which have double chambers, each containing a single seed. Stiff hooked spines surround the burrs, which stick to fur, hair, wool, or clothing, and can be quite difficult to extract (Fig. 1).

Xanthium seeds and seedlings are toxic and cause severe damage to livestock that ingests it. Some species of cockelbur have a highly toxic glycoside (carboxy-atractyloside; Fig. 3) in their seeds, killing livestock – hogs, cattle, goats, horses, sheep – and poultry. Some domestic animals will avoid consuming the plant if other forage is present, but less discriminating animals, such as pigs, will consume the plants and then sicken and die (Masvingwe and Mavenyengwa, 1998).

Seckbach (1963, 1965) and Khan (1963, 1975) investigated two sesquiterpenoid lactones: xanthinin ($C_{17}H_{22}O_5$, mol. wt. 306) and its deacetyl form, xanthatin ($C_{15}H_{18}O_3$, mol. wt. 246) from *X. pensylvanicum* (Fig. 2). Xanthatin is restricted entirely to the young leaves, while xanthinin is more generally distributed throughout the plant. These compounds have physiological and toxic features. Both exhibit antifungal activity (Lavault et al., 2005). Seckbach (1965, 1963) and Khan (1963, 1975) discovered and described a new enzyme, xanthinin deacylase, which catalyzed the hydrolysis of xanthinin to xanthatin and acetate (Fig. 2). Recently, in Japan, Yokoe et al. (2008) synthesized the first enantioselective xanthatin.

Xanthium is known to be a SD plant (one long night of photoperiod is sufficient to cause its flowering). The youngest upper leaves in the plant control lateral bud growth. The level of xanthatin decreases as the leaves become older. An inverse correlation exists between the degree of branching (under various light conditions) and the xanthatin content of young leaves. Incandescent or far-red light inhibited lateral bud development and caused an increase in xanthinin content of young leaves, while fluorescent or red-light stimulated lateral bud

Figure 1. Leaves and young burrs of *Xanthium*; at right is a ripe cocklebur fruit. (The single burr photo: courtesy of Professor Subhuti Dharmananda.)

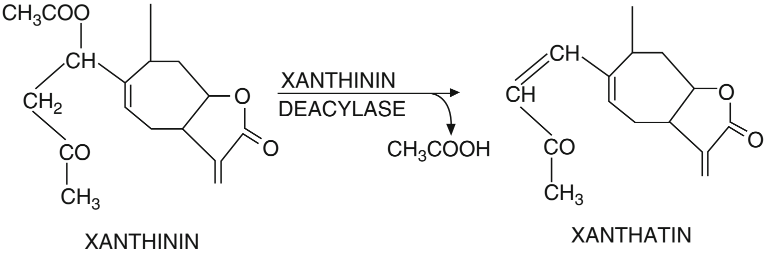

Figure 2. Conversion of xanthinin to xanthatin and acetate by the enzyme xanthinin deacylase (Data from Khan, 1975; Seckbach, 1965).

branching, decreasing the xanthatin content. This indicates the involvement of the phytochrome system. Xanthatin is a strong inhibitor of seed germination (Khan 1963), while xanthinin (acetyl-xanthatin) is less toxic. Crude extract of *Xanthium* and xanthatin have cytotoxic activity (Roussakis et al., 1994). In addition, *Xanthium* plants have bioactivities, such as hypoglycemic, antitussive, antioxidant, antidiarrheal, antibacterial, stomachic, antifungal, and antimalarial compounds Akter et al. (2009). Seckbach (1969, 1971, 1972, 1982) has shown that *Xanthium* accumulate high levels of iron (as phytoferritin in its leaves) after starvation of iron in its nutrient solution. The phytoferritin buffers the toxic ferric ions, which the starved plants absorbed.

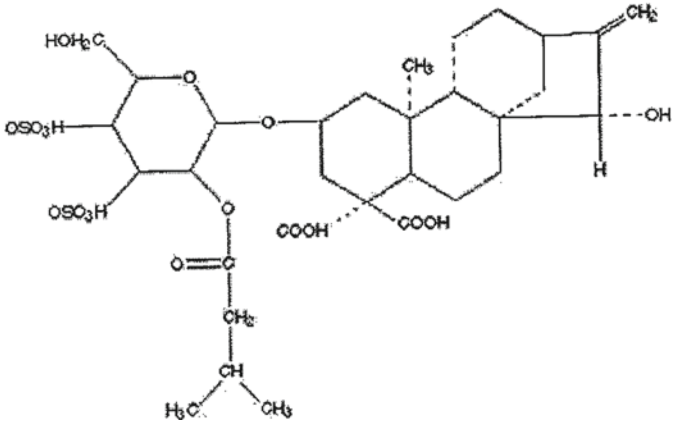

Figure 3. Chemical structure of carboxyatractyloside.

3. The Beneficial Features of *Xanthium* as a Medicinal Plant

Ginesta-Peris et al. (1994) reported on the antimicrobial activity of xanthatin from *X. spinosum,* while others (Cerdeiras et al., 2007; Olivaro and Vazquez 2009) also described this activity from *X. cavanillesii.* The evaluation of antimicrobial activity from *X. strumarium* and the presence of the toxic carboxyatractyloside (Fig. 3) analysis were recently published by Scherer et al. (2009). *Xanthium* extracts and its infusion serves as skin antiseptic.

Furthermore, *X. strumarium* is also a beneficial weed, which repels armyworms and other pests from nearby domesticated plants. Recently, antioxidant and anti diarrheal activities of the extract of *X. Indicum* leaves have been demonstrated (Akter et al., 2009; Raushanara et al., 2009). Favier et al. (2005) reported on the antiulcerogenic activity of sesquiterpenes from *X. cavanillesii* in rats. Talakal et al. (1995) studied the antitrypanosomal activity of *X. strumarium* leaves. *Trypanosoma* is a parasitic flagellate protozoan transmitted to the vertebrate bloodstream, lymph, or spinal fluid (via certain insects), and may cause human diseases such as sleeping sickness. Lavault et al. (2005) exhibited antileishmanial (*Leishmania* is a flagellate protozoan that causes leishmaniasis) and antifungal activity from seven compounds of fruits from *X. macrocarpum.* The crude extract of *Xanthium* has potential anticancer agents (Roussakis et al., 1994) and anti ulcergenic activity. A recent report by Kovács et al. (2009) has shown that $CHCl_3$ extract of the leaves of *X. italicum Moretti* led to the isolation of four xanhanolies (including xanthatin). These isolated compounds have shown cell-growth inhibitory activity against human cervix adenocarcinoma, skin carcinoma, and breast adenocarcinoma cells. Ramírez-Erosa et al. (2007) showed in vitro cytotoxic activity from xanthatin and xanthinosin of *X. strumarium* in human cancer cell lines of colon, breast, and lung. Similarly, Ancuceanu and Istudor (2004) reported on a pharmacologically active compound of a derivative of xanthatin (from extracted *X. strumarium* leaves) which exhibited a strong inhibition of cultured human lung cells. Antimalarial activity from extract of *Xanthium* has been reported by Joshi et al. (1997).

4. Photoperiodism in Plants and Animals

4.1. PHOTOPERIOD IN PLANTS

The ability to distinguish between daily cycles of light and dark is related to the circadian clock. Plants are divided for flowering induction into SD, LD, and ND plants. Some plants (such as the *Xanthium* spp., a SD plant) are satisfied with only one cycle of their photoperiod requirement. Plants that were not grown in their favorite light regime would not flower. It has been shown elsewhere that the phytochrome (red–far-red system) is involved in several processes in plants (see, Tucker and Mansfield, 1971). There are also the cryptochrome chromophores involved in the photoperiod and circadian rhythm in plants and animals (Cashmore et al., 1999; Levy et al., 2007). Cryptochromes are blue light-ultraviolet-A photoreceptors of plants and animals. They regulate germination, elongation, photoperiodism, and other responses (for more data, see Cashmore et al., 1999; Levy et al., 2007).

4.2. PHOTOPERIOD AMONG ANIMALS

It is noteworthy that animals share with plants several photoperiod-related phenomena. Long-day regimes in animals have many lines of effects. In chickens, additional artificial light stimulates egg production; while in birds, the long day light increases sex activities. Birds under certain light regime begin to molt, with influence on fur and feather colors. Other activities such as the reproductive cycle, migration in butterflies and birds, hibernation, and sex behavior are all connected to the circadian rhythms. The biological clock in animals regulates the hormonal activities of the body. Antlers in LD regime grow, and in SD, they fall off. Long day illumination increases estrus and spermatogenesis; while in insects, SD periods cause diapause (period of dormancy). Such photoperiodism affects plants, invertebrates, annelids, amphibian, mollusks, arthropods, bony fish, frog, turtle, and mammals.

5. Summary and Conclusions

Xanthium spp. are SD plants and will flower after a single regime of this photoperiod. They show the typical relation between the plant world and animal and human life. We conclude that the cocklebur illustrates the multi-level plant–animal interactions.

Their fruit (burrs) are equipped with hooked spines; these specialized structures are designed to use zoochory (dispersion by means of tangled attachment to the surface of animals or human clothes) and may also be distributed through water.

These herbs defend themselves by producing toxic compounds against grazing animals, harmful protozoans, and other soil enemies. *Xanthium* plants contain xanthinin and xanthatin, and the highly toxic glycoside carboxyatractyloside that kills livestock, such as cattle, pigs, sheep, and goats, as well as poultry.

Xanthium plants contain several pharmacological beneficial compounds that have shown to contain medicinal compounds against several diseases and tumors. Among them are antimicrobial, fungal, ulcerogenic, trypanosomal, leishmanial, epileptic, syphilitic, diuretic, and anticytotoxic activities. In addition, *Xanthium* spp. have shown antitumor activities (tested in human colon cancer, breast and lung cancer cell lines), and their leaves produce compounds involved in sedative, diaphoretic, antibilious, emollient, laxative, hypothermic, astringent, and antidiuretic activity.

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PART 6:
MARINE ENVIRONMENTS

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THE DIVERSITY OF EPIZOIC DIATOMS

Relationships Between Diatoms and Marine Invertebrates

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1. Introduction

The adhesion of microalgae to living substrata is well known in marine and freshwater environments (Wahl, 1989; Prieur, 1991; Carman and Dobbs, 1997; Di Camillo et al., 2005; Romagnoli et al., 2007). Therefore, a number of phyla of marine organisms commonly host on their surface a complex community of microorganisms composed of bacteria, fungi, and protists.

A number of benefits for microalgae are related to the epizoic *modus vivendi* (Round, 1981; Gaevskii et al., 2004; Romagnoli et al., 2007), such as protection against grazing, which is hardly to make on live animals, a nutritional advantage, as the epibiontic microalgae can exploit the host catabolites and CO_2 supply for their growth. Moreover, microalgae may also benefit from having an elevated position avoiding the resuspension of sediments.

Diatoms represent a major component of benthic marine microalgae. They occur either as free living on sediments and hard surfaces or as attached growth forms, comprising the adnate or adpressed (i.e., cells adhering to substratum through their valve face and having a limited movement) and erect (i.e., cells attaching to the substratum by one valve pole either through mucus pads or stalks). Growth forms of epizoic diatoms correspond to those of the epiphytic and epilithic ones, and express the capability of diatoms to live in a solid/liquid interface (Round, 1971).

Several animal phyla are known to host diatoms as epibionts: ciliates, sponges, hydrozoans, mollusks, crustaceans, and also vertebrates (Round, 1981; Round et al., 1990). The first report about the colonization of marine invertebrates by benthic microalgae dates back to the nineteenth century, when Giesbrecht (1892) observed the presence of stalked epizoic diatoms on the marine planktonic copepod *Corycaeus limbatus* from the Adriatic Sea. Further studies confirmed the occurrence of diatoms (which have been finally attributed to *Pseudohimantidium pacificum*) associated with several copepods and having a wide range of geographical distribution (Gibson, 1978 and references therein).

Hard-shelled metazoan groups (crustaceans, mollusks) generally support higher densities of epiphytes. The degree of epibiosis is inversely related to the motility of animal host, as actively moving animals are less colonized than sedentary ones (Round, 1981).

Also, several marine vertebrates may host an epibiontic flora. Cribb (1969) reported crusts of calcareous algae and a fur of filamentous forms on the carapace of marine turtle, *Eretmochelys*. A specific diatom flora has been reported to be associated with the skin of whales, represented by *Benettella ceticola* and *B. constricta* (both formerly reported as *Cocconeis ceticola*), *B. wheeleri,* and *Epipellis oiketis* which occur together with other species (Bennett, 1920; Hart, 1935; Holmes, 1985). The massive development of diatoms produces yellow patches on the whale skin, and the composition of epizoic diatoms has been related to the time spent by cetaceans in the cold Antarctic waters (Bennett, 1920). Dense diatom fur has also been reported on the ventral feathers of marine birds (Croll and Holmes, 1982; Holmes and Croll, 1984).

The relationships between diatoms and heterotrophic organisms are not restricted to the epibiontic ones. In the 1980s, diatoms have also been reported as endosymbionts in Foraminifera (Lee et al., 1979, 1980a, b, c, 1982; Lee and Xenophontes, 1989) and turbellarians (Apelt and Ax, 1969). When living inside their hosts, diatoms do not form frustules, which is normally built when they are isolated and cultured.

2. Host Diversity

2.1. PORIFERA

Marine sponges commonly host photosynthetic endosymbionts, which are often cyanobacteria (Burja and Hill, 2001; Thacker and Starnes, 2003), or less commonly dinoflagellates (Garson et al., 1998), cryptomonads (Wilkinson, 1992), or even diatoms (Cox and Larkum, 1983; Hamilton et al., 1997; Webster et al., 2004). Cox and Larkum (1983) reported high densities of small pennate diatoms found as symbionts in three species of encrusting sponges from the Great Barrier Reef. Diatoms were localized extracellular, throughout the sponge mesohyl, partially surrounded by sponge cells. Unlike other cases of endosymbiosis, they maintained their frustules.

Recently, several studies highlighted the existing relationships between marine diatoms and Porifera, particularly from Antarctic areas. Several Antarctic sponges normally host dense diatom communities (*Fragilariopsis curta*, *Thalassiosira* cf. *gracilis,* and *T. perpusilla*) due to their ability to incorporate planktonic algae through the exopynacocytes of the sponge surface or the endopinacocytes of the inhalant canals. The trend of chlorophaeopigments and diatoms suggests that microalgae may remain alive for a long time inside the sponge tissues before being probably utilized as a food source (Gaino et al., 1994; Cerrano et al., 2004a,b; Totti et al., 2005).

The diatom *Porannulus contentus* has been reported only in association with a few Antarctic sponges, such as *Mycale acerata* (Hamilton et al., 1997; Cerrano et al., 2004b), where it forms dense mats over the sponge surface (see Fig. 1).

A complex diatom community lives epibiontic on the sponge *Sphaerotylus antarcticus* (Totti et al., 2005), a species characterized by a dense spicule fur that represents a complex three-dimensional microhabitat for benthic organisms: diatoms are represented by the centric *Hyalodiscus* sp. forming short moniliform chains, *Diploneis crabro*, *Entomoneis paludosa*, *Trachyneis aspera*, and *Pleurosigma intermedium,* potentially representing a source of dissolved organic matter for the sponge (Fig. 1 a–f).

2.2. HYDROZOANS

Benthic hydroids and diatoms are primarily related by trophic relationships. Several studies from temperate and polar seas have shown that polyps can capture large amounts of planktonic prey, including diatoms (Coma et al., 1999). The diet of the sub-Antarctic species *Silicularia rosea* is almost exclusively composed of benthic diatoms, which are filtered when they are resuspended by the water movement from bottom sediments (Gili et al., 1996). A number of diatoms have also been detected in the coelenteron of the Antarctic species *Hydractinia angusta* (Cerrano et al., 2000b). The tropical hydroid *Nemalecium lighti* largely feeds on algal cells (Gili et al., 1998) and diatoms have been suggested as the main food source for this species in the Réunion Island reefs (Gravier-Bonnet and Mioche, 1996).

Besides such classic trophic ties, a number of examples of epibiosis are known involving diatoms and hydroids. Hydrozoans represent a highly suitable substratum for diatom colonization (Bavestrello et al., 2008). Round et al. (1961) described diatom development on *Amphisbetia* (formerly *Sertularia*) *operculata* in Lough Ine (Ireland), and reported that different diatom communities developed, depending on the current velocity and sedimentation regimes. A community rich in attached forms (mostly *Grammatophora* followed by *Licmophora*, *Striatella*, *Synedra*, *Cocconeis,* and tube-dwelling *Navicula*) developed in a site exposed to strong currents, whereas dominance by the adnate *Cocconeis scutellum*, forming a complete coating over the perisarc was observed when the hydroid was transferred to a sheltered zone.

A required condition for the settlement of diatoms is the presence of the perisarc, an exoskeleton composed of polysaccharides and proteins, which covers hydrorhiza, stem, branches (of thecate and athecate species), and hydrothecae (of thecate species). The perisarc is easily overgrown by diatoms, while they do not settle on the naked epithelium of the polyps (Di Camillo et al., 2005).

Epibiontic diatom communities seem to gain advantage from the presence of the host. On comparing the trends of diatom densities on living colonies and

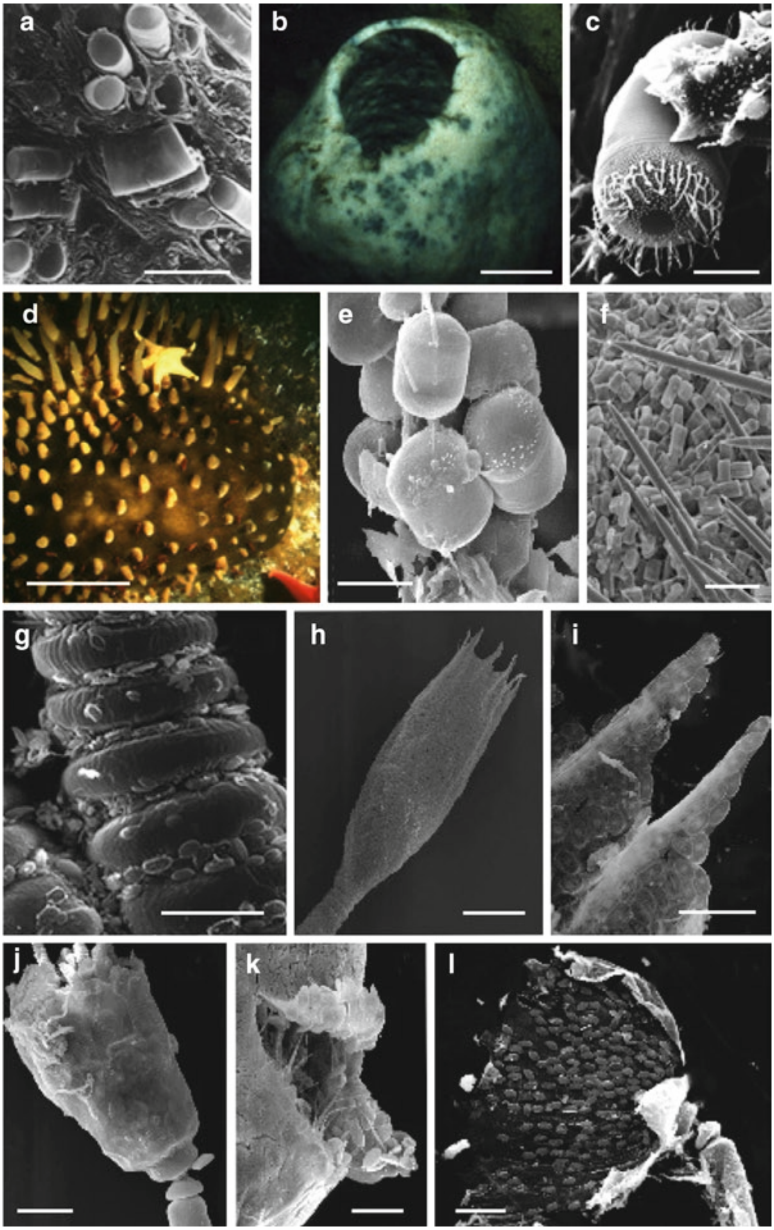

Figure 1. (**a**) Living cells of *Fragilariopsis curta* embedded in the tissues of an Antarctic sponge; (**b**) the volcano sponge *Scolymastra joubini* showing the green spots due to the parasitic invasion of the diatom *Melosira* sp.; (**c**) Particular of the benthic diatom *Melosira* sp.; (**d**) the Antarctic sponge *Sphaerotylus antarcticus* with a brown-green surface due to a coat of epibiontic diatoms; (**e**) detail of the spicule fur of *S. antarcticus* with several cells of *Hyalodiscus* sp. settled on a spicule; (**f**) the extremely dense coat of *Porannulus contentus* settled on the surface of the Antarctic sponge *Mycale acerata*; (**g**) the annulated stalk of the Mediterranean hydroid *Clytia linearis* showing cells of *Cocconeis* sp.

mimic substrata, it was observed that significantly higher abundance and biomass values of epiphytes were recorded in living host, suggesting that diatoms may benefit from the polyp catabolites (Romagnoli et al., 2007). Nutritional and metabolic relationships between epibiontic microalgae and their hosts have been interpreted in several ways. In the case of epiphytic communities, a number of studies consider macrophytes only as a neutral substrate for the epiphyte growth, or even if a host effect occurs, it can be masked by other important environmental factors (Sullivan, 1979; Thomas and Jiang, 1986; Snoeijs, 1994, 1995), while others report the existence of a clear host preference (Pinckney and Micheli, 1998). On the contrary, in the case of epizoic diatoms, there is a general agreement about the fact that algal growth is stimulated by waste products of host (Round, 1981; Gaevskii et al., 2004; Romagnoli et al., 2007). The degree of host specificity has been related to the trophic conditions of water column, as the host effect decreases under eutrophic conditions (Eminson and Moss, 1980; Burkholder et al., 1990; Jones et al., 2000).

2.3. MOLLUSKS

The fact that hard shells of gastropod or bivalve mollusks represent a suitable habitat for the settling of diatoms has been known since 1949, when Politis described an extensive diatom flora on the bivalve *Pinna nobilis* from Bosphorous and Marmara Sea (Round, 1971 and references therein). The epizoic communities on mollusks were compared with that on the brown seaweed *Cystoseira* from the same area, and a difference in terms of species composition has been found, as in the seaweeds stalked growth forms occurred, which were absent in animals.

Gillan and Cadée (2000) investigated the community of microorganisms epibionthic on the gastropod *Hydrobia ulvae* from the Wadden Sea. They reported that a dense coverage of the diatoms *Cocconeis placentula* and *Achnanthes lemmermanni* occurred together with bacteria and cyanobacteria. The composition of epibiontic assemblage did not show any relationship with the shell size of the hosts. The algal mat appeared to be encrusted with a mineral rich in Fe(III). The biofilm formed by EPS (extracellular polymeric substances) secreted by a microbial community may act as a scavenging site for the heavy metal. The authors remarked on the importance of taking into account the epibionts when the ecology of animal hosts is studied.

◄

Figure 1. (continued) settled in the grooves between annuli; (**h**) the theca of *C. linearis* completely covered by diatoms; (**i**) enlargement of the teeth of *C. linearis* with a continuous sheet of cells of *Cocconeis pseudonotata*; (**j**) the thecal external surface of the hydroid *Campanularia hincksii* free of diatoms; (**k**) Enlargement of a broken theca of *C. hincksii* showing the diatom *Cylindrotheca* sp. present on the inner wall; (**l**) a broken portion of the theca of *C. hincksii* showing the monospecific assemblage of *Cocconeis notata* organized in a continuous layer on the inner side of the theca. Scale bars: **a**, 20 μm; **b**, 10 cm; **c**, 10 μm; **d**, 10 cm; **e**, 20 μm; **f**, 50 μm; **g**, 50 μm; **h**, 200 μm; **i**, 50 μm; **j**, 100 μm; **k**, 50 μm; **l**, 100 μm."

The epizoic diatom communities were investigated during summer 2006 in several gastropod species from Mediterranean Sea (Cante et al., 2008; D'Alelio et al., 2010), indicating that the shell morphology affected the epiphytic composition, with higher biodiversity occurring in shells with a more articulated structure. Diatom communities were dominated by adnate life forms (*Amphora* and *Cocconeis*), whose rounded cells are able to colonize the shell microenvironments. An *Amphora* species was also observed living as endolithic into the calcareous shell, perhaps as a strategy to elude other competitors and grazers.

Although not quite an epibiosis, an interesting example of interaction between diatoms and mollusks is offered by the tychopelagic marine diatom *Haslea ostrearia*, which proliferates typically in oyster ponds, and has the distinctive feature of synthesizing and releasing marennine, a blue-green water-soluble polyphenolic pigment (Pouvreau et al., 2006a,b). This pigment is responsible for the greening of oyster gills, raising the product's pre-market values by 40% (Muller-Feuga, 2000), and has an inhibitory effect on the growth of several planktonic and benthic diatom species, prolonging the dominance of *H. ostrearia* in its environment (Pouvreau et al., 2007).

2.4. ARTHROPODS

The first historical records about the epizoic diatom communities concern examples of diatom species living epizoic on planktonic copepods (Hiromi et al., 1985). Until now, at least five different diatom taxa have been reported as epizoic of marine copepods, showing a high degree of host selection (see below). In freshwater bodies of inland ecosystems, common symbiotic relationships are reported involving zooplanktonic cyclopoids and microalgae, including both euglenoids (*Colacium calvum* and *C. vesiculosum*) and diatoms (*Synedra cyclopum*) living as epibionts on their exoskeleton (Chiavelli et al., 1993; Gaiser and Bachmann, 1994). Often the development period of epibionts paralleled that of crustacean hosts (Gaevskii et al., 2004). Some epibiontic species are shown to be facultative epibionts as they can also occur free in the plankton (Gaiser and Bachmann, 1994), whereas other cannot grow anywhere but on copepods, as they are not able to survive in culture (Russell and Norris, 1971).

The interactions between planktonic copepods and their epibiontic diatoms involve several ecological aspects. The benefits for diatoms are related to living over a moving host, gaining advantage when compared with planktonic species, due to the possibility of active moving in the water column with a continuous replenishment of nutrients, and obtaining CO_2, and catabolite products from crustaceans. Zooplankters benefit from the oxygen supply, which may be limiting during periods of intensive bloom. However, epibionts may impede their movement and reduce the buoyancy, the capability in avoiding predators, and obtaining food, and affect their reproductive rate interfering with the host mating, which may sharply alter the copepod population (Gibson, 1979; Chiavelli et al., 1993 and references therein).

Horseshoe crabs act as moving substrata hosting diversified communities of marine organisms. Patil and Anil (2000) reported a complex community epibiont on *Tachypleus gigas* from India, consisting of macroepibionts (coelenterates, bryozoans, and bivalves) and diatoms (mainly the pennate *Navicula* spp., *Nitzschia* spp., and the centric *Skeletonema* sp.).

2.5. BRYOZOANS

Bryozoan colonies are a favorable microenvironment for benthic microorganisms (Wuchter et al., 2003). Their surface is often highly sculptured and offers protection against predators, and the feeding currents generated by the polyps provide a permanent supply of nutrients (Scholz, 1995). Therefore, bryozoans often host extensive microbial mats which are the results of a specific interaction between bryozoans and microorganisms and appear to differ both from the community of the surrounding substratum and between various bryozoan species (Scholz and Krumbein, 1996).

Wuchter et al. (2003) investigated the epibiontic diatoms on four bryozoan species from the German Bight, and observed that each bryozoan species hosted a specific diatom community (see below). The extent and the composition of the diatom flora were affected by intrinsic factors, such as surface structure, antimicrobial substances, and extrinsic ones such as physicochemical parameters and grazing pressure.

3. Ecology

3.1. ABUNDANCES

Only a few studies investigated the diatom abundances on animal substrata. However, it seems that the density of epibiontic diatom communities is very variable according to the nature of hosts and environmental conditions.

Cell densities around three orders of magnitude per square millimeter were recorded on gastropod shells: 1,000 cells mm^{-2} were reported on *Hydrobia ulvae* from the Wadden Sea by Gillan and Cadée (2000), while densities from 1,128 to 4,421 cells mm^{-2} were reported on the Mediterranean Gastropods *Jujubinus striatus* and *Bittium reticulatum* (Cante et al., 2008; D'Alelio et al., 2009). The highest abundances were recorded in bigger shells indicating that shell size influenced the diatom communities. Abundances, one order of magnitude lower, were reported on mussel shells from the Black Sea by Bodeanu (1987–1988).

On the bryozoans *Electra pilosa*, *Membranipora membranacea*, *Flustra foliacea*, and *Alcyonidium gelatinosum* from the North Sea, 31 species belonging to 26 diatom genera were found with densities of 71–547, 77–110, and 1–27 cells mm^{-2}, respectively for *Electra pilosa*, *Membranipora membranacea,* and *Alcyonidium* *gelatinosum,* while on *Flustra foliacea*, diatoms were virtually absent, with fewer than 5 cells mm^{-2} (Wuchter et al., 2003).

Diatom abundances reported on *Tachypleus gigas* by Patil and Anil (2000), i.e., 5,000 cells dm^{-2}, were significantly lower than those reported for other epizoic coverage (Gillan and Cadée, 2000; Romagnoli et al., 2007). In fact, the community growing on horseshoe crabs normally experience a wide range of environmental conditions, from the tidal limit to over 35 m depth, as these organisms migrate periodically from deep regions to near-shore waters. Therefore, epibionts must tolerate desiccation periods due to prolonged subaerial exposure, which occurs during nesting periods. Diatoms may tolerate such extreme conditions due to the production of EPS, but probably the long periods of light reduction that they experience at high depth limit their proliferation. The significant higher abundances observed on male than on female carapaces have been explained, considering both that male carapace is more hydrophobic and has a surface rougher than the female one, and that toxic compounds associated with eggs may act as a control limiting epibiosis.

In temperate lakes, the observed abundance of *Synedra cyclopum* reached up to 4,000 cells cladoceran^{-1}, exhibiting a clear seasonality, with higher abundances in spring due to the optimal light and nutrient availability as well as the abundance of *Daphnia* spp. which represent their preferred hosts (Gaiser and Bachmann, 1994).

Very high densities of epibiontic diatoms were recorded for the communities settled on marine hydroids: an average of 45,000 cells mm^{-2} (with a maximum close to 100,000 cells mm^{-2}) was reported for *Eudendrium racemosum* from the Mediterranean Sea, with strong seasonal variations. On colonies of this species, diatoms and cyanobacteria exhibited opposite seasonal trends with respect to abundance, with diatoms mainly occurring in winter and spring and cyanobacteria in summer. Within the diatom assemblages, all the growth forms, i.e., motile, adnate, and tube-dwelling, showed a different annual trend. The adnate diatoms seem to be the group that better-followed the annual cycle of the host (Romagnoli et al., 2007), as they may easily benefit from the nutrient exchange with the host because of their intimate association with it (Sullivan, 1984). On the other hand, biraphid taxa are superior competitors for light and nutrients due to the ability of active moving on substrata (Hudon and Legendre, 1987; De Nicola and McIntire, 1990), and erect diatoms benefit from a better light exposition (Tuji, 2000).

Antarctic sponges normally host large amount of diatoms both on their surface and inside their tissues, as a result of an active process of concentration due to the sponge activity, with strong differences according to the seasonal cycle (up to 1,217 ± 178 cells × 10^6 g^{-1} dw). Cerrano et al. (2004a) investigated the temporal variations of diatom abundance inside the sponge tissue from November 2001, before the ice melting, to February 2002, with weekly frequency. Diatom concentration in the sponge tissues was very low at the beginning of austral summer, but quickly increased reaching the maximal values at the end of January and decrease again during the austral winter thus indicating a relationship with the summer phytoplankton bloom.

3.2. SPECIFICITY

Although, in general, the microflora living on benthic animals reflects that of the substrata where the animal host lives (Bodeanu, 1987–1988), a number of specific associations between microalgae and animal hosts have been reported. The existence of host specificity was suggested for diatoms by Sullivan (1984), as certain epiphytic species were distributed only on particular macrophytes, but these have also been interpreted as a response to different physical conditions (Sullivan, 1981).

The interactions between marine copepods and their epibiontic diatoms offer several interesting examples of host specificity. *Pseudohimantidium pacificum* lives on a few marine crustaceans belonging to cyclopoid (*Corycaeus* spp., *Farronula gracilis*) and harpacticoid (*Euterpina acutiformis*) copepods, from a wide range of marine areas, attaching to the host exoskeleton by a mucus stalk. *Protoraphis atlantica* lives mainly on pontellid and candacid copepods, while *Falcula hyalina* was found mainly on *Acartia* species from Japan and *Sceptronema orientale* on *Euterpina acutifrons* (Gibson, 1978, 1979; Prasad et al., 1989). In temperate lakes of Europe and North America, *Synedra cyclopum* is a common epibiont of cladoreran and copepod crustaceans, showing marked host selectivity and attaching only on particular *Daphnia* species (Gaiser and Bachmann, 1994). The degree of host specificity found in epibionts varies not only between different epibiont species, but sometimes between populations of the same species in different systems (Chiavelli et al., 1993).

A marked specificity for the host was often reported for marine hydroids. Siqueiros-Beltrones et al. (2001), described diatom colonization on the hydroid *Campanularia integra* (formerly *Eucopella caliculata*) living epibiont on the apical frond of the giant kelp *Macrocystis pyrifera*, and reported that monospecific communities of *Cocconeis notata* occurred inside the hydrotheca, while a multispecific community of small naviculoids forms lived inside the gonangia. Unfortunately, it was not possible to establish if diatoms colonized the hydroid before or after its death. Similar results were obtained by Di Camillo et al. (2005) who ascertained that diatoms associated with Mediterranean hydroids colonized living specimens.

Marked host specificity has been found by Wuchter et al. (2003) for diatoms epibiontic on bryozoans. Some bryozoan species, such as *Flustra foliacea* and *Alcyodinium gelatinosum,* seem to be an unfavorable substratum for the growth of epibionts due to their morphological characteristics (smooth surface), as well as a possible production of antimicrobial products. On the contrary, other species such as *Electra pilosa* and *Membranipora membranacea* host a rich and diversified diatom flora.

Marine sponges offer several examples of host specificity. In Antarctic environment, large populations of the diatom *Melosira* sp. were recorded as specific parasites of the hexactinellid sponge *Scolymastra joubini.* Diatoms formed large mats that are embedded in the sponge body destroying the living tissue of the hosts and appearing as green spots on the sponge surface (Cerrano et al., 2000a). A parasitic behavior of diatoms has also been hypothesized in a number of Antarctic sponges hosting larger amounts of diatoms that appear to utilize the products of metabolism of their host as an energy source (Bavestrello et al., 2000).

Hamilton et al. (1997) described a new diatom genus *Porannulus* (type species *P. contentus*) that was found only associated with several Antarctic sponges, such as *Axocella flabellata*, *Sphaerotylus antarcticus*, *Gellius phakelloides*, *Mycale acerata*, *Mixilla* sp., and *Polymastia isidis* (Cerrano et al., 2004b). Owing to the morphological simplicity of their frustule, lacking perforations and sculptures, suggesting an adaptation to a nutrient-enriched environment, such association has been initially interpreted as an endosymbiosis (Hamilton et al., 1997). Later, Cerrano et al. (2004b) observed that high cell density values of *P. contentus* occurred on the surface of *M. acerata* (377×10^6 frustules g^{-1} dw) before the beginning of November, i.e., very early in the austral spring–summer, indicating that the bloom of this species occurs at least one month before the ice melt. This study concluded that *Porannulus* lives epibiontic on the ectosome of *Mycale acerata*, actively reproducing on the sponge surface, probably benefiting from the enrichment of the microenvironment due to sponge excretion, and that the sponge may utilize it as a trophic source, by incorporating the cells into the choanosome.

The fact that *P. contentus* lives in association with some sponge species (Hamilton et al., 1997) and not with others of the same area may be related to the ability of some sponge species to produce bioactive secondary metabolites (Kelman et al., 2001; Laport et al., 2009). As the species was never recorded in the plankton, it is possible that it crosses the austral winter in the sponge tissues as a resting stage.

3.3. SPATIAL DISTRIBUTION

Sometimes, animals represent an amazing mosaic of different microhabitats exploited by different species of diatoms.

In the diatom colonization of marine copepods, a different spatial distribution on male and female specimens has been reported in several cases, with diatoms concentrating in part of the exoskeleton and not in others. Such kind of distribution has been related to the copulatory behavior of the copepod hosts (Russell and Norris, 1971; Hiromi and Takano, 1983; Hiromi et al., 1985).

On the branched colonies of hydroids, diatoms occupy each portion of the colony-like base of the stem, branches, pedicels, and, in thecate hydroids, the inner space between the polyp and the theca. When the colonies of *Eudendrium racemosum* are exposed to direct light, their stem and branches become covered with an extremely rich diatom assemblage, mainly composed of erect *Licmophora* spp. (mainly *L. oedipus* and *L. flabellata*) forming fan-shaped colonies. As a rule, *Licmophora* spp. colonized the upper part of the hydroid, while *Cocconeis* and *Amphora* settled in the lower portion of stem (Fig. 2). In any case, the abundance

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Figure 2. (continued) *E. racemosum*, growing in the annulated stalk where they gain protection against grazing; (**i**) branch of *E. racemosum* showing colonization of the adnate *Cocconeis* sp. and erect *Tabularia tabulata*; (**j**) settling of erect *Tabularia tabulata* and adnate *Cocconeis* sp. and *Amphora* sp.; (**k**) cells of *Synedra* sp. an erect form attached to the substrata through a mucus pad, secreted by the apical pore fields. Scale bars: **a**, 200 μm; **b**, 200 μm; **c**, 20 μm; **d**, 50 μm; **e**, 2.5 μm; **f**, 10 μm; **g**, 50 μm; **h**, 5 μm; **i**, 10 μm; **j**, 10 μm; **k**, 20 μm.

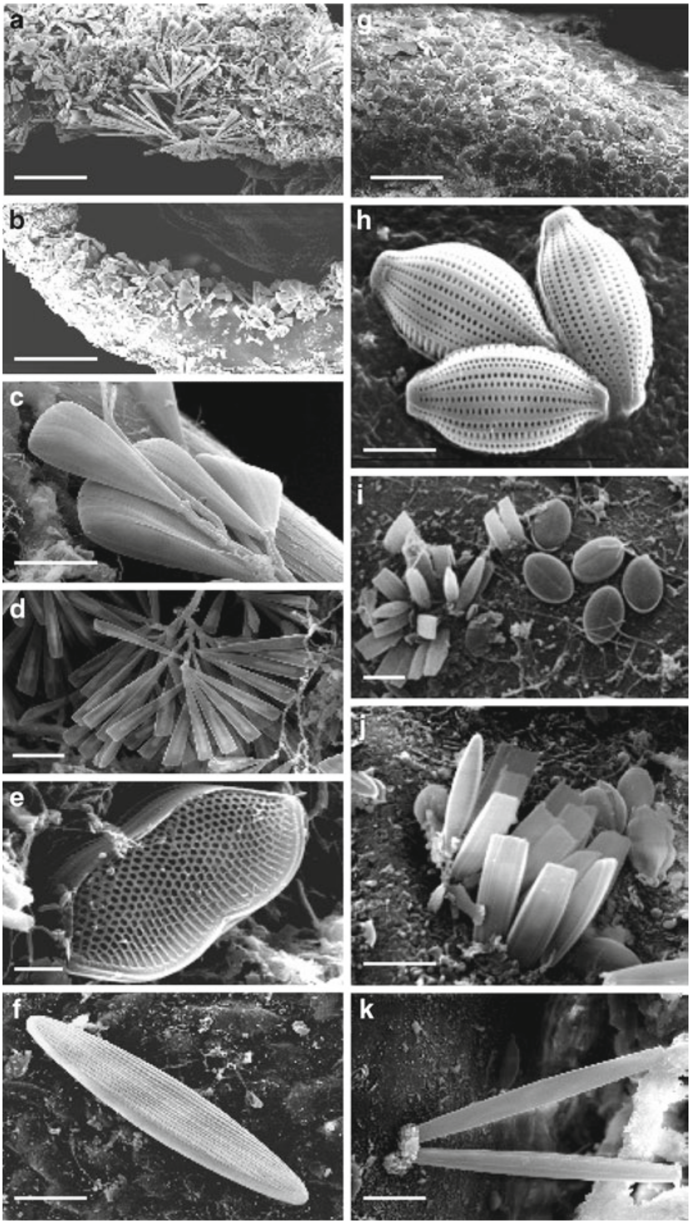

Figure 2. (**a**) Rich colonization of diatoms on a branch of the marine hydroid *Eudendrium racemosum* showing colonies of erect *Licmophora* spp.; (**b**) colonization of *L. oedipus* and *L. abbreviata* on a pedicel under polyp; *Licmophora* spp. form fan-shaped colony attached to the substrata by a mucilaginous stalk; (**c**) particulars of *L. oedipus*; (**d**) fan-shaped colony of *L. flabellata*; (**e**) *Psammodictyon mediterraneum*, a motile form living on *E. racemosum* colony (**f**) *Navicula* sp., a motile form that move along the *E. racemosum* colony; (**g**) a rich coverage of an adnate *Cocconeis* spp. that colonize mainly the basal part of the *E. racemosum*; (**h**) the adnate *Amphora coffeaeformis* settled in the middle and the basal part of the

of diatoms increases from the base to the apex of the colony, suggesting a certain degree of interaction between algae and polyps (Romagnoli et al., 2007). The diatom covering stopped at the base of pedicels, sometimes colonized by the ciliate *Ephelota* sp. (Di Camillo et al., 2005).

In some hydroids like *Campanularia hincksii*, it has been shown that no diatoms settle on the external side of the theca, while a monospecific diatom assemblage of *Cocconeis notata* living only on the inside of the theca was recorded (Di Camillo et al., 2005). On the external side of the theca of *Clytia linearis*, colonization by *Cocconeis pseudonotata* starts from the vertical grooves present in each tooth, and subsequently continues, completely covering the theca and pedicel with a continuous layer. A monospecific community formed by a *Cylindrotheca* sp. settled in the internal side of the theca. The green color of the cells clearly indicates that they are living and that, apparently, their presence did not produce damage to the polyps, which were always observed alive (Fig. 1 g–l). A similar situation was observed on *Synthecium evansi,* where *Cocconeis neothumensis* var. *marina* colonized the perisarc surrounding stem, branches, and thecae, while *Navicula* sp. was localized exclusively inside the thecae (Di Camillo et al., 2005).

4. Conclusions

Data presented in this chapter illustrate the breadth of the relationships established between diatoms and benthic organisms. Although endobiotic species lacking frustule were occasionally described, the majority of the reported relationships between diatoms and marine invertebrates involve epizoic processes. This capability is enhanced by the widespread ability of benthic organisms to produce external protective coats, both organic and mineral, such as perisarc, cuticule, tubes, or shells, which are easily colonized by benthic diatoms. Often, the colonized animals represent an increase in the available substrata for benthic diatoms, and many epizoic diatoms are therefore benthic forms only slightly adapted for the life in close relationships with animals. The life on non-cellular structures avoiding any contact with the living tissues of the host decreases the effect of antifouling substances widely produced by benthic organisms (Pawlik, 1993).

On the other hand, other epizoic species express marked adaptations and selective relationships with their hosts. Although no experimental data are currently available, it seems that diatoms obtain some benefits from the host, using some products of its catabolism as nutrients, as suggested for hydroids (Romagnoli et al., 2007), crustaceans (Gaevskii et al., 2004), and mollusks (Cognie and Barillé, 1999).

A different mechanism is hypothesized for the relationships involving diatoms and sponges. Planktonic diatoms are filtered, retained alive for a certain time inside sponge tissues, and finally probably exploited as food by sponges themselves. Their ability to survive for a long time and reproduce inside the sponge tissues (Cerrano et al., 2004a, b) is probably explained by a mixotrophic lifestyle (Gaino et al., 1994; Bavestrello et al., 2000) and by the possibility to obtain light through the siliceous spicules of the host acting as optical fibers (Cattaneo-Vietti et al., 1996). Beside such examples, which are common and widespread particularly in Antarctic areas, almost two cases of more stable relationships are known: *Melosira* sp. has been found living as a parasite in volcano sponges (Cerrano et al., 2000a), while *Porannulus contentus* appears to be subjected to a sort of cultivation by the sponges, as it may live as epibionts on sponge surfaces reaching extremely high concentration, and, on the other hand, it may serves as food for its host (Cerrano et al., 2004b).

Further studies are needed to deepen the existing relationships between diatoms and their living hosts. It seems clear that the research on benthic diatoms may not neglect the contribution of epizoic component, and at the same time, the studies of the ecology of marine organisms should consider the presence of their microepibionts, which may markedly affect their biology and lifecycles.

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EPIZOIC DIATOMS ON GASTROPOD SHELLS

When Substrate Complexity Selects for Microcommunity Complexity

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1. Introduction

Complexity pervades biological systems at any scale: from microbes to higher organisms, from individuals to populations, from communities to ecosystems. The degree of organization of plant and animal populations, their trophic relationships, and ecological associations is currently explored in ecology. Biodiversity and community structure are strongly influenced by the complexity of those relationships, in addition to the interplaying between multiple environmental conditions. On the other hand, complexity in microcommunities has not been solved yet. For instance, the role of substrates in shaping the diversity and structure of biofouling communities is virtually unexplored.

Benthic microalgae living in shallow coastal regions give a reliable contribution to the dynamics of the aquatic ecosystems, in terms of primary production, oxygenic activity, and trophic processes (Mac Intyre et al., 1996). Attached communities of microalgae can develop in different benthic microenvironments: from detritus (epipelon and endopelon), rocks (epilithon and endolithon), and sediment sands (epipsammon), to microalgal and seagrasses turfs (epiphyton) and animals (epizoon) (Round, 1971). The peculiar microenvironments provided by marine animals – i.e., growing in size, metabolite-rich, and potentially grazers-free – are successfully exploited by an important lineage of benthic microalgae, the diatoms (Round, 1981).

1.1. THE DIATOMS

Diatoms (Bacillariophyta) constitute a successful lineage of unicellular Heterokonts (Falkowski et al., 2004), both planktonic and benthic, which is responsible for the

20% of the global primary production (Smetacek, 1999). Diatoms represent the most intensively studied marine microalgae. To date, more than 10,000 diatom species have been described (Mann, 1999), but the evaluation of the total number of existing species is still on debate due to both cryptic diversity within and the capability of this algae to spread around the world in every aquatic environment. Another unique feature of diatoms is the capability to take silicon from water and convert it into a highly ornamented siliceous cell wall, the frustule, in which the protoplast is enclosed. The frustule is constructed like a pill-box, consisting of two valves, with the ventral (hypovalve) slightly smaller than the dorsal (epivalve), joined together by a series of silica bands (copulae) that form the lateral walls of the box (girdle or cingulum) (Round et al., 1990; van den Hoek et al., 1996) (Fig. 1a). Cell division occurs with the formation of a new valve inside each of the two mother valves, so that one daughter-cell has the same size as the mother, whereas the other is smaller. This modality of division is called "size reduction" as it leads to a progressive diminishing of the size of the population. The size reduction ends with a meiotic process, which produces two gametes. Gametes fuse and form a diploid cell (auxospore), which lacks the silica frustule at the first stages of development, and it is thus able to re-establish the original maximum cell size (Round et al., 1990).

Diatoms possess a number of chloroplasts that vary from one to many per cell, depending on the species (Fig. 1). Chlorophyll *a*, c_1, c_3, and c_2 are the most important photosynthetic pigments. Carotenoids and xanthophylls, mainly fucoxanthin, are the most abundant accessory pigments. Diatoms have an enormous ecological significance; they are ubiquitous in the plankton and benthos of marine and freshwater environments, from the tropics to the Polar Regions, as well as in temporarily humid surroundings, such as damp moss or rocks (Round, 1981; Mann, 1999). Diatoms are crucially involved in the ocean biological pump, and frustules form a consistent part of organogenic sediments in marine bottoms (Smetacek, 1999).

Traditionally, two main orders have been considered within diatoms, Centrales and Pennales, showing radial and bilateral symmetry of the valves, respectively (Hustedt, 1959). However, we must point out that this classification is often arbitrary. Some centric diatoms do not possess radial symmetry and some pennates are not bilaterally symmetric. Round et al. (1990) proposed a different classification based on three classes: Coscinodiscophyceae (corresponding to centric diatoms, Fig. 1b), Fragilariophyceae (araphid pennate species, Fig. 1c), and Bacillariophyceae (raphid pennate species, Fig. 1d), with a total of 44 orders and 91 families.

Species ascribed to Bacillariophyceae have a pennate symmetry and are characterized by a peculiar structure, the raphe, which is a deep slit running across the valve from pole to pole, and from which mucous is emitted. Raphid diatoms (mono- or biraphid, whether the raphe is borne in only one or both the valves) use this structure for locomotion, i.e., they slide above the mucous trails released by the raphe itself (Fig. 1e, f). Species ascribed to Fragilariophyceae have

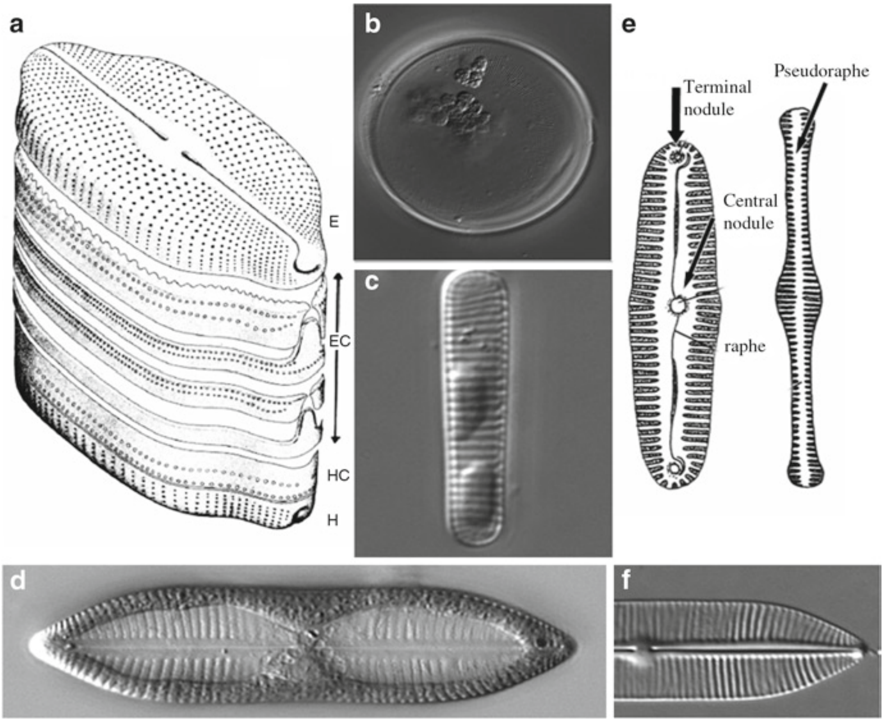

Figure 1. (**a**) The diatom frustule; (**b**) a centric diatom showing several chloroplasts; (**c**) a pennate araphid diatom showing two chloroplasts; (**d**) a pennate raphid diatom showing two chloroplasts; (**e**–**f**) a schematic view and a light microscopy image (LM) of the raphe in a pennate diatom, respectively. (**a**) and (**e**) modified from Round et al., 1990.

a pennate symmetry too, but lack the raphe, and are generally attached to the substrate by means of mucous pillows or stalks/peduncles extruded by specific structures located at the valve apices (apical pore fields).

1.2. EPIZOIC DIATOMS: GLASS CELLS LIVING ON WET FLESH

Among those characterized by a benthic life mode, many diatoms are reported to colonize sponges (Cerrano et al., 2004a, b), hydrozoans (Bavestrello et al., 2008; Romagnoli et al., 2006), bryozoans (Wuchter et al., 2003), crustaceans (Ikeda, 1977), bivalves (Round, 1981), and vertebrates (Round, 1981; Round et al., 1990), with a high degree of specificity for some hosts. This is possible because of the presence of different and sometimes *ad hoc* strategies used by these microalgae to keep in connection with, as well as to gain “hospitality” by, their substrate. The main strategy accounts for the development of differentiated growth forms (Fig. 2a–f), such as:

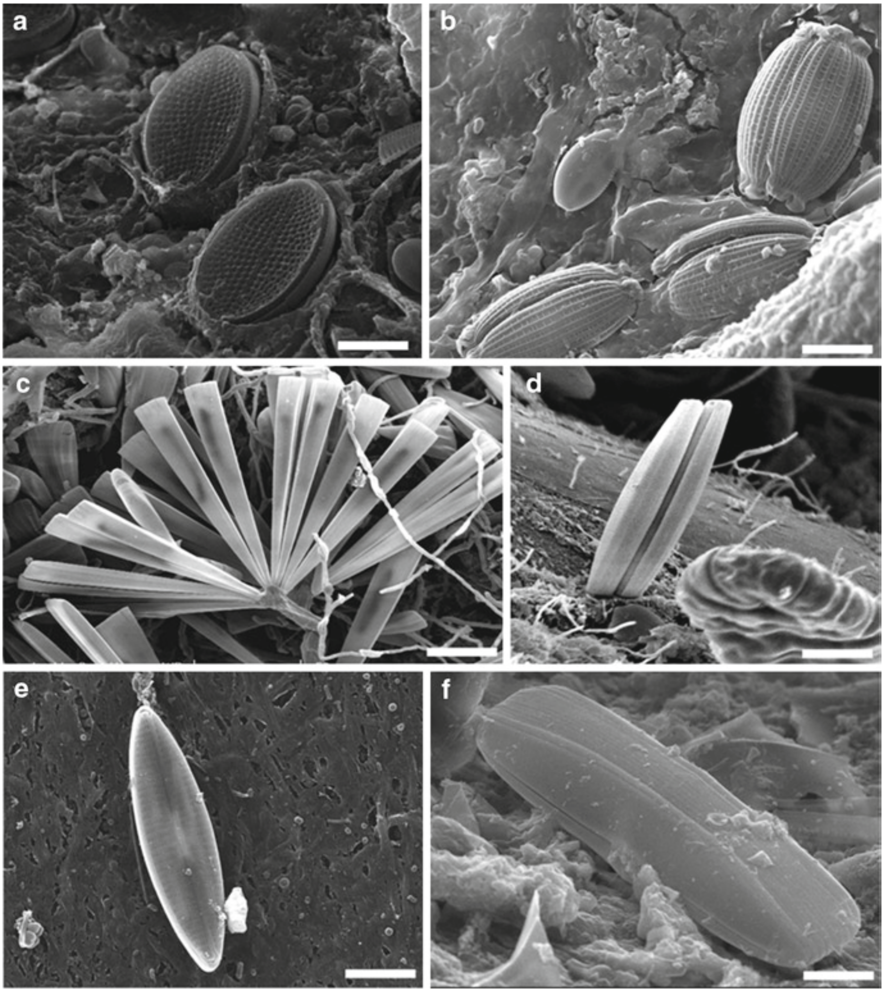

Figure 2. (**a**) and (**b**) Adnate diatom cells adhering horizontally to a substrate (Scanning Electron Microscope, SEM, scale bars = 10 and 5 μm, respectively); (**c**) and (**d**) erect diatom cells adhering vertically to a substrate (SEM, scale bar = 10 and 10 μm, respectively) – a peduncle connecting the colony to the host surface is visible in C; (**e**) and (**f**) motile diatom cells (SEM, scale bars = 5 and 2 μm, respectively).

1. Adnate cells (both biraphids and monoraphids diatoms), strongly adhering horizontally to the substrate by means of the raphidic valve (Fig. 2a, b)
2. Erect cells (araphid, biraphid, and monoraphid diatoms), adhering vertically to the substrate by means of mucous pillows or stalks/peduncles (Fig. 2c, d)
3. Motile cells (mostly biraphid, some monoraphid diatoms), having high movement capability enabling them to glide and spread above the substrate (Fig. 2e, f)

The succession of benthic diatom communities is well explained by the dynamics of colonization of a new substrate by the above-mentioned growth forms. Populations of adnate forms, strongly attaching to the substrate, represent the first encrusting and more stable component in the diatom assemblage. Populations of erect forms, by adhering to a smaller surface, are less stable and colonize the substrate after the adnates. Eventually, populations of motile forms, only partially adhering, spread more effectively above the substrate than the other forms, but are less stable and can be easily removed by water movements. Thus, while a young community is mainly dominated by adnates, an older and well-structured community should include the three growth forms, all equally important in quantitative terms (Round et al., 1990).

Despite the general ecological succession explained earlier, epizoic diatom communities have a complex dynamics strongly depending on the nature and dynamics of their substrate, e.g., the animal's growth, position in the water column, swimming behavior, exposition to streams, and so on.

The interaction between diatoms and Porifera has been recently well documented. Many Antarctic sponges can host rich communities of these microalgae due to the capability of pinacoderms cells to incorporate them for a certain time before their digestion (Cerrano et al., 2004a, b; Totti et al., 2005). Monospecific assemblages of the diatom *Porannulus contentus* Hamilton, H. Klöser, and M. Poulin have been observed in some species of Antarctic sponges. A dense mat of *P. contentus* was detected on the outer surface of the sponge *Mycale acerata* Kirkpatrick; this mat is incorporated within the animal tissues, thus suggesting that the sponge itself can feed on this diatom (Cerrano et al., 2004a). A much more complex diatom community is associated with the sponge *Sphaerotylus antarcticus* Kirkpatrick, a species externally covered by a dense layer of spicules. This envelop represents a three-dimensional habitat richly colonized by a diversified community of benthic diatoms – e.g., the centric diatom *Hyalodiscus sp.*, the pennate *Diploneis crabro* Ehrenberg, *Entomoneis paludosa* (W. Smith) Reimer, *Trachyneis aspera* (Karsten) Hustedt, *Pleurosigma intermedium* Smith – and a high density of planktonic species, such as *Fragilariopsis curta* (Van Heurck) Hustedt (Totti et al., 2005).

A number of examples of epizoic associations are known for marine hydroids, as their episarc represents a very suitable habitat for diatom growth and it often looks brown for the presence of adnate and pedunculate diatoms. A dense assemblage of the pennate *Cocconeis notata* Petit was reported on the hydroid *Campanularia integra* MacGillivray, which is in turn an epibiontic of the brown seaweed *Macrocystis pyrifera* (Linnaeus) C. Agardh (Siqueiros-Beltrones et al., 2001). Round et al. (1961) documented different kinds of diatom assemblages lying on hydroids ascribed to *Sertularia operculata* Linnaeus and exposed at different hydrodynamic conditions. Recently, Di Camillo et al. (2005) reported several examples of host specificity for epibiontic diatoms and hydroids. A clear spatial distribution of epibiontic diatoms has been reported for the marine hydroid *Eudendrium racemosum* Gmelin (Romagnoli et al., 2006): adnate diatoms were distributed mainly in the basal and central part of the hydroid colonies, while erect forms were in the apical part only.

Interesting epizoic associations are also known for planktonic crustaceans, both in freshwater and marine environments, which carry epibiontic diatoms or cyanobacteria on their exoskeleton and appendages (Ikeda, 1977; Round, 1981; Hiromi et al., 1985; Gaiser and Bachmann, 1994). For example, diatoms belonging to the genus *Pseudohimantidium* can produce monospecific and stable assemblages on the marine copepod, *Coryacaeus* (Gibson, 1978).

2. Diatom–Gastropod Interactions

The hard shells of mollusks are reported to provide a substrate for the settling of microphytobenthos, and sometimes a high degree of specificity has been found between epizoic diatoms and their animal hosts. For instance, a rich community including several benthic diatom genera (e.g., *Licmophora*, *Achnanthes*, *Cocconeis*) was reported on the bivalve *Pinna nobilis* Linnaeus, with a species composition differing from that seen in co-occurring thalli of the brown algae *Cystoseira* (Round, 1971 and references therein). A dense assemblage of *Cocconeis placentula* Ehrenberg has been reported on the shells of *Hydrobia ulvae* Pennant, with no relation between the size of the shell and the density of the diatomaceous layer (Gillan and Cadée, 2000).

In a recent study, gastropod species were sampled from a coastal site in the Bay of Naples during summer 2006 (Cante et al., 2008). The sampled specimens were highly representative for inshore Mediterranean waters and differed in dimension, gross-, and fine-scale morphology of the shell. By means of a multi-approached research, based on Scanning Electron Microscopy and computer-based image analysis, we revealed that the degree of complexity of epizoic diatom mats (in terms of species composition and biodiversity indexes) is mainly influenced by the complexity of the host shell, despite its overall dimension. The following sections open a detailed window about the above-mentioned study.

2.1. GASTROPOD SHELLS AS SELECTIVE MICROENVIRONMENTS FOR DIATOM COMMUNITIES

In order to decrypt reliable microselective constraints in the building process of diatom communities on the shell surface, the following gastropod species were extensively investigated:

1. *Columbella rustica* Linnaeus (Gasteropoda, Prosobranchia, Caenogastropoda, Neogastropoda, Columbellidae)
2. *Alvania lineata* Risso (Gasteropoda, Prosobranchia, Caenogastropoda, Mesogastropoda, Rissoidae)
3. *Nassarius incrassatus* Stroem (Gasteropoda, Prosobranchia, Caenogastropoda, Neogastropoda, Nassariidea)

4. *Bittium reticulatum* Da Costa (Gasteropoda, Prosobranchia, Caenogasteropoda, Mesogasteropoda, Cerithiidae)
5. *Clanculus cruciatus* Linnaeus (Gasteropoda, Prosobranchia, Archaeogastropoda, Vetigasteropoda, Trochidea)
6. *Gibbula adansoni* Payraudeau (Gasteropoda, Prosobranchia, Archaeogastropoda, Vetogastropoda, Trochidea)
7. *Jujubinus striatus* Linnaeus (Gasteropoda, Prosobranchia, Archaeogastropoda, Vetogastropoda, Trochidea)

The above-mentioned gastropods, shown in detail in Figs. 3 and 4, were ordered according to their shell fractal dimension (*D*) – i.e., the theoretical dimension measuring the degree of irregularity and interruption of an object (Mandelbrot, 1977). *D* is commonly used to quantify the degree of discontinuity of landscapes. Basically, the higher the *D,* the more chaotic and discontinuous is the surface. In turn, more organized (i.e., regular) landscapes show a relatively lower value of *D*. *D* value ranges 1.460–1.635 for the above-mentioned gastropods species. However, the one-dimensional shell size is more variable, ranging 4–20 mm (Table 1).

The first three species, *A. lineata*, *Co. rustica*, *N. incrassatus*, showing the highest values of *D* and a low degree of fine-scale ornamentation, represent highly irregular microscopic landscapes (Fig. 3a–i). The following two species, *B. reticulatum* and *C. cruciatus*, with a value of *D* approaching 1.56 and a moderate degree of ornamentations, represent a better organized and more regular landscape (Fig. 4a–f). *Gibbula adansoni*'s landscape was comparable with those made by the previous two species in terms of regularity, though this species was weakly ornamented on one side, and relatively smooth on the other side of the shell (Fig. 4g–i). Finally, *J. striatus*, characterized by the lowest fractal dimension and the highest density of radial swirls, represented the more regular and organized landscape among those under examination (Fig. 4j–l).

The substrate provided by gastropod shells is characterized by different kinds of structures lying on a spatial scale comparable to the cell size (20–100 μm). The simplest substrates, with a chaotic arrangement, are generally deprived of stable structures; the more organized ones show a regular succession of "mountain-like" swirls, deep "canyons," and smooth and protected "valleys". Such a "microscopic landscape" can be differentially exploited by encrusting organisms. Different diatom species (and different growth forms, see Section 1.2) can buildup different communities, depending on the actual microenvironments they develop on.

Adnate forms took advantage from the different shell morphologies, because they effectively exploited the microhabitats provided by shells with a more organized landscape. For instance, the genera *Amphora* and *Cocconeis* adhered preferentially to surfaces with protuberances and reticulations (e.g., *Bittium reticulatum*), and small *Cocconeis* species (e.g., *C. neothumensis* Krammer) managed to adhere to surfaces with many microhabitats (e.g., *Clanculus cruciatus*).

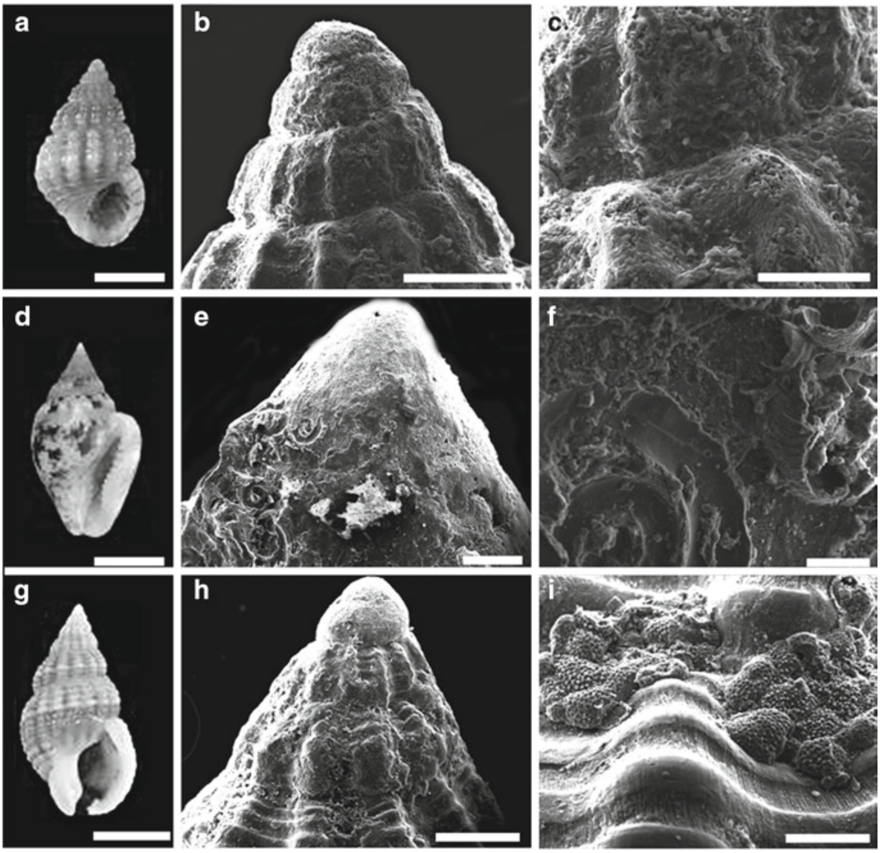

Figure 3. (**a**) *Alvania lineata* (optical image, OI, scale bar = 1 mm); (**b**) *A. lineata* (SEM, scale bar = 300 µm); (**c**) *A. lineata*, shell surface with few ornamentations (SEM, scale bar = 150 µm); (**d**) *Columbella rustica* (OM, scale bar = 6 mm); (**e**) *C. rustica* (SEM, scale bar = 400 µm); (**f**) *C. rustica*, surface with calcareous incrustations (scale bar = 50 µm); (**g**) *Nassarius incrassatus* (OI, scale bar = 5 mm); (**h**) *N. incrassatus* (SEM, scale bar = 500 µm); (**i**) *N. incrassatus*, shell surface with spiral bands and canyons (scale bar = 250 µm).

Some adnate taxa, colonizing shells with complex morphological structures, slid into the canyons and formed endolithic structures, which eventually became integrated into the shell surface. This peculiar behavior might represent a strategy to avoid grazers as well as competition for space with other diatom species. Erect diatoms, in turn, could only hardly cope with water movements (e.g., by means of elastic fluctuations of their peduncle), because their adhesion to the shell was partial. Finally, the almost absolute absence of motile species in nearly all the shells under analysis should be due to the scarce capability of this substrate to keep them safe from water turbulence and mechanical stress provoked by streams and animal motion.

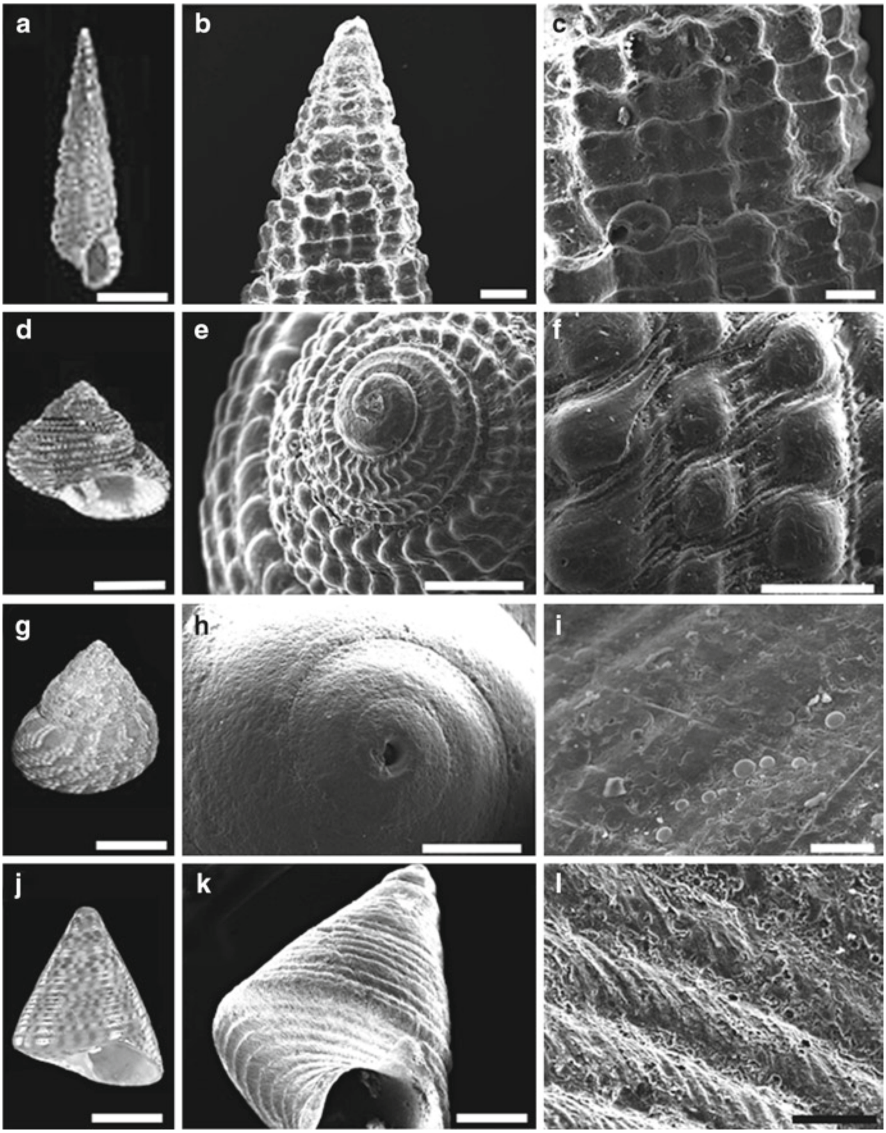

Figure 4. (**a**) *Bittium reticulatum* (OI, scale bar = 4 mm); (**b**) *B. Reticulatum* (SEM, scale bar = 300 µm); (**c**) *B. reticulatum*, shell surface with many ornamentation and a complex reticulation (SEM, scale bar = 150 µm); (**d**) *Clanculus cruciatus* (OM, scale bar = 5 mm); (**e**) *C. cruciatus* (SEM, scale bar = 600 µm); (**f**) *C. cruciatus*, shell surface with small canyons among granulated swirls (scale bar = 250 µm); (**g**) *Gibbula adansoni* (OI, scale bar = 6 mm); (**h**) *G. adansoni* (SEM, scale bar = 700 µm); (**i**) *G. adansoni*, shell surface with coarse spiral bands and few canyons (scale bar = 100 µm); (*J*) *Jujubinus striatus* (OI, scale bar = 5 mm); (**k**) *J. striatus* (SEM, scale bar = 1 mm); (**l**) *J. striatus*, shell surface with narrows swirls (scale bar = 100 µm).

Table 1. Combined characteristics of gastropods and relating epibionthic diatom communities. Gastropods having different gross shell morphology, dimension, and ornamentation are ranked according to their fractal dimension (*D*), from the highest to the lowest value. The diatom communities are characterized by the overall cell abundance (A, cells per square millimeter), the species richness (S), and community evenness (P).

Gastropods	**Gross shell morphology**	**Shell dimension (mm)**	**Ornamentation**	**(*D*)**	**(*A*) (c·mm^{-2})**	**(*S*)**	**(*J*)**
A. lineata	Globular/ tower-like	4	Spiral rounds with radial swellings	1.653	448	7	0.30
Co. rustica	Globular	17	None	1.653	829	13	0.32
N. incrassatus	Globular	15	Radial protuberances and spiral bands	1.650	531	17	0.60
B. reticulatum	Elongated	13	Radial swellings and bands	1.563	1,467	9	0.61
C. cruciatus	Spinning top-shaped	10	Spiral rounds and granulated bands	1.557	684	25	0.80
G. adansoni	Spinning top-shaped	11	Few rounds, smooth	1.551	1,323	25	0.72
J. striatus	Spinning top-shaped	10	Many spiral rounds	1.460	350	16	0.70

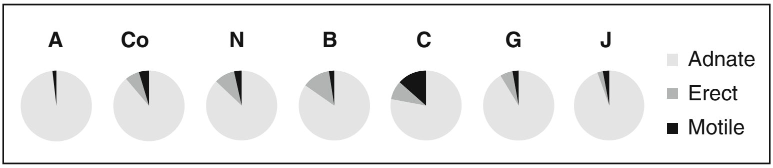

Figure 5. Distribution of the main diatom growth forms (adnate, erect, and motile) above each gastropod shell (indicated by the bold capital letter above each pie: A = *Alvania lineata*, Co = *Columbella rustica*, N = *Nassarius incrassatus*, B = *Bittium reticulatum*, C = *Clanculus cruciatus*, G = *Gibbula adansoni*, J = *Jujubinus striatus*).

The adnate genus *Amphora* (biraphid) and *Cocconeis* (monoraphid) were the primary component of shell-diatom communities. Another component more differentiated in terms of genera, which may be defined as secondary, included erect genera, mainly *Tabularia*, *Grammatophora*, *Rhabdonema,* and *Licmophora*. A fraction of motile diatoms, rarely becoming numerically important, was represented by the genera *Navicula, Diploneis,* and *Nitzschia*. Moreover, the presence of conspicuous bacterial and fungal mats competed against diatoms for space. Thus, the peculiar substrate provided by gastropods could have fostered the dominance of adnate against erect and motile species (Fig. 5).

Alvania lineata, *Columbella rustica*, and *Nassarius incrassatus* showed a globular and heavy shell with few ornamentations (Fig. 3a–i), such as coarse spiral bands (Fig. 3b, c, h, i) and calcareous incrustations (Fig. 3f), and they all hosted a community prevalently made by adnate species (Fig. 6). Those shells were also differentially colonized. While the apical portion of *A. lineata* was poorly colonized, the central and inferior sides showed a uniform distribution of *Amphora* cf. *helenensis* (Fig. 6a–c). The central portion of *Co. rustica* showed scales and rich assemblages of adnate (dominated by *Amphora* cf. *helenensis*) and some erect taxa accumulated among adjacent scales (Fig. 6d–f). Finally, *Amphora* cf. *helenensis* dominated in the apex, while *Cocconeis* spp. formed the central portion of *N. incrassatus* (Fig. 6h, i).

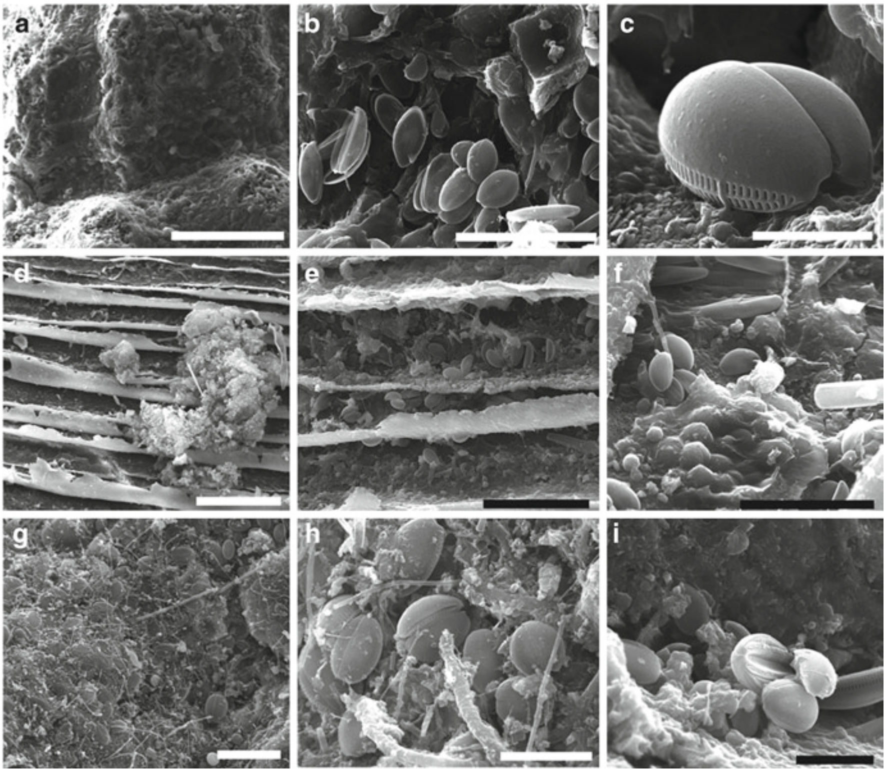

Figure 6. SEM pictures of *Alvania lineata* (**a**–**c**), *Columbella rustica* (**d**–**f**), and *Nassarius incrassatus* (**g**–**i**) at different magnifications. (**a**) shell surface with few ornamentation (scale bar = 150 µm); (**b**) microcommunity of adnate dominated by *Amphora* cf. *helenensis* (scale bar = 20 µm); (**c**) two cells of *Amphora* cf. *helenensis* (scale bar = 10 µm); (**d**) shell surface with scales (scale bar = 150 µm); (**e**) a rich assemblage of adnate and some erect taxa among adjacent scales (scale bar = 50 µm); (**f**) cells of *Amphora* cf. *helenensis* (scale bar = 25 µm); (**g**) shell surface with small canyon (scale bar = 30 µm); (**h**) microcommunity of adnates (scale bar = 10 µm); (I) cells of *Amphora* cf. *helenensis* (scale bar = 10 µm).

Bittium reticulatum and *Clanculus cruciatus* showed many ornamentations in their shells, which created microhabitats that favored the adhesion of several adnate species, some erects, and the movement of motile taxa (Fig. 4a–f). *Amphora* cf. *helenensis* and *Cocconeis scutellum* effectively colonized *B. reticulatum,* while other *Cocconeis* spp. grew inside the shells forming a rich endolithic community (Fig. 7a–c). A copious diatom community, made up of adnate (dominant), erects, and motile, developed on *C. cruciatus* where many small cells (e.g., *C. neothumensis*) slid into small canyons among granulated swirls (Fig. 7e, f). Moreover, many motile species found protection within little and narrow valleys where they could keep moving in and reproduce.

Gibbula adansoni showed a highly organized shell which was, however, rather smooth at the spatial scale of the cells (Fig. 4g–i). Only a small number of ornamentations and coarse spiral bands with few canyons were present and the central portion of the shell showed some grooves hosting rich assemblages of *Cocconeis* spp. (Fig. 7g–i). However, the remnant portion of the shell showed a scattered colonization, with single cells or small groups of two or three specimens (e.g., *Amphora* cf. *helenensis*).

Jujubinus striatus shell showed dense, homogenously distributed, and narrow swirls. However, though describing a regular and highly organized landscape, it lacked a complex reticulation at the scale of the cells (Fig. 4j–l). The diatom community, dominated by adnate species, was not copious and cells were single or in small groups of two or three (Fig. 7j, k). Among adnate, *Cocconeis peltoides* Hustedt and *Cocconeis distans* (Gregory) Grunow (which are also epipsammic species) were dominant (Fig. 7l).

2.1.1. *How Substrate Complexity Influences Community Structure*

One would expect that gastropod shells should select for diatom communities based on their size, with smaller shells hosting extremely simple communities, both in terms of absolute abundance and species composition, and large shells hosting more structured communities. In fact, in our research, *Bittium reticulatum*, *Gibbula adansoni*, *Columbella rustica,* and *Clanculus cruciatus* hosted a high quantity of epizoic diatoms, while the smallest shells, *Jujubinus striatus*, *Alvania lineate,* and *Nassarius incrassatus*, hosted a lower quantity. However, shells with a similar size, such as *B. reticulatum* and *J. striatus*, hosted far different diatom communities: *B. reticulatum* showed the highest abundance with a low diversity, whereas *J. striatus* showed the lowest abundance, but high species richness. Moreover, *Gibbula adansoni*, *Clanculus cruciatus,* and *Jujubinus striatus* hosted the highest specific diversity, even though they had different dimension and ornamentations (Table 1). In addition, while the specific composition did not vary significantly in different shells, the absolute and relative abundances of diatom community were more variable (Table 1). The Pielou index of evenness (J, a number ranging 0, for a community dominated by only one species, and 1, for a richer community where all species are equally represented; Pielou, 1966) varied significantly among the investigated shells (Table 1). From the comparison of J and D in different

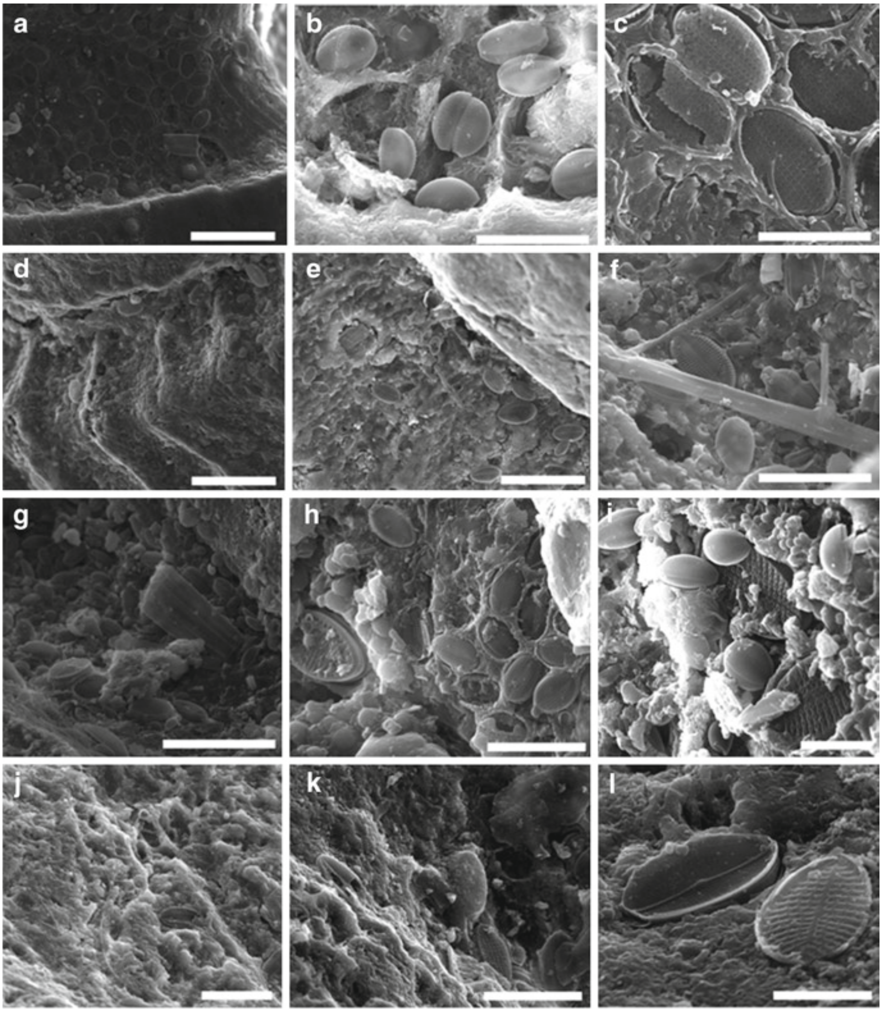

Figure 7. SEM pictures of *Bittium reticulatum* (**a**)–(**d**), *Clanculus cruciatus* (**e**) and (**f**), *Gibbula adansoni* (**g**)–(**i**), and *Jujubinus striatus* at different magnifications (**j**)–(**l**). (A) Shell surface with deep canyons (scale bar = 150 μm); (**b**) assemblage of *Amphora* cf. *helenensis* (scale bar = 60 μm); (**c**) endolithic communities of *Cocconeis scutellum* (scale bar = 30 μm); (D) small canyons among granulated swirls (scale bar = 120 μm); (**e**) diatom communities dominated by adnate (scale bar = 40 μm); (**f**) few cells of *Cocconeis* spp. (scale bar = 30 μm); (**g**) shell surface with a small number of ornamentation (scale bar = 60 μm); (**h**) endolithic community of *Cocconeis* spp. (scale bar = 40 μm); (**i**) small group of *Amphora* cf. *helenensis* and a couple of endolithic cells in the background (scale bar: 10 μm); (**j**) shell surface with few ornamentation (scale bar = 90 μm); (**k**) small group of two or three cells (scale bar = 30 μm); (L) two cells of *Cocconeis* spp. (scale bar = 15 μm).

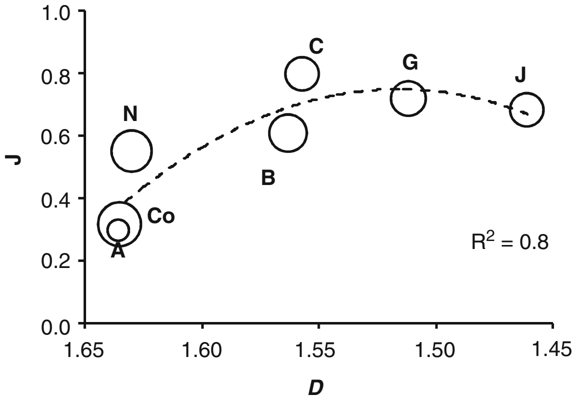

Figure 8. A comparison between the evenness (*J*) of diatom communities on gastropods shells (capital letters, as in Fig. 5) and shell fractal dimension (*D*). The dimension of each ball represents the individual shell size. The scale of the *y*-axis is inverted for graphical reasons.

shells, an interesting pattern emerged: the diatom community structure in different shells seems to be influenced by the complexity of the substrate. The more organized is the gastropod shell (i.e., the lower the *D*), the more complex (in terms of species richness and biodiversity indexes) is the diatom community. For instance, the Pielou index well correlates with the shell fractal dimension, with the more irregular shells having a low evenness, and the latter parameter increasing with the increasing substrate complexity (Fig. 8).

The diatom community encrusting the irregular and discontinuous substrate provided by *A. lineata* was composed by only seven species (*S* = 7, Table 1), four of which were adnate, two motile, and only one erect; in such shells, adnate cells occurred in large majority (90% of the total cells, Fig. 5) and *J* for this community was close to 0 (Table 1). An analogous community (*J* = 0.32) – made by 13 species, among which 6 were adnate, 3 erect, and 4 motile-colonized *Co. rustica*, which also showed a chaotic shell arrangement. As an exception, *Nassarius incrassatus* and *B. reticulatum* showed similar evenness (*J* approaching 0.60), though having far different shell-fractal dimensions (1.65 vs. 1.56, respectively); also, both the shells' diatom communities were dominated by adnate taxa (11 out of 17 in *N. incrassatus* and 7 out of 9 in *B. reticulatum*). On the contrary, well-structured diatom communities colonized the more organized shells of *C. cruciatus*, *G. adansoni,* and *J. striatus.* These three shells showed the higher specific diversity (*S* = 25, 25, and 19, respectively) and evenness (*J* = 0.80, 0.72, and 0.70, respectively). Adnate taxa were dominant in all the three (57%, 65%, and 68% of the total species, respectively), but erect and motile together accounted for 43% of the total species in *C. cruciatus*, 35% in *G. adansoni*, and 22% in *J. striatus*. Moreover, *C. cruciatus* hosted a large amount of motile cells (Fig. 5), which might successfully exploit the fine-arranged microenvironment provided by this shell and spread into it.

In comparison with other animal-derived microenvironments, and based on the scarce literature available, diatom communities colonizing vagile mollusks, such as gastropods, seem to be different from those developing on sponges, hydrozoans, and crustaceans. In particular, gastropod shells and hydroid colonies, for which a comparable amount of data has been collected, host different diatom communities. The global dominance of adnate taxa in any microenvironment available on gastropod shells contrasts with the reliable spatial segregation of adnate and erect taxa detected in hydroids, with the first dominant on the basal and central side of the host, and the second, on the apical side, probably due to a combined effect of differential grazing and hydrodynamic conditions (Romagnoli et al., 2006).

The data included in this section, though preliminary, clearly suggest that, besides the overall dimension, the gastropod shell morphology represents a stronger constraint in the developing and structuring of epizoic diatom communities. Adnate taxa are largely dominant in those substrates, while erect and motile forms occur generally with low abundances, probably due to the effect of particular hydrodynamic conditions (such as microturbulence) that may limit the colonization of the taxa having reduced adhesion surface (erect) or being partially detached from the substrate (motile). Adnate taxa, in turn, can strongly adhere to the substrate – and sometimes “inside” the substrate in a sort of endolithic behavior – and grow in number within shell ravines.

3. Chapter’s Conclusions

In general terms, community complexity emerges from a combination of differential environmental conditions on substrates and the capability of organisms to adapt to them: the more complex the substrate, the more complex the dynamics of colonization. As a cascade effect, the more diverse the way organisms stabilize into an environment, the higher the biodiversity and the evenness of colonizing communities.

The chapter shed light upon animal–plants interaction at the microscale. Our final conclusion is that microscopic “plants” like diatoms can successfully exploit animal-derived microenvironments to grow and build different kinds of communities whose organization is strongly selected by the organization of the substrate itself.

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MANAGING THE INTERACTIONS BETWEEN PLANTS AND ANIMALS IN MARINE MULTI-TROPHIC AQUACULTURE

Integrated Shrimp and Valuable Low Food Chain Organisms with Seaweeds

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1. Introduction

China has long been known for manure-based integrated multiple species fish farming technology. Commonly known as "polyculture," this traditional Chinese system is practiced in pond aquaculture where several fish species are reared together, creating a multi-output production structure. For example, polyculture of carps with animal and plant husbandry is based on the efficient utilization of organic manures in the ponds. Typically, the manures are supplied from animal and poultry houses built directly over the ponds at the edge, or from large numbers of ducks allowed to swim freely on the ponds. All these practices have been common in China for centuries and have long since been adopted by other countries in Asia and Central Europe. In Israel and several Central Europe countries, cattle and pig manures and litter from poultry houses are also transported from external animal houses and spread in the fish ponds (Billard, 1995).

Adaptations of this principle to the treatment of intensive marine aquaculture effluents in both inland and coastal areas was proposed, and quickly followed by the integration to their system of carnivorous fish and the macroalgivore abalone (Tenore, 1976; Robertson-Andersson et al., 2008). The inception of the modern concept of integrated aquaculture has been the work of Ryther et al. (1975), who approached the integrated use of extractive organisms – shellfish, microalgae, and seaweeds – in the treatment of household effluents. They described the concept and provided quantitative experimental results of integrated waste-recycling marine aquaculture systems. A domestic wastewater effluent, mixed with seawater, was the source of nutrients for phytoplankton culture, which in turn was fed to oysters and clams. Other organisms were cultured in a separate food chain, based on the organic sludge of the farm. Dissolved remnants of nutrients in the final effluent were filtered by seaweed (mainly *Gracilaria* and *Ulva*) biofilters. If properly selected and placed, co-cultured species will have accelerated growth from the uptake of extra nutrients provided by the fed culture species. Typically, the fed culture species (i.e., upper trophic level) in these systems have been carnivorous fishes, whose wastes augment the natural food supply or nutrient uptake of co-cultured extractive species. Fish excrete soluble ammonia and phosphorus (orthophosphate), which are inorganic nutrients readily available to inorganic extractive species such as seaweeds. Fish also release organic solids which can become food for shellfish and deposit feeders, the organic extractive species (Troell et al., 2003). Not all supplemental nutrients flow directly from the waste by-products of the fed species. For example, some ammonia may be generated by organic extractive species (e.g., shellfish) and also extracted by seaweeds. Waste feed may also be a source of additional nutrients; either directly available for consumption by organic extractive species (e.g., deposit feeders) or from the release of soluble nutrients by decomposition, for inorganic extractive species. This increases the overall environmental assimilative capacity of a site, thereby reducing the potential for negative environmental impacts.

2. Aquaculture in the Tropics

In tropical and subtropical coastal areas worldwide, no economical activity has evolved as quickly as shrimp farming in the last 15 years. World's shrimp production from aquaculture was estimated at 855,500 t in 2001, and such an enormous development has been accompanied by strong controversies on the environmental, economic, and social impacts of shrimp farming. In Mexico, production area and total shrimp harvest from aquaculture have been increasing every year, with a record 47,450 t obtained in the year 2001 (SAGARPA, 2002). Total production and number of farms are expected to continue to grow, responding to demands from local and export markets, primarily in the United States. Mexico, as well as several nations from Asia and Latin America, has experienced an increased expansion that concerns governmental and nongovernmental organizations (Páez-Osuna, 2001).

The shrimp-farming industry is generally perceived as an activity that negatively impacts the environment. By-products and wastes in water outflows are discharged to farms' surrounding water bodies or land-producing diverse environmental impacts depending on several factors: (a) location of farms; (b) management and use of technology during pond operation; (c) culture surface and scale of production; and (d) depurative capacity of receiving water body.

2.1. ENVIRONMENTAL IMPACTS OF SHRIMP FARMING

Historically, massive mortality and low growth problems in shrimp farming in certain regions of Asia and America seemed to be related to deteriorated water quality (Qingyin et al., 1997). Several authors recognized that the concentration of aquaculture facilities and the destruction of mangrove were aggravating factors (Phillips et al., 1993; Primavera, 1998). The direct discharge of waste nutrients from shrimp farms into adjacent waters has raised global concerns regarding adverse environmental impacts from such practices (Naylor et al., 2000). In addition to environmental issues, the discharge of untreated pond effluents represents an economic loss of costly nutrients, thereby reducing farm profitability (Smith et al., 2002).

To give an idea of nutrient enrichment produced by shrimp farming, Bergheim et al. (1993) found that intensive shrimp farms produce a total waste equivalent to 73–85 people for N and 101–161 people for P per ton of shrimp, while land-based marine salmon farms produced wastes equivalent to 17 people for N and 12 people for P per ton of fish. Hopkins et al. (1993) suggests that typical water exchange rates used in intensive shrimp farms (25% day^{-1}) may be drastically reduced (2.5% day^{-1}) resulting in a cost savings to farms and reduced potential for environmental impact from the effluent. However, by doing so, total ammonia, N, and total suspended solids will be only reduced from 92.6 to 42 kg ha^{-1} crop and 11,289 to 6,662 kg ha^{-1} crop, respectively, while reactive orthophosphate will increase from 38.6 to 40.9 kg ha^{-1} crop.

The evolution of most of the water quality variables in shrimp ponds is strongly influenced primarily by the quality of inflow water, and secondary by pond management practices. When organic wastes (unconsummated feed pellets, feces, etc.) accumulated in the sediment are degraded, ammonia, nitrate, and nitrite are formed (Martin et al., 1998). Furthermore, ammonia is released in the water through the excretion of the shrimp being the major nitrogenous compound excreted by crustacean. These nutrients support primary production and phytoplankton growth, and increase in chlorophyll *a* and particulate N occurs. Concerning the dissolved elements, the effluents show a decrease in inorganic nutrients, especially ($NO_2^- + NO_3^-$) that are used by primary producers. Meanwhile, particulate and dissolved organic matter in the sediments can be mineralized and the residues are rich in $NH_4^+ + NH_3$ that accumulate in the water column.

Several studies on nutrient mass balances in shrimp ponds have shown that the major source of nutrient input (e.g., N and P) is the formulated feed (Jackson et al., 2003). Intensive shrimp cultivation can generate ~1,500 kg total N and 400 kg total P per hectare of pond per year (Briggs and Funge-Smith, 1994). More recently, Páez-Osuna et al. (1997) found that for the 1,822 kg ha of shrimp harvested, only 35.5% and 6.1% of N and P input to the ponds were recovered as shrimp biomass, respectively. In addition, Funge-Smith and Briggs (1998) have shown that only 18–27% of N and 6–11% of carbon applied to the pond was assimilated in Thai intensive shrimp ponds. This may cause wastage of nutrients, of which 24% is in the sediments and 27% is water discharged. Thus, there is an enormous risk of eutrophication in receiving water bodies of farm effluents.

2.2. INTEGRATED MULTI-TROPHIC AQUACULTURE

Simultaneous culture of several species in the same water body with the objective of optimizing the use of space and nutrients is termed as integrated aquaculture, polyculture, or co-culture. Integrated aquaculture is traditionally used in the fresh-water pond aquaculture; however, developing sustainable approaches is recently becoming one of the main goals in coastal aquaculture (Troell et al., 2003). Multi-trophic refers to the incorporation of species from different trophic or nutritional levels in the same system (Chopin, 2006). This is one potential distinction from the age-old practice of aquatic polyculture, which could simply be the co-culture of different fish species from the same trophic level. In this case, these organisms may all share the same biological and chemical processes, with few synergistic benefits, which could potentially lead to significant shifts in the ecosystem. Some traditional polyculture systems may, in fact, incorporate a greater diversity of species, occupying several niches, as extensive cultures within the same pond. The Integrated Multi-trophic Aquaculture (IMTA) refers to the more intensive cultivation of the different species in proximity of each other, but not necessarily right at the same location, connected by nutrient and energy transfer through water. The co-cultured species should be harvestable crops of commercial value. Therefore, the system should result in greater production for the overall system, based on mutual benefits to the co-cultured species and improved ecosystem health.

The use of seaweed as biofilters in marine aquaculture was proposed at the end of the 1970s. Several studies have already demonstrated that it is possible to cultivate economically valuable seaweeds using wastewaters from intensive and semi-intensive aquaculture, improving its water quality and allowing re-circulation or discharge into the sea (e.g., Buschmann et al., 1996; Jiménez del Rio et al., 1996; Troell et al., 1997; Neori et al., 1998; Neori and Shpigel, 1999). There are relatively few studies investigating the feasibility or application of integrated cultures of seaweeds and shrimps (Lin et al., 1993; Enander and Hasselstrom, 1994; Phang et al., 1996; Primavera, 1998; Jones et al., 2001), although this approach has been regarded as promising (Primavera, 1998; Nelson et al., 2001).

3. Integrated Multi-trophic Aquaculture: *Gracilaria corneal Farfantepenaeus brasiliensis*

The biological nutrient removal of shrimp effluent by the economically important red seaweed *Gracilaria cornea* as an alternative management strategy to reduce nutrient wastes from shrimp farming has been studied in tropical coastal areas. This proposal incorporates other valuable low food chain organisms (e.g., sea cucumber) to such systems. Most existing IMTA studies have been developed for fish farms and tested in temperate waters (see review by Troell et al., 2003). The rapid development of tropical coastal aquaculture, particularly shrimp farming, requires the urgent adaptation of such systems using tropical species. Commercial modern marine and brackish water aquaculture is usually practiced as large monocultures. This is also the case for the commercial shrimp cultivation worldwide. Although previous studies have showed enhanced growth of seaweeds and shrimp in co-culture (Chiang, 1981, Shan and Wang, 1985; Wei, 1990), integrated aquaculture of shrimp and seaweed requires fine tuning because it is highly site-specific (e.g., latitude, climate) and species-specific (e.g., strains). In order to arrive at the optimal system, experimental research and developments are still needed. As a first step in this direction, we have tested the applicability of the economically important agar producing red seaweed *Gracilaria cornea* in

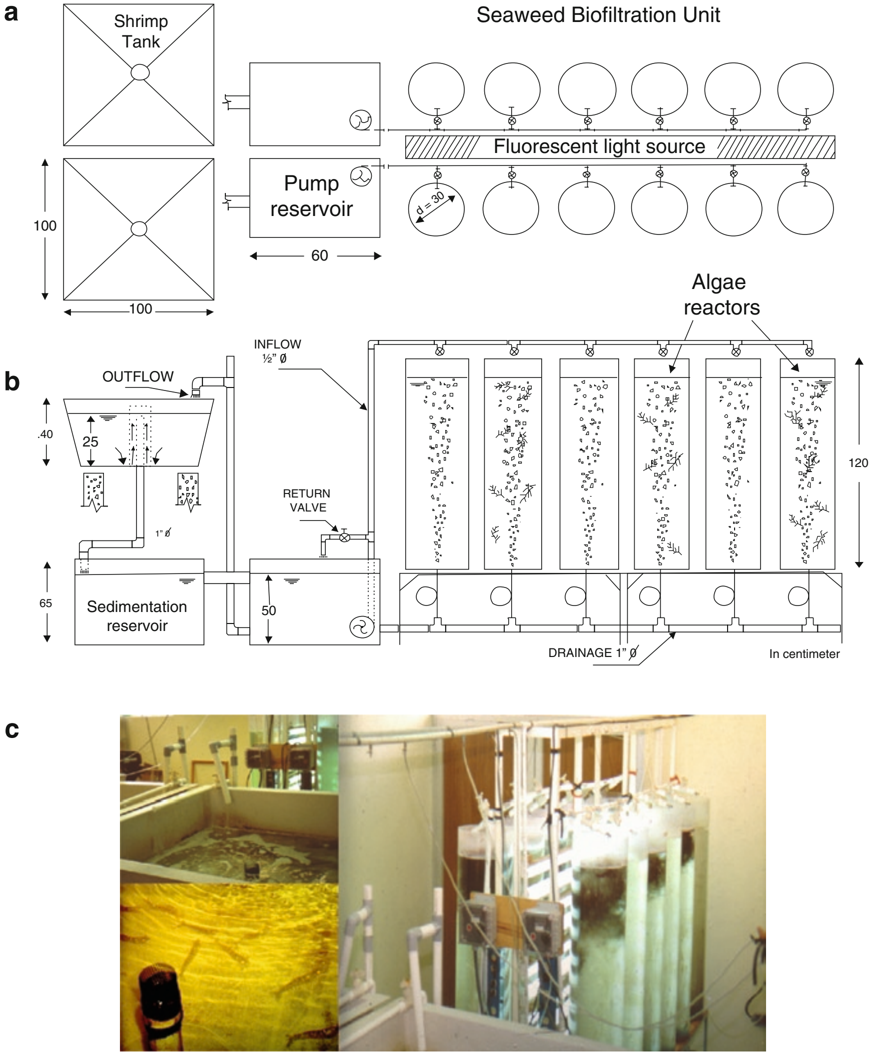

Figure 1. Integrated cultivation of shrimp with seaweed. Top (**a**) and side (**b**) view of experimental system consisting of one shrimp tank (1 m^2), sedimentation tank, and a pumping reservoir attached to six 90-l algae reactors. Photographic details of shrimp tanks and the seaweed biofilter units (**c**) kept indoors under controlled conditions 100 μmol m^{-2} s^{-1} PFD, 30°C. The effluent from shrimp tanks with an intensive cultivation density (100 individuals m^{-2}) was recirculated into seaweed biofilter unit at a seawater exchange rate of 1 volume day^{-1}.

an experimental biofilter for the cultivation of the shrimp *Farfantepenaeus brasiliensis* (Fig. 1). Different species release wastes of different quality and quantity, but generally most of the nutrients added through feed are released to the environment. Many commercially important aquaculture species release a large amount of wastes, such as bivalves at 75% and abalone at 60–75% (for both N and phosphate (PO_4^{3-})), salmon at 70–75% and shrimps at 77–94% (only for N), which were mostly added through feed to the coastal environment (Robertson and Phillips, 1995; Neori et al., 2000). Shrimps are among the less efficient converters of feed, thereby increasing the concentration of phytoplankton, bacteria, nutrients, and suspended solids.

In an aquaculture system, most of the N and P come from animal excretion and is available in the form of ammonium (NH_4^+) and PO_4^{3-}. In intensive aquaculture systems, where water is re-utilized, biological filters perform nitrification, oxidizing ammonia to form nitrate (NO_3^-) (Troell et al., 2003). Therefore, in these systems, N also becomes available in the form of NO_3^-. In Fig. 2a, we can observe the daily fluctuation of the N compounds derived from shrimp metabolic activity. Most of the N excreted by shrimps is in the form of NH_4^+ (42%) with a highest content of 47.5% during early morning (between 6 and 8 a.m.).

Excretion of shrimp at intensive cultivation densities generated an inlet ammonium concentration (NH_4-N) and DIN at seaweed biofilter of 40 and 104 μM, respectively. During an 8-week period, all experimental densities (2.5, 4, 6, and 8 g l^{-1}) received the same inorganic nitrogen load (DIN) from the shrimp tanks. Inorganic N availability, as total ammonium nitrogen (TAN), at the recirculating system (outflow) was influenced by seaweed density. In terms of removal efficiencies, the highest values were obtained at seaweed densities from 6 to 8 g l^{-1}, around 85–90% day^{-1}, respectively (Fig. 2b). Lower removal efficiencies were observed during afternoon hours, although light regime was set for continuously photoperiod (24 h).

At the exchange rates currently practiced in intensive shrimp farms (1–20 volumes day^{-1}), treatment systems for low intensity farms must be designed to remove particulate N, while treatment systems for high-intensity farms must remove both particulate and dissolved N (Lorenzen et al., 1997). Therefore, effluent treatment must primarily remove particulate N (e.g., by filter-feeding or deposit-feeding organism), but also dissolved N (e.g., seaweed) where farming intensity exceeds the pond carrying capacity. For assessment of water quality, the effects of nutrients on seaweeds may be more relevant than the instantaneous physical concentrations. Particularly relevant are the different sources of N (NH_4^+, NO_2^-/NO_3^-, or organic forms such as urea) in the water column. These forms of N are the preferred sources of these nutrients for many seaweed species (Lobban and Harrison, 1994; Troell et al., 2003). In particular, studies on *Gracilaria cornea* showed that it grows equally well on NO_3^- or NH_4^+, both forms simultaneously or even with organic N forms such as urea (Navarro-Angulo and Robledo, 1999). However, in the seaweed biofilter under experimental conditions, the preference for NH_4^+ was evident under all stocking densities, and thus being reflected in the DIN removal efficiency (Fig. 3).

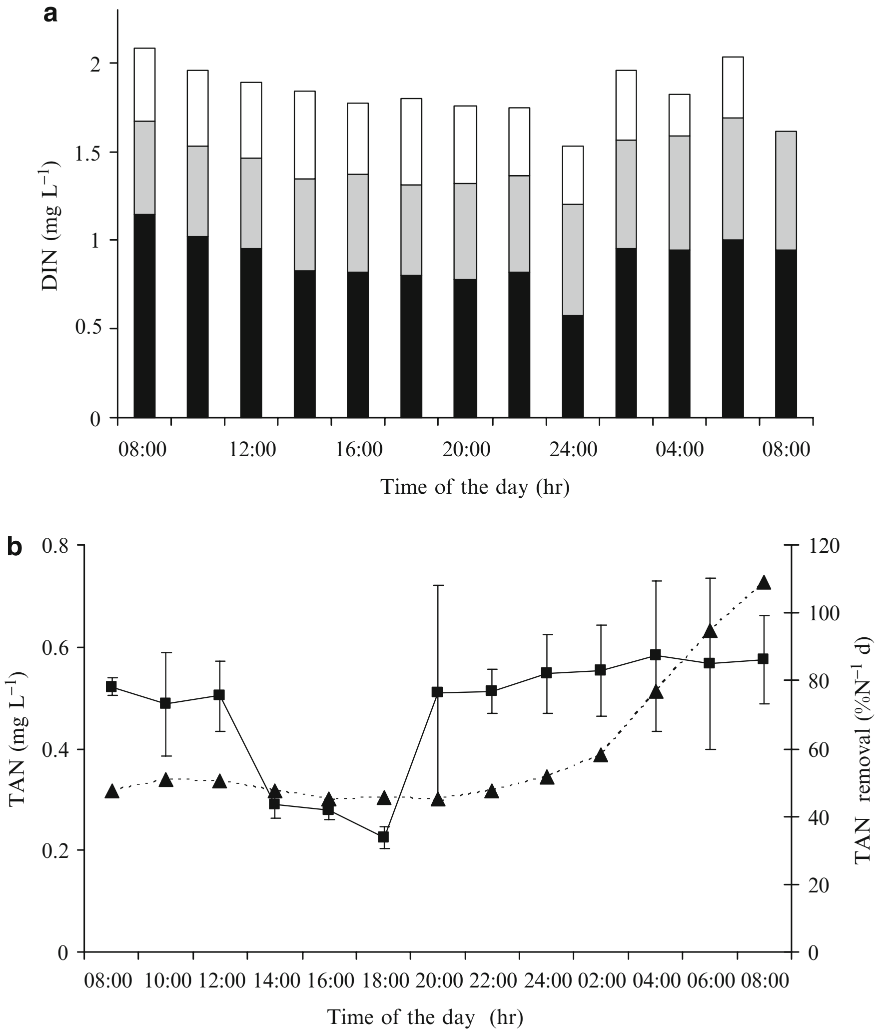

Figure 2. Experimental integrated cultivation of shrimp with seaweed. (**a**) Excretion of dissolved inorganic nitrogen (DIN = Ammonia; ■, Nitrite; and Nitrate ☐☐) by shrimp over a 24-h period. (**b**) Total ammonia nitrogen (TAN) availability in the system (--☐▲--) and TAN reduction efficiency by seaweeds (-☐■-).

Seaweed pigment, amino acids, and tissue N content have been observed by many researchers to respond to changes in the water column N availability (Horrocks et al., 1995). The amino acid composition is the least studied of these physiological characteristics, although the results from Jones (1996) indicate a strong correlation ($r^2 = 0.84$) with the concentration and source of available N. The metabolic profile

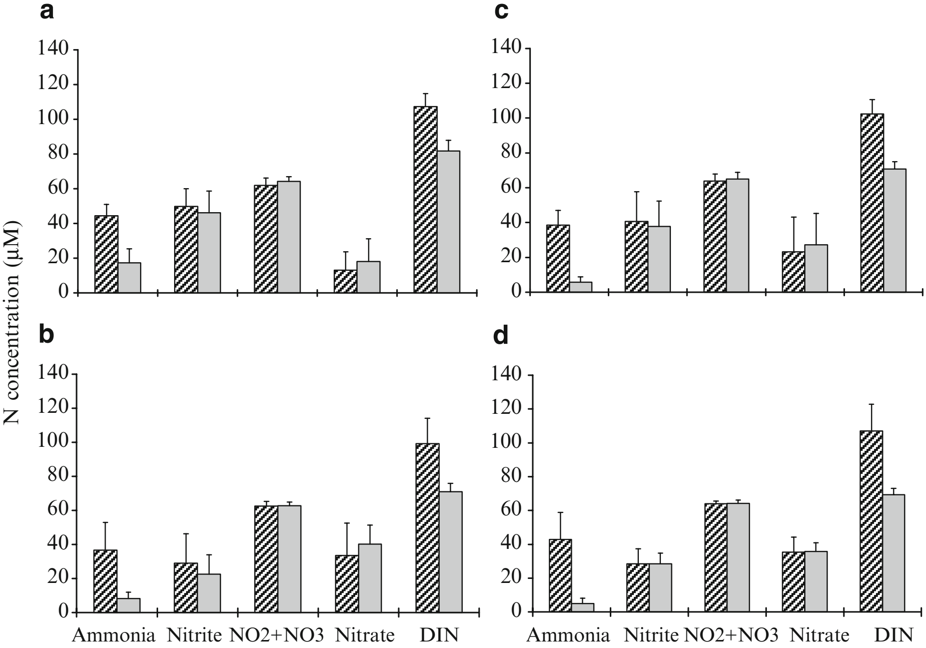

Figure 3. Algal stocking density, growth rate, biomass yield, and nitrogen uptake were evaluated for optimized bioremediation capacity of the seaweed infiltration unit. Nutrient water concentrations at inflow (*stripped bars*) and outflow (*gray bars*) of the experimental integrated cultivation of shrimp with seaweed at (**a**) 2.5, (**b**) 4, (**c**) 6, and (**d**) 8 kg l^{-1} stocking density. DIN.

of *Gracilaria cornea* grown in an experimental biofilter showed the dominant source of N being assimilated by the red algae. Seaweeds assimilate and store large concentrations of nutrients in their tissue in the form of amino acids and proteins. These N stores act as reserves for times of nutrient limitation. Therefore, if N is available at concentrations such as that other factors become limiting to growth, they will start to store these reserves in the form of free amino acids and pigments. In the seaweed biofilter, the density affected protein and carbohydrate contents; with the maximal protein levels obtained at densities of 6 g l^{-1} with values of 7.1%. In contrast, the densities of 4 g l^{-1} registered the maximal levels of carbohydrates with values of 44.8%.

The above-mentioned result exemplifies how economically important red seaweeds could be an efficient biological nutrient removal system; however, the feasibility of seaweed cultivation at the commercial level in shrimp farm effluents requires the solution of pond management practices. It has been shown that the nutritional composition of aquaculture effluents depends on various factors related to hydraulic management, oxygen, and feeding practices. Not only daily fluctuations of nutrient loads are important, but seasonality will significantly affect nutrient removal efficiencies. Low water movement and large amount of suspended particles in effluents contribute to seaweed biomass reduction due to deposition of a thin layer of silt over thalli, which eventually block the incident light, thus affecting growth performance. Moreover, various chemicals (e.g., calcium hypochlorite) currently used in shrimp aquaculture as cleaning agents could be toxic not only to animals but also to plants. In order to efficiently utilize seaweeds as biofilters for shrimp intensive aquaculture, further research toward the improvement of seaweed removal efficiency outdoors is under study. On this regard, Troell et al. (2003) made a comprehensive analysis of research needs for the IMTA using seaweed to be effectively used at commercial scale.

4. Development of Integrated Multi-trophic Aquaculture: *Gracilaria cornea*/Sea cucumber

Increased demand for bêche-de-mer and worldwide declines in stocks of tropical sea cucumbers have encouraged aquaculture, stock restoration, and enhancement programs for holothurians (Battaglene and Bell, 1999). The species can yield returns of up to US $12 per kg "green" weight if appropriately processed. Sea cucumbers are a valuable food item and dietary supplement in the People's Republic of China, Hong Kong, Taiwan, Singapore, and Korea (Conand, 2000). The high value of sea cucumber in the international fish trade – up to US $60 kg^{-1} at market for the premium species – ensures that fisheries are heavily exploited as a part of the traditional and commercial fisheries that exist in many coastal nations as it is occurring in the coastal areas of Mexico.

Deposit-feeding holothurians ingest sediment-bearing organic matter, including bacteria, protozoa, diatoms, and detritus of plants or animals (Yuan et al., 2006). On the other hand, the distribution of adults is well documented: they are associated most commonly with soft sediments in the vicinity of mangrove–seagrass habitats and lagoonal coral reef flats (Dance et al., 2003). Other Asian species preferentially inhabits the bottom in flourishing large algae, rich detritus of which provide sea cucumber with main organic nutrient (Yuan et al., 2006).

Land-based intensive culture – a new promising cultural method of this animal, however, has not been drawn much attention so far (Chang, 2003; Kang et al., 2003). One of the problems encountered in this cultural method is that there is no proper diet. In the practice of hatchery-produced juveniles, newly settled larvae were commonly fed with diatoms, and then in nursery tanks, powdered algae were added for holothurian juveniles (Battaglene et al., 1999). Recent studies have also shown that the food residue and feces of marine animals, and even sea cucumber's own feces, in which an increase in suitable bacteria has occurred, are also important to nutrients of sea cucumbers (Yang et al., 2001; Kang et al., 2003).

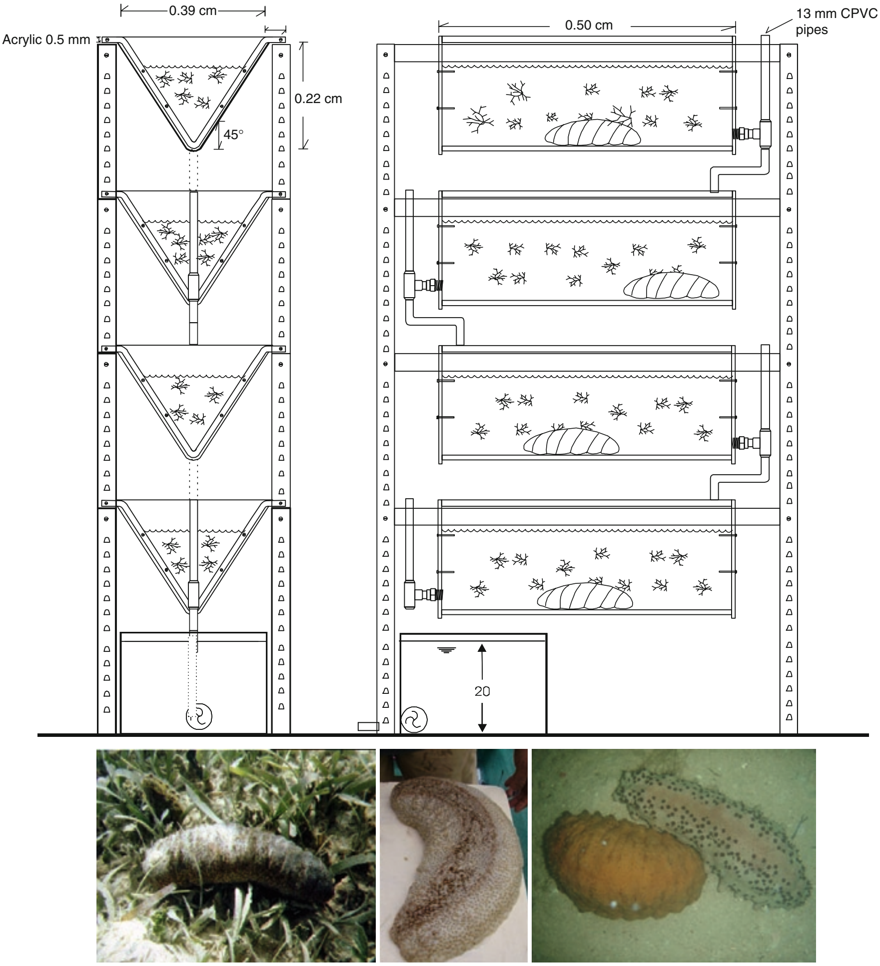

Figure 4. Details of the experimental sea cucumber-seaweed biofiltration unit, using effluents from shrimp-fish cultivation. Valuable tropical sea cucumber species under study (*from left to right*). *Holothuria mexicana, Astichopus multifidus, and Isostichopus badionotus.*

Following the concept of integrated multi-trophic aquaculture, we are presently testing a system to evaluate a new component into the shrimp-seaweed biofilter unit, introducing local holothurians species (Fig. 4). The integration of these organisms will improve the assimilation of wastes, reducing the discharges to the receiving environment, while at the same time, producing valuable aquatic species that can be marketed.

Bivalves, such as mussel, oyster, scallop, etc., are active filter-feeders. They possess highly efficient filtering mechanisms that enable them to concentrate a large amount of phytoplankton and other suspended particulate matter from the pelagic system and reject undigested organic and inorganic material as feces and pseudofeces. Yang et al. (2001) demonstrated that the sea cucumber *Apostichopus japonicus* could utilize feces and pseudofeces of scallop *Chlamys farreri* and grow well in simulated polyculture system. Recently, Zhou et al. (2006) found that the same species could be co-cultured and grown well with bivalves in suspended lantern nets.

The ability that sea cucumbers have to use various kinds of organic materials from sediments makes them very appealing for polyculture systems. Previous studies have showed that sea cucumbers can be co-cultured with abalone, and observed that the sea cucumber *Stichopus japonicus*, co-cultured with the abalone *Haliotis discus hannai* in a static culture system, could improve water quality by ingesting food residue and feces (Kang et al., 2003). Ahlgren (1998) found the red sea cucumber *Parastichopus californicus* co-cultured in salmon net pens could convert the net from self-fouling to self-cleaning and could turn fouling debris (fish feces, excess fish food, phytoplankton, and other particulate organic matter) into a marketable product (sea cucumber biomass). In addition, sea cucumbers, e.g., *S. japonicus* and *Holothuria scabra*, can also be co-cultured with shrimp in ponds (Pitt et al., 2004). Sea cucumbers also survived well when reared with juvenile *Litopenaeus stylirostris,* but their growth was significantly slower due to increased levels of ammonia from shrimp (Purcell et al., 2006). These studies provided evidence that sea cucumbers are an efficient deposit feeder and consequently can be a good co-culture candidate with other marine aquaculture species.

Existence of cultured animals at different trophic levels can be the basis of environment-friendly polyculture systems (Neori et al., 2004). The introduction of sea cucumber together with seaweeds could reduce the inorganic wastes while improving the performance of shrimp-sea cucumber polyculture. Culturing organisms at different trophic levels with two or more ecologically compatible species can reduce the impact of aquaculture activities. The integration of fed aquaculture with extractive organisms (seaweeds and deposit-feeding invertebrates) can ensure long-term industry sustainability. Seaweeds can remove a great deal (up to 90%) of inorganic N excreted from shrimp, and bottom-feeder invertebrates, such as sea cucumber, can reduce organic matter converting wastes into a biomass, which can be removed and managed as a valuable byproduct resulting in both environmental and economic advantages (Chopin et al., 2001).

5. Conclusions

A more holistic assessment of plant–animal interactions constitutes an option for mitigation of adverse environmental impacts of intensive animal farming systems. Concerns about adverse environmental impacts due to discharging effluent from aquaculture ponds directly into adjacent waterways have prompted the search for cost-effective methods of effluent treatment. Under these circumstances, basic and applied research can help promote:

1. Less polluting production systems and efficient waste management
2. Production systems that can deliver environmental goods while supporting economically viable animal production
3. Integration of underutilized resources to improve efficiency of such production systems

One method of reducing the level of these components is to flow the effluent through treatment ponds of raceways stocked with seaweeds, which act as natural biofilters of soluble inorganic (and organic) nutrients. In addition to improving effluent water quality prior to discharge, the use of natural biofilters provides a method for capturing otherwise wasted nutrients. For a balanced ecosystem approach, extractive aquaculture should be integrated with fed aquaculture as an innovative aquaculture development. The IMTA represents a clear case of mutual benefits for the co-cultured organisms. Production of fish or crustaceans generates particulate organic waste (fecal material and uneaten food) that can be utilized by sea cucumber (deposit feeders) and soluble inorganic excretory waste that seaweeds utilize efficiently. Economic diversification in aquaculture could integrate other high-valuable low-chain organisms, such as sea cucumber, which could be integrated into the system to provide additional income and serve as natural scrubbing for organic deposition.

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MARINE MICROALGAE/CYANOBACTERIA–INVERTEBRATE SYMBIOSIS

Trading Energy for Strategic Material

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1. Introduction

Photosymbiotic associations between unicellular algae, cyanobacteria, and invertebrates such as corals, sea anemones, bivalves, sponges, foraminiferans, flatworms, and hydra, are found in seawater and freshwater (Muscatine, 1971; review by Trench, 1992; Stat et al., 2006; Venn et al., 2008; Table 1). The most common and prominent association is the exclusive marine symbiosis of coelenterates with zooxanthellae that are located in vacuoles (symbiosomes), usually within the host's endoderm cells, while in other hosts, such as ascidians and tridacnids, the photosymbionts are extracellular (Trench, 1987; Yellowlees et al., 2008).

The symbiotic algae are named according to their pigment color: (1) Zooxanthellae for yellow-brown algae (Brandt, 1882, 1883). They are taxonomic, belonging to the Chromophyta, which includes diatoms (= Bacillariophyta) and dinoflagellata. From the latter, they mainly belong to the genus *Symbiodinium* sp. Phylogenetic classification of the genus *Symbiodinium* contains several lineages: clade A in cnidarian and molluscan hosts; clade B in cnidarian; clade C in cnidarian, molluscan, foraminifera, and others; clade D in coral sponge foraminifera; clade E found in one sea anemone species; clade F in foraminifera and, rarely, in coral; clade G in foraminifera, sponges, octocorals, and scleractinian corals; and clade H, found only in foraminifera (Rowan and Powers, 1991; Carlos et al., 1999; Pochon and Pawlowski, 2006 (also as review)). (2) The green ones, known as zoochlorellae, belong to the chlorophytes found in sea anemones, sponges, nudibranchs, and hydra. (3) The blue-green algae, which are the cyanobacteria named zoocyanellae, are found in sponges, foraminifera, and nudibranchs (Table 1). *Prochloron sp*. is an obligate symbiont in didemnid ascidians (Munchhoff et al., 2007), and is also found in association with nudibranchs.

The genetic diversity within photosymbioses, specifically in *Symbiodinium*, is a part of the adaptation of the symbionts to the environment and seems likely to correlate to the diverse range of physiological properties in the host–symbiont

Table 1. The invertebrates, their photosymbionts, their habitat, the type of photosynthetic export products from the photosymbionts to the host, and the energy support by the photosymbiont to the host.

Host organisms	Habitat	Symbionts	Photosynthetic export products	Evidence for energy support
Cnidaria Hard and soft corals (scleractinia and octocoral)	Seawater	Zooxanthellae (*Symbiodinium*, clades A, B, C, D, and F (rare)	Glycerol, glucose, amino acids, lipids, C4 acids, organic acid, lipids (review by Venn et al., 2008; Yellowlees et al., 2008)	Up to 90% is translocated. Photosynthesis is more than the respiration needed by the whole colony (Davies, 1984; Muscatine et al., 1984; Falkowski et al., 1984). About 40% of the photosynthesis might be lost as mucus (Crossland et al., 1980 and many others)
Cnidaria Hydrocoral *Millepora*	Seawater	Zooxanthellae (*Symbiodinium*, *Amphidinium*)		Translocation of 75%. Less than 2% excretion as DOC (Schonwald et al., 1997)
Hydroid *Myrionema amboinense*	Seawater	Zooxanthellae		Zooxanthella translocation rates is 92% in shade-adapted and 88% at high-light adapted coral (Fitt and Cook, 2001)
Cnidaria Sea anemones	Seawater	Zooxanthellae (*Symbiodinium*, clades A, B, C, E) and zoochlorellae	Glycerol, glucose, organic acid, amino acids, lipids (review by Venn et al., 2008; Yellowlees et al., 2008)	9–412% depend on the sea anemone (review by Muller-Parker and Davy, 2001)
Cnidaria Hydrozoans	Seawater	Zooxanthellae (*Scrippsiella*, *Gloeodinium*)	Glycerol, glucose, alanine (review by Venn et al., 2008)	Banaszak et al., 1993
Cnidaria Medusa (jellyfish)	Seawater	Zooxanthellae	Glycerol, glucose, alanine (review by Venn et al., 2008)	A total of 5–10% net algal photosynthate appears to be released *in vivo* to the host (Hofmann and Kremer, 1981)
Zoanthids	Seawater	Zooxanthellae (*Symbiodinium*)	Glycerol, organic acid, amino acid, sugars (Trench, 1971; Venn et al., 2008)	

Cnidaria Hydra *Hydra viridis*	Fresh water	Zoochlorellae (*Chlorella*)	Maltose (review by Venn et al., 2008; Yellowlees et al., 2008)	
Bivalves *Tridacnid*	Seawater	Zooxanthellae (*Symbiodinum*)	Glucose, glycerol, fatty acid (review by Venn et al., 2008; Yellowlees et al., 2008)	CZAR mean of 82% (Fisher et al., 1985). Depending on the *Tridacna* species, CZAR of 151 or 186% (Jantzen et al., 2008)
Bivalves *Anodonta*	Fresh water	Zoochlorellae (*Chlorella*)		
Mollusca Nudibranch	Seawater	Rhodophyceae *Grifithsis* (in *Sacoglossa*) Zoocyanellae	Glucose, amino acid (review by Venn et al., 2008)	
Portifera Sponges	Seawater	Zoocyanellae Zooxanthellae dinoflagellates (*Symbiodinium* clades D, G), chlorophytes and diatoms rhodophytes, and cryptomonads (review in Wilkinson, 1992; Taylor et al., 2007; Usher, 2008)	Glycerol (Wilkinson, 1992)	50% (Wilkinson, 1983, 1992)
Portifera Sponges	Fresh water	Zoochlorellae (review in Wilkinson, 1992; Taylor et al., 2007; Usher, 2008)	Glycerol, glucose, (review by Venn et al., 2008)	
Ascidians	Seawater	Zoocyanellae Prochloron	Glycolate (review by Venn et al., 2008; Yellowlees et al., 2008)	
Flatworms *Convoluta roscoffensis*	Seawater	Zoochlorellae (*Platymonas convolutae*)	Amino acid, alanine (review by Venn et al., 2008)	Muscatine et al., 1974
Foraminifera	Seawater	Chloroplasts, zooxanthellae, zoochlorellae and zoocyanellae (clades C, D, F, G, and H), and rhodophyceae (Lee, 2006; Pochon and Pawlowski, 2006)		
Radiolarian	Seawater	Zooxanthellae (*Symbiodinium*)		Swanberg and Anderson, 1985

assemblages (Stat et al., 2008). The host can acquire its symbionts either from its parents, such as in the case of asexual fragmentation reproduction, or from the surrounding environment. When the symbionts are transferred directly from the host to offspring, the process is known as vertical, or closed-system, transmission. However, in most species, including coral colonies that are broadcast spawners, scyphozoans, and tridacnid bivalves, each generation must acquire new zooxanthellae from the surrounding seawater or, in rare cases, from a secondary host, in a process called open-system or horizontal transmission (see Karako et al., 2002; Barneah et al., 2007a, b; Huang et al., 2008).

The morphology of the animal host, specifically in the case of cnidaria and porifera, contributes to the distribution of symbionts in their tissues. The large surface area to volume and the only two layers of tissue (ectoderm and endoderm) allow the symbionts to capture maximum light (Venn et al., 2008).

In the tissue of many anthozoans, the algal cells are arranged in a monolayer. As a result, the reef corals average millions of dinoflagellates per square centimeter of coral-colony surface (Drew, 1972). The average cell-specific density (CSD) ranges from 1.11 to 2.1. While in some species, e.g., *Stylophora pistillata*, the variation in distribution of number of algae per host cell is minimal, in others, such as *Condylactis gigantea*, significant variation can be found (Muscatine et al., 1998). The dinoflagellates occupy most of the interior of the macerated host cells, leaving the host cytoplasm and cell membrane as a thin outer layer. As such, the symbiotic zooxanthellae in cnidarians live within an osmotically different environment from that of free-living dinoflagellates (Goiran, et al., 1997; review by Mayfield and Gates, 2007). This spatial arrangement may support diffusion and transport of CO_2, bicarbonate ions, and nutrients from the environment to the algae (Muscatine et al., 1998). Marubini et al. 2008.

The symbiosis is based on fluxes of carbon and nutrients between the host, the algae, and the environment (Muscatine, 1990). This mutualistic relationship allows corals and coral-reef communities to succeed in spite of the low concentrations of nitrogen and phosphorus typical of the “blue deserts” – the oligotrophic waters surrounding the reefs (Muscatine and Porter, 1977). Both the cnidarian host and the algae are capable of ammonium assimilation from the surrounding environment, having the enzymes glutamine synthetase (GS) and glutamate dehydrogenase (Rahav et al., 1989). A variety of ammonium transporters have recently been found in *Symbiodinium* with similarity to bacterial transporters (Leggat et al., 2007, see below). Both partners benefit from nitrogen (N) recycling between animals and microorganisms. The host benefits because the microbial symbionts act as a sink for potentially toxic nitrogenous waste compounds, and the symbionts benefit from access to the N source for growth (Douglas, 2008).

By their photosynthesis, the symbionts provide to the host a major fraction of the metabolic requirements of the animal host (Muscatine, 1990). This dependence on photosynthesis sets depth limits to zooxanthellate corals, restricting most species to the photic depth, with reef growth and species diversity declining with light. The photic (also called euphotic) depth is usually set at 1% of subsurface light.

In addition, the algae create oxidized surroundings for the coral animal. It should be noticed that harboring photosynthesizing microalgae inside the tissue, which produce upon illumination great amounts of oxygen, is an advantage while the free-radical reactive oxygen species (ROS) that are also produced can be harmful to the host (Mayfield and Gates, 2007; Merle et al., 2007.

The photosynthetic algae pass up to 95% of their photosynthetic products to their animal host (Muscatine et al., 1984) primarily as glycerol, but also in the form of peptides, amino acids, glucose sugars, complex carbohydrates, and lipids (e.g., Trench, 1979; Swanson and Hoegh-Guldberg, 1998; review by Venn et al., 2008, Table 1). Host release factor (HRF) probably controls the translocation of the photosynthetic product from the zooxanthellae to the host (Trench, 1971; Gates et al., 1995, 1999; Withers et al., 1998; Cook and Davy, 2001; Grant et al., 2006a, b; Biel et al., 2007).

In return, the algae are exposed to higher levels of nutrients from the digestion of zooplankton by the whole coral, and to higher CO_2 from respiration, when compared with sea concentration. By growing in the coral tissue, indicating loss of the ability to move, the algae lose their flagellate (although it exists in culture), change their lifecycle (Freudenthal, 1962), and mainly reduce their growth rate. Existence in the tissue protects the algae from grazing by other organisms.

Several major metabolic processes exist in the holobiont: respiration by both the host and the symbionts, photosynthesis (by the zooxanthellae), and calcification in all the organisms that build skeletons, such as corals, mollusca, and foraminifera.

$$6CO_2 + 12H_2O \Leftrightarrow C_6H_{12}0_6 + 6C0_2 + 6H_2 0(\text{photosynthesis} \Leftrightarrow \text{respiration})$$

$$H_2O + CO_2 \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO_3^- \Leftrightarrow 2H^+ + CO_3^{2-} \left(\text{bicarbonate system}\right)$$

$$Ca^+ + 2HCO_3^- \Leftrightarrow^- CaO_3 + CO_2 + H_2O$$

$$Ca^{2+} + 2HCO_3^- \Leftrightarrow Ca\left(HCO_3\right)_2 \Leftrightarrow CaCO_3 + H_2CO_3$$

$$Ca^{+2} + H_2O + CO_2 \Leftrightarrow CaCO_3 + 2H^+ \left(\text{calcification}\right)$$

These three processes involve diffusion and transport of molecules from the seawater inside the holobiont in the exoderm, the endoderm, the zooxanthellae, and vice versa (Gattuso, 1999). The availability of CO_2 to the bicarbonate system has a major influence on the process and, in some cases, will limit them due to competition between the host and the symbionts.

In the sea anemone, *Anemonia viridis*, and the coral, *Stylopora pistillata*, the dissolved inorganic carbon (DIC) is absorbed from the seawater by the ectodermal cell layer and then transferred to the endodermal cell layer, where the zooxanthellae are located. In the latter, HCO_3^- is dehydrated to CO_2 and used for symbiont photosynthesis and OH^-, which is secreted within the coelenteric cavity. This process leads to a functional polarization of the oral layers, with the endodermal face being alkaline. The major supply of DIC then results from a

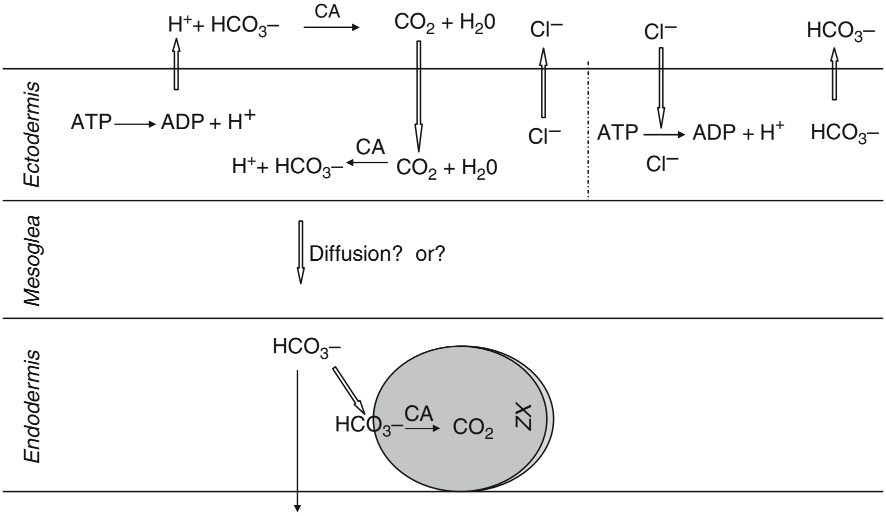

Figure 1. Model of inorganic carbon uptake by cnidarians. (After Furla et al., 2000a, b).

transcellular transport of HCO_3^-, whereas a fraction (20%) is supplied by passive diffusion (Benazet-Tambutte et al., 1996; Furla et al., 1998, 2000a, b, Fig. 1). The HCO_3^- absorption by ectodermal cells is carried out by H^+ secretion and by H^+-ATPase, and the formation of carbonic acid in the surrounding seawater, which is quickly dehydrated into CO_2 by a membrane-bound carbonic anhydrase (CA) (Furla et al., 2000a, b). *Symbiodinium* sp. have high CA activity and specific transporters for the delivery of bicarbonate ions to the host cells, keeping the partial pressure of CO_2 in the immediate surroundings of the symbiont cells high enough to support photosynthetic carbon fixation (Allemand et al., 1998). Corals growing in seawater at a reduced pH of 7.2 calcified at half the rate of corals at pH 8.0, indicating that coral growth is strongly dependent on the concentration of CO_3^{2-} ions in seawater (Marubini and Atkinson, 1999; Marubini et al., 2008).

Environmental changes that alter the metabolite exchanges between host and symbionts and – by that – the osmolyte pool levels might cause an osmotic stress response that will be followed by ROS formation, protein damage, photoinhibition, and even bleaching (Mayfield and Gates, 2007).

During the last decades, global climate change has caused an increase in sea temperature, *p*CO2, and acidification that led to zooxanthellae being expelled and/or losing their pigments, resulting in the bleaching of holobiont coloration and the death of entire reefs. These relationships between the marine microalgae/cyanobacteria and invertebrate symbiosis depend on the host genotype, the symbiont genotype, and environmental conditions. As these relationships develop during evolution time, stressful conditions such as high temperature, high light intensity, UV, and eutrophication break them down (see Stambler and Dubinsky, 2004; Grottoli et al., 2006; Stat et al., 2006; Carpenter et al., 2008; Day et al., 2008; Stambler, 2010).

2. Scleractinia – Hard Corals

Photosynthetically fixed carbon is translocated from zooxanthellae to the host. Under high-light conditions, translocation may amount to some 95% of the photosynthetic production of the zooxanthellae (Falkowski et al., 1984; Muscatine et al., 1984). The contribution of zooxanthellae to animal respiration (CZAR) is up to 100% of daily metabolic requirements, and in some cases, they provide more than the total metabolic needs of the host animal (Muscatine et al., 1981, 1984; Falkowski et al., 1984; Grottoli et al., 2006). In *Pocillopora damicornis* and *Fungia scutaria*, the CZAR is in the order of 63–69% (Muscatine et al., 1981). The energy input from photosynthesis in the coral *Pocillopora eydouxi* is about twice the amount that the animal needs for respiration (Davies, 1984). In the coral *Porites porites*, the animal used only 33% of the energy translocated from the zooxanthellae (Edmunds and Davies, 1986). In *Stylophora pistillata*, a product of algal photosynthesis could provide between 143% and 58% of the total carbon and energy requirements of the high- and low-irradiance-adapted colonies, respectively (Falkowski et al., 1984; Muscatine et al., 1984, Fig. 2).

This energy is used by the host for metabolism including respiration, production, planula, larvae, and mucus release. Energy in the form of translocated carbon from the symbiont often covers the necessity for the daily needs of some scleractinians (Davies, 1991), and excess carbon can be stored as lipid reserves in concentrations of 10–40% of total biomass (Edmunds and Davies, 1986; Stimson, 1987; Harland et al., 1992, 1993). Up to 50% of the photosynthetically fixed carbon exported to the host is released from the host as mucus (Crossland et al., 1980; Davies, 1984; Crossland, 1987; Wild et al., 2004).

A compound described as HRF, which stimulates the release of photosynthate from symbiotic algae, has been found in the host tissue of several symbiotic cnidarians (e.g., Muscatine, 1967; Grant et al., 2006a). The HRF controls the amount of carbon translocated from the zooxanthellae to the host. In the case of the scleractinian coral *Plesiastrea versipora* (Lamarck), the HRF, which has a low molecular weight, stimulates the release of glycerol from its symbiotic dinoflagellate, which can then be utilized by the animal host for its own needs. This diversion of glycerol from the algae results in a partial decrease in the algal synthesis of triacylglycerol (TG) and starch (Grant et al., 2006a).

Under oligotrophic conditions, zooxanthellae are nitrogen- and phosphorus-limited, and can multiply. Owing to the nitrogen limitation, much of the carbon fixed in photosynthesis can translocate to the host. The result is that the growth rate of the zooxanthellae is extremely slow, with doubling times as long as 70–100 days (growth rate, $\mu = 0.007–0.001$ day^{-1}) in the common Red Sea coral *Stylophora pistillala* (Falkowski et al., 1984) when compared with the much higher growth rate

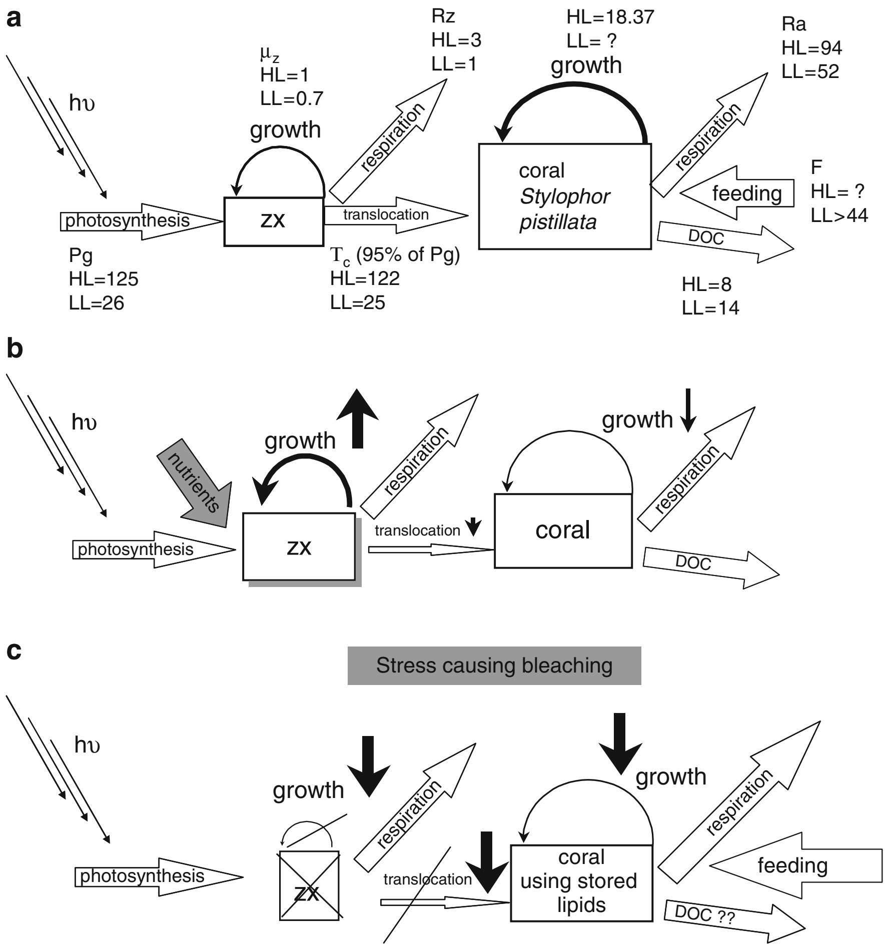

Figure 2. Energy flow in the symbiotic association between zooxanthellae and coral. (**a**) High and low light for the coral *Stylophora pistillata* in μg C cm^{-2} day^{-1}. (After Muscatine et al., 1984; Falkowski et al., 1984.) (**b**) A model of carbon fluxes under eutrophication conditions. (**c**) A model of carbon fluxes under stress conditions which cause bleaching, such as high temperature and high UV.

of zooxanthellae cultured from the coral *Acropora* sp. (0.33–0.48 day^{-1}) (Taguchi and Kinzie, 2001). The mechanism for regulating algal–cnidarian symbiosis is by the expulsion of dividing algal cells and not by digestion (Baghdasarian and Muscatine, 2000). Once supplied with additional nutrients, either as inorganic compounds, such as ammonium and phosphate or via zooplankton consumption by the host animal (e.g., Dubinsky et al., 1990; Falkowski et al., 1993; Dubinsky and Jokiel, 1994), the zooxanthellae retain most of their photosynthetic products. This photosynthetic carbon is now utilized for the synthesis of zooxanthella biomass, accelerating their growth rates and increasing their densities up to fivefold (Dubinsky et al., 1990).

This growth results in the following adverse effects on the overall carbon and energy flux within the association: the zooxanthellae significantly reduces their photosynthetic rates per algal cell due to carbon limitation in the super-dense, multilayered algal population (Dubinsky et al., 1990). However, based on the area, photosynthesis increases when multiplied due to the increase in algal numbers. In addition to the above-described measured effect, the following two effects were also inferred: the rapidly multiplying algae retain a much higher fraction of photosynthate, rather than translocating it to the animal (Muscatine et al., 1989; Falkowski et al., 1993), and nutrient enrichment causes an imbalance in coral growth between organic tissue and carbonate skeleton (Tanaka et al., 2007). Because of these process changes under eutrophication, symbiosis breaks down (Stambler et al., 1991; Dubinsky and Stambler, 1996).

Size growth in the solitary coral *Fungia concinna* is not limited strictly by energy availability, but by not recognizing physiological and/or ecological constraints (Elahi and Edmunds, 2007). It might be controlled by the availability of nutrients and other components necessary for animal growth. Corals, which are heterotrophic organisms, use multiple heterotrophic inputs as food sources, including zooplankton (e.g., Sebens et al., 1996; Ferrier-Pages et al., 2003; Palardy et al., 2006), particulate organic matter (Anthony and Fabricius, 2000), and bacteria (Ferrier-Pages et al., 2003). These provide the association nutrients, such as nitrogen and phosphorus (e.g., Muscatine and Porter, 1977; Szmant-Froelich and Pilson, 1980). A high percentage of 66% of coral skeletons is based on heterotrophic inputs (Grottoli and Wellington, 1999). The ratio between the heterotrophic and autotrophic energy contribution, which depends on the coral and zooxanthella species, changes under different conditions, such as food availability and light intensity (Porter, 1976; Falkowski and Dubinsky, 1981; Falkowski et al., 1984; Anthony and Fabricius, 2000; Palardy et al., 2005; Grottoli et al., 2006). *Montipora capitata*, a tropical coral, was more resilient to bleaching by increasing its food ingestion. The colonies of *Montipora capitata* that had been bleached started to recover, increasing the feeding rates (fivefold higher in bleached versus non-bleached), and by that, the percentage of the contribution of heterotrophically acquired carbon to daily animal respiration (CHAR) supplied more than 100% of their daily metabolic energy requirements and allowed them to survive. This was not the case with *Porites compressa* and *Porites lobata* (Grottoli et al., 2006). Metabolic changes in lipids, such as triacylglycerol, phospholipid, monoacylglycerol, diacylglycerol, and free fatty acid show that during bleaching and recovery, *Montipora capitata* corals switch between heterotrophy and photoautotrophy, while *Porites compressa* corals rely mostly on photoautotrophy (Rodrigues et al., 2008).

Coral energy balance is a function of (1) heterotrophy and phototrophy, which are influenced by bleaching status and light regime, and (2) energy losses that are also functions of temperature and light (e.g., Anthony and Connolly, 2004; Grottoli et al., 2006). The interaction between temperature, light, and feeding controls growth, zooxanthella density, and asexual reproduction (Rodolfo-Metalpa et al., 2008a, b). Chlorophyll concentrations increased under low light and temperature, probably in order to maintain a sufficient level of autotrophy (Rodolfo-Metalpa et al., 2008a). In the case of the temperate coral *Cladocora caespitosa*, feeding was especially important for growth at low temperatures (Rodolfo-Metalpa et al., 2008b). The translocation rate of the zooxanthellae was higher in winter than in summer (Peirano et al., 2005). It seems that at high temperature, some of the heterotrophic energy supply is used by the host for other metabolic processes, such as sexual and asexual reproduction (Rodolfo-Metalpa et al., 2008b). The glycerol translocated to the host is usually rapidly respired, although the host maintains temporally dynamic pools of both glycerol and amino acids within its tissues. In response to temperature increase, these pools decrease due to shifts in the symbiotic metabolism (Gates and Edmunds, 1999). While irradiance had no effect on the temperate coral *Cladocora caespitosa*, high temperature and food supply increased coral growth rates. The effect of feeding was greater for corals maintained at low temperatures, suggesting that heterotrophy is important during the cold season. At low temperatures, samples that were fed exhibited higher zooxanthella density and chlorophyll concentration. Sexual reproduction level was higher during high temperatures and zooplankton availability (Rodolfo-Metalpa et al., 2008). There is some evidence that zooxanthellae under low temperature become heterotrophic (Dimond and Carrington, 2008).

In the field, in response to temperature stress, some symbiont communities change in reef-building corals, suggesting a population-wide acclimatization to increased water temperatures, creating new, more thermally tolerant holobionts (Jones et al., 2008; Maynard et al., 2008). This change supports the adaptive bleaching hypothesis (Buddemeier et al., 2004). As Jones et al. (2008) pointed out, the advantage of more temperature tolerance might result in certain disadvantages, such as slower growth rate of the holobiont (Little et al., 2004). This could be due to a reduction in the photosynthetic rates of the new symbionts (Rowan, 2004) using more energy for zooxanthella growth and less energy reserves (Hoogenboom et al., 2006; Loram et al., 2007).

Total lipid concentrations decline in some species when zooxanthella and/or chlorophyll *a* concentrations are low (Rodrigues and Grottoli, 2007; Rodrigues et al., 2008). Part of the coral recovery after bleaching is due to increasing lipid concentration, mainly from heterotrophic feeding. Storage lipids depend on the coral species. While *Montipora capitata* corals switch between heterotrophy and photoautotrophy, *Porites compressa* corals rely on photoautotrophy (Rodrigues et al., 2008).

Reduced photosynthesis as a result of bleaching leads to reduced energy reserves for maintenance and growth and/or tissue biomass (see Fitt et al., 2000; Grottoli et al., 2004; Anthony and Connolly, 2007). During the course of a bleaching event, energy reserves may thus fall to the point at which resources for maintenance and growth are compromised, leading to increased risk of mortality (Anthony and Connolly, 2007).

In the coral *Acropora pulchra*, during algal photosynthesis, more than 70% of organic N synthesized from NO_3^- by zooxanthellae is produced and immediately translocated to the host coral. The organic matter translocated to the host is similar, at least in C:N ratios, to that found in the algal cells. However, NO_3^--derived N accumulates more in the zooxanthellae when compared with the alga:coral N ratio, probably followed by a reduction in the organic matter translocated to the host with increasing NO_3^- availability for the symbionts. Once incorporated, organic compounds of higher C:N ratios are consumed more rapidly than those of lower C:N ratios in both the host coral and zooxanthellae. The coral–zooxanthella symbiotic system could be highly conservative for N (Tanaka et al., 2006).

Nutrients, including nitrogen and phosphate, are a limiting factor in the oligotrophic seas (e.g., Jackson and Yellowlees, 1990). The taking up, retention, and recycling of dissolved inorganic and organic nutrients by the symbioses have contributed to the success of coral reefs in nutrient-depleted tropical seas (Muscatine and Porter, 1977).

Many aspects of the nitrogen cycle were studied (Fig. 3, Yellowlees et al., 2008). The efficiency with which nitrogen from predation was fully incorporated into the zooxanthella *Oculina arbuscula* was nearly 100%, when compared with only 46% for corals containing few zooxanthellae. In *A. pallida*, symbiont density

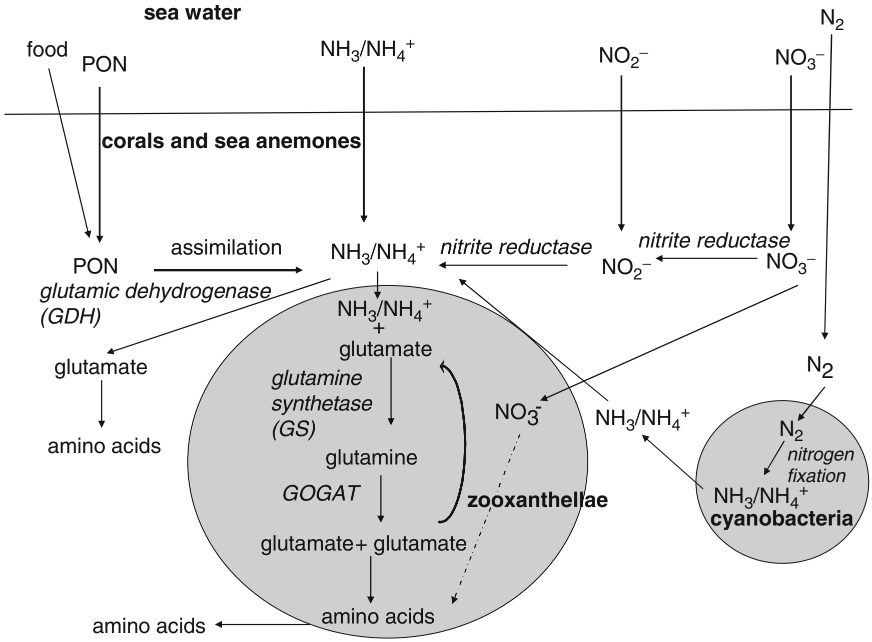

Figure 3. Model of the nitrogen cycle in the cnidarian association based on Crossland and Barnes (1977), Rahav et al. (1989), Markell and Trench (1993), Falkowski et al. (1993), Wang and Douglas (1999), Fagoonee et al. (1999), Roberts et al. (2001), Grover et al. (2003), Lesser et al. (2004), Tanaka et al. (2006), Davy et al. (2006), Leggat et al. (2007), Lesser et al. (2007), and Yellowlees et al. (2008).

had no effect, and N assimilation was 23–29% (Piniak et al., 2003). The host and alga are capable of ammonium assimilation from the surrounding seawater, with both possessing the enzymes GS and glutamate dehydrogenase (Rahav et al., 1989). Some ammonium transporters were found in *Symbiodinium* (Leggat et al., 2007). The host ammonium assimilation is high and requires a constant supply of carbon skeletons, presumably from photosynthesis, for ammonium assimilation. Some nitrogen is assimilated by *Symbiodinium* and transported back to the host, in particular, the essential amino acids (Wang and Douglas, 1999). *Symbiodinium* transporters for nitrate and nitrite have been found (Leggat et al., 2007). *Symbiodinium* are probably capable of utilizing nitrate as an N source (Fagoonee et al., 1999). The intact cnidarian symbiosis removes nitrate from the water column (Crossland and Barnes, 1977). Nitrate is converted to nitrite and, furthermore, to ammonium, through the action of the enzyme nitrate reductase (Leggat et al., 2007). This nitrogen can be quickly translocated to the host (Tanaka et al., 2006), presumably as amino acids (Yellowlees et al., 2008). Nitrate and ammonium can serve as nitrogen sources. The cnidarian symbioses are capable of reasonably high rates of nitrate uptake from the very low concentration in the water, although they prefer ammonium (Grover et al., 2003). Zooxanthellae may use the nitrogen for their growth and release some of it back to the host in the form of amino acids (Markell and Trench, 1993). Nitrogen fixation also occurs inside the coral by intracellular cyanobacterial symbionts that fix N_2 (Lesser et al., 2004, 2007). In temperate areas where the nutrients are a less limiting factor when compared with tropical areas, the zooxanthellae probably store nitrogen for use when nutrients and food are less available (Davy et al., 2006).

Few studies have been carried out on phosphate, and its cycle in the holobiont is not known. Only by symbiont cnidarians, not aposymbiont cnidarians, phosphate uptake occurs. The uptake rates by intact symbiosis are higher in the light than in the dark (D'Elia, 1977).

Photosynthesis and calcification in the reef-building corals *Pontes compressa*, *Porites porites*, and *Acropora* sp. are affected by bicarbonate concentrations. *Porites porite*s increases the calcification rate in response to the addition of $NaHCO_3$, reaching saturation at 6 mM while the photosynthesis saturation is 4 mM HCO_3. *Acropora* sp. calcification and photosynthesis are higher than those of *Porites porite*s: photosynthesis saturates at 4 mM, while calcification continues to increase even above 8 mM HCO_3 (Marubini and Atkinson, 1999; Herfort et al., 2008).

Owing to global changes, it is predicted that in the next century, $p\mathrm{CO}_2$ will increase by 15% (0.3 mM) the oceanic HCO_3^- concentration (based on Herfort et al., 2008) and this could stimulate photosynthesis and calcification of hermatypic corals. Unfortunately, the increase in $p\mathrm{CO}_2$ will cause acidification of the seawater, which will cause a decrease in coral growth calcification, decalcification of the coral, coral death, and might irreversibly change the entire reef (Fine and Tchernov, 2007; Hoegh-Guldberg et al., 2007; Jokiel et al., 2008; Veron, 2008a, b). Coral symbiosis has developed for over 250 million years, and is usually exposed to slow changes in the environment. Bleaching events caused by increasing sea temperature already cause and will probably contribute to the death of the entire reefs (Hoegh-Guldberg, 1999, 2005; Hoegh-Guldberg et al., 2007; Veron 2008a, b). We hope that the host, the symbionts, and the holobiont will be able to adapt. Some corals are able to recover and survive bleaching. In some cases, such as in the case of *Pocillopora verrucosa*, bleaching sensitivity might not be associated with clade specificity (Richier et al., 2008).

Some bleached and recovering corals increase their feeding and, in that way, obtain heterotrophic carbon for daily animal respiration (CHAR) (Grottoli et al., 2006). Coral species with a heterotrophically high energy source and lipid storage capabilities during bleaching and recovery will be able to survive bleaching events over the long term, and might become the dominant coral species on reefs (Grottoli et al., 2006; Anthony and Connolly, 2007; Rodrigues et al., 2008). The ability of the host to change its energy source, together with changing the symbionts associated with the host, might prevent the total global extinction of reefs.

3. Hexacorallia – Sea Anemones

Temperate and tropical sea anemones harbor zooxanthellae and/or zoochlorellae in their tissues. In most cases, this symbiosis is similar to that in hard and soft corals (e.g., see section on coral for the N cycle, Fig. 3), but there is a unique symbiosis of the sea anemone *Phyllactis* (= *Oulactis*) *flosculifera*, which has developed specialized behavioral, structural, and chemical adaptation. *Phyllactis* (= *Oulactis*) *flosculifera* cultivates the zooxanthellae in specialized areas of the body, and then breaks them down and uses them as a source of nutrition (Steele and Goreau, 1977).

The photosynthetic products of the algae transfer to the host. For example, in the temperate sea anemone *Anemonia viridis*, glucose and succinate/fumarate are the important photosynthetic compounds transferred from the *Symbiodinium* cells to the host tissues (Whitehead and Douglas, 2003, Table 1).

The temperate sea anemones *Anthopleura elegantissima* and *Anthopleura xanthogrammica* from the northern latitudes can harvest both the zooxanthella *Symbiodinium muscatinei* (dinoflagellate) (LaJeunesse and Trench, 2000) and the zoochlorella Coccomyxa (chlorophyte) (Verde and McCloskey, 2002, 2007; Lewis and Muller-Parker, 2004). In the intertidal sea anemone *Anthopleura elegantissima*, both the zooxanthellae and zoochlorellae translocate 30% of photosynthetically fixed carbon in freshly collected anemones, although the zoochlorellae fixed and translocated less carbon than the zooxanthellae (Engebretson and Muller-Parker, 1999).

Only 50% of the photosynthetically assimilated carbon is utilized by the zooxanthellae of the sea anemone *Anemonia sulcata* from the Israeli Mediterranean coast, while the rest is translocated to the animal tissue. The percentage of carbon translocated to the anemone tissue is independent of light intensity and, therefore,

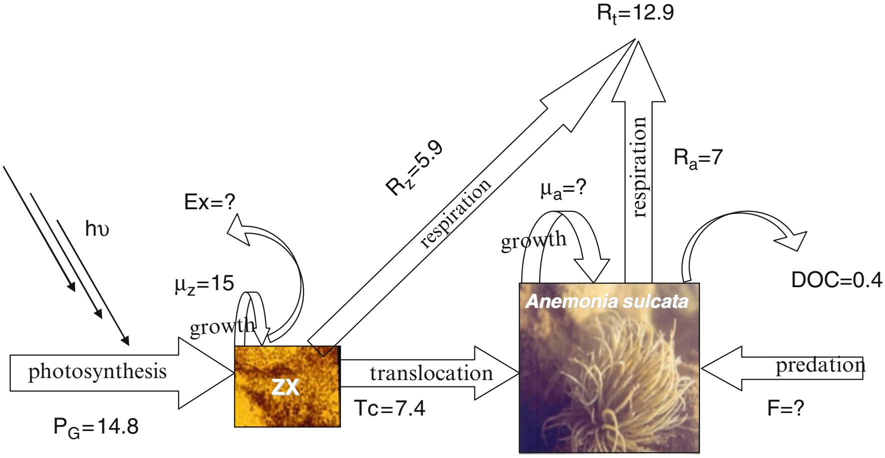

Figure 4. Energy flow in the symbiotic association between zooxanthellae and *Anemonia sulcata* in cal g^{-1} wet weight day^{-1} (After Stambler and Dubinsky, 1987).

does not depend on the total amount of fixed carbon. This translocation of the photosynthetic products from the algae to the animal tissue may provide up to 116% of the animals' respiratory needs under natural conditions (Fig. 4). Under starvation conditions, the percent of translocation increases up to 70%. The energy contribution of zooxanthellae to the anemone allows its survival and maintenance under starvation conditions, but for growth, the anemone also needs essential nutrients, such as nitrogen and phosphate, which have to be obtained through predation (Stambler and Dubinsky, 1987). The CZAR in *Anemonia viridis* from different locations along the coast of Ireland is 140.6–142.9% at 1.5 m on sunny days, but less than 100% under low light. Zooxanthellae of the sea anemones *Cereus pedunculatus*, *Anthopleura ballii*, and *Anemonia viridis* at 1.5 m on sunny days, requires 1.80–5.89% of the carbon fixed in photosynthesis for respiration and growth, and translocates 94.11–98.20% to the host. At 9 m on cloudy days, zooxanthellae use 38–88% of the fixed photosynthesis carbon, leaving 12–62% for translocation.

The CZAR is measured at 72.6% in *Anthopleura ballii* and 72.1% in *Cereus pedunculatus*, at 1.5 m on sunny days, and decreases to just 2.1% and 0.7%, respectively, at 9 m on cloudy days (Davy et al., 1996). In the anemone *Anthopleura elegantissima* from the low-intertidal, the CZAR is 34–42%, decreasing to 17% in high-intertidal anemones (Zamer and Shick, 1987). In the dark, starved anemones lose weight at a higher rate than in the light (Smith, 1939; Taylor, 1969; Tytler and Davies, 1986). In the anemone *Anthopleura elegantissima*, it is 34–56% when starved, but only 8–9% in fed animals (Fitt et al., 1982). Generally, a translocation rate of more than 90% in both tropical and temperate zooxanthellate anemones is found under well-light conditions (Muller-Parker and Davy, 2001).

Irradiance regulates both the photophysiology and metabolism of this alga–sea anemone association. Regardless of light intensity, algal densities remain stable for *Anthopleura elegantissima,* harboring zooxanthellae or zoochlorellae. Net photosynthesis, the potential carbon contribution of the algae to animal respiration (CZAR), and the mitotic indices of both the symbionts, vary with light intensity, with no change in the chlorophyll per algal cell. Zooxanthella photosynthetic rates are consistently higher than those of zoochlorellae (McCloskey et al., 1996; Verde and McCloskey, 2002). No matter which algae are associated with the sea anemone, net photosynthesis is always higher during spring and summer. In addition, the zooxanthella net photosynthesis is higher than that of the zoochlorellae. Anemone respiration is also higher during the spring and summer. As a result, the CZAR does not show a clear relationship with season; however, the CZAR for zooxanthella anemone is greater than for zoochlorella anemone (Verde and McCloskey, 2007). Lower zooxanthella growth rates, higher photosynthetic rates, and CZAR give an advantage to the *Anthopleura elegantissima* association with zooxanthellae at high-light intensity when compared with zoochlorellae, and can be seen by the higher densities of zooxanthellate anemone in the shallow water, while *Anthopleura elegantissima* with zoochlorellae is found primarily under low-light conditions in shaded habitats (McCloskey et al., 1996; Verde and McCloskey, 2002). In the sea anemone *Aiptasia pulchella*, zooxanthellae become heterotrophic under low-light conditions (Steen, 1987). Changes in CZAR, with environmental variation, were found in other symbioses, such as in the zoanthid *Isozoanthus sulcatus*. In the zoanthid, the translocation rate exceeded 95% under high- and low-light conditions, while the CZAR was 181.5% at 1.5 m on sunny days, but less than 100% at 9 m on cloudy days (Davy et al., 1996).

Owing to temperature increase, bleaching events were observed in the Mediterranean Sea anemones *Anemonia sulcata var. smaragdina* and *Anemonia rustica*. Both the species lost 90% of their zooxanthellae (Leutenegger et al., 2007). Aposymbiont anemones depend on heterotrophic feeding.

Condylactis gigantean, which are similar genetic individuals, harbor either *Symbiodinium* clades A or B, which are functionally different. At 25°C, there is no significant difference in the clade photosynthesis. Temperature changes the carbon fixation rates per algal cell. For symbioses harboring clade A, the total fixation rates are higher at 30°C when compared with 25°C, while the opposite is found for symbioses harboring clade B. Clade A incorporation of algal photosynthetic carbon into animal lipids and amino acid pools is significantly higher when compared with clade B. This difference may be due to the difference in the amount of compounds translocated to the animal tissues or a difference in the metabolic processing of the mobile compounds by the animals (Loram et al., 2007).

4. Octocorallia – Soft Corals

Few studies have been carried out on soft corals and their symbionts. The assumption is that the relationship between the host and the zooxanthellae is very similar to that of the hard coral and sea anemone, even though predation may play a major role in this soft-coral symbiosis. The soft coral, octocoral *Sinularia lochmodes*, controls the cell division of *Symbiodinium* by arresting the algae in the cell-dividing, non-motile stage via chemical signaling (Koike et al., 2004). This signal causes the translocation of the photosynthesis product from the algae to the host. In the soft coral *Capnella gaboensis*, photosynthetically fixed carbon by zooxanthellae is incorporated into the coral tissues as glycerol, glucose, succinate, citrate, fumarate, glycolic acid, malate, aspartate, glycine, serine, and alanine. In this case, about 10% of the total fixation was translocation (Farrant et al., 1987).

5. Mollusca, Bivalvia, Clams

The giant clam family Tridacnidae contains large numbers of *Symbiodinium* sp. The zooxanthellae, which live in the clam's siphonal mantle (hypertrophied siphonal tissues), are important for its nutrition. The existence of zooxanthellae in the mantle tissue demands its exposure to light and causes the clams to be permanently sessile. This increases the risk of attracting predators and causes the development of a very unique system of light sensors and a mechanism for retraction of the mantle tissue, allowing closing of the shell valves against the predators. In another photosymbiotic bivalve, *Corculum cardissa*, which harvests *Symbiodinium corculorum* zooxanthellae, the algae are also located in a zooxanthellal tubular system that is associated with the hemocoel and is similar to that seen in the tridacnine clams (Farmer et al., 2001).

Tridacnidae zooxanthellae live within a branched, tubular structure that has no direct connection to the hemolymph. The fact that there is no connection between the hemolymph and the stomach via the tubes associated with the zooxanthellae prevents digestive enzymes from entering the hemolymphatic system. The entire branched tubular system associated with the zooxanthellae communicates with the stomach via a single opening, which is visible in clams that are only a few weeks old. This would appear to explain the initial entry of zooxanthellae into the mantle. While it is unlikely that intracellular digestion occurs in the zooxanthellal tube, the epithelial cells of the zooxanthellal tube might have been misidentified as hemocytes engulfing algal cells (Norton et al., 1992).

On the one hand, healthy zooxanthellae observed in the *Tridacna* stomach (Fitt et al., 1986) pass through the intestine and rectum and are released in the feces; thus, these algae, by a still unknown mechanism, are able to resist host digestion (Trench et al., 1981). This route is also available for the mass expulsion of zooxanthellae from clams exposed to elevated environmental temperatures (Estacion and Braley, 1988). On the other hand, there are some indications that some zooxanthellae in the clams *Tridacna derasa* are digested by host clams (Maruyama and Heslinga, 1997). Although in the zooxanthellal tubes, zooxanthellae usually have intact ultrastructures, suggesting that they are photosynthetically active, the stomach always contains degraded zooxanthellae that were probably discharged from the zooxanthellal tube. In four *Tridacna* species, symbiotic algae are capable of providing 2–4 times more carbon than required by the host for respiration. The CZAR increases with clam size in all species, except in *Hippopush hippopus*, which has a comparatively high and more constant CZAR of 340%. The lowest CZAR value is 186% in the smallest *Tridacna squamosa* (Klumpp and Griffith, 1994). In the Red Sea, similar CZARs of 186% in *Tridacna maxima* and 151% in *Tridacna squamosa* were found (Jantzen et al., 2008).

Degraded zooxanthellae are always found in the stomach of veligers and *Tridacna crocea*, *Tridacna derassa*, and *Tridacna squamosa.* They seem to be digested with other stomach contents, such as diatoms. Giant clams probably digest zooxanthellae directly, and ingest the secreted photosynthates from them. Thus, the giant clams probably utilize the zooxanthellae not only as photosymbionts, but also, directly, as foods. There may be a selection mechanism to discharge unhealthy zooxanthellae from the mantle into the stomach. In addition to zooxanthellae, digested diatoms and other unidentified digested materials in the stomach suggest that filter-feeding also contributes to giant-clam nutrition. However, symbiosis with zooxanthellae supplies the host with a photosynthetic product, while the digestion of zooxanthellae may also supply nutrients for the giant clams (Hirose et al., 2006).

The advantage of having zooxanthellae in the tissue of a giant clam such as *Tridacna crocea*, is the receiving of glucose release by the zooxanthellae (Ishikura et al., 1999). The glucose release is controlled by the host release factor (Muscatine, 1967). The zooxanthellae supply as much as 100% of the daily respiratory carbon requirements of the clam *Tridacna gigas* (Fisher et al., 1985) and more than 50% of the carbon resources required by other host clams (Trench et al., 1981; Klumpp and Lucas, 1994).

Zooxanthellae in giant clams use CO_2 as the primary source of their carbonate while the symbionts in corals use bicarbonate (Leggat et al., 2000; but see Furla et al., 2000a, b, Fig. 1).

6. Mollusca, Nudibranchs, Sea Slugs

Photosymbiosis is found in the gastropod. Live photosynthetic dinoflagellates are also found in the hepatopancreas and gonads of the Red Sea snail *Strombus tricornis*. They are found within the upper whorls of the snail's shell, where light penetration is 5–15% of the incident light reaching the shell (Berner et al., 1986). However, in this taxonomy group, most of the symbiosis is found in nudibranchia, where the light is not blocked by a shell.

Nudibranchia are associated with zooxanthella *Symbiodinium*, zoochlorellae, and *Prochloron*. The nudibranchs take up the algae through their prey, mostly by feeding on soft corals, and, in some cases, also on hard corals. Stable symbiosis and long-term retention of zooxanthellae are found with *Aeolidoidea* and *Dendronotoidea* (Burghardt et al., 2008). In the aeolid nudibranch *Aeolidia papillosa*, zooxanthellae and zoochlorellae obtained by the ingestion of *Anthopleura elegantissima* remain photosynthetically active within the cerata, and it is likely that they derive some benefit from these algae. The zooxanthellae and zoochlorellae may survive the nudibranch cerata as heterotrophic (Mcfarland and Muller-Parker, 1993). In other nudibranchs, even after 200 days of starvation, the number of zooxanthellae is high and dividing zooxanthellae detected (Burghardt et al., 2008). Burghardt et al. (2008) suggest that the uptake of zooxanthellae via the prey and the ensuing enhancement of cryptic appearance might represent the beginning of the evolution of nudibranch–zooxanthellae symbioses. A branched digestive gland has a larger surface area for the exchange of metabolites and gases, with large exposure to the light due to body and transparent ceras wall. This allows high photosynthetic utilization (Burghardt et al., 2008). The additional products produced by the symbionts allow the slugs to survive with food shortages lasting from weeks to months (see in Burghardt et al., 2008).

Sacoglossan mollusks maintain photosynthetic plastidin/chloroplast in their cells, a phenomenon known as kleptoplasty (Waugh and Clark, 1986). The sacoglossans incorporate the chloroplasts into their digestive cells through phagocytotic feeding (e.g., Rumpho et al., 2000; Evertsen et al., 2007; Casalduero and Muniain, 2008). Functional chloroplasts photosynthesize inside the mollusk cells and produce oxygen, carbohydrates, lipids, and proteins (Greene and Muscatine, 1972; Trench et al., 1972). The ability to retain functional chloroplasts (RFC) varies among seven sacoglossans from the Indo-Pacific and Mediterranean Seas: *Plakobranchus ocellatus* – 11 months, *Elysia timida* – 3 months, and *Elysia* sp., *Elysia tomentosa*, *Thuridilla carlsoni*, *Thuridilla lineolata*, and *Elysiella pusill* – 15 days. The variations are based on the quantum yield of charge separation in photosystem II in dark acclimated cells measured by pulse amplitude modulated (PAM) fluorometry (Evertsen et al., 2007). The survival rates of *Elysia timida*, after being kept in the dark for 28 days, are up to 30% lower when compared with the rates of those that are kept in the light, and exhibit size decrease, even though chlorophyll concentration values are similar in both the cases. The kleptoplasts provide the mollusks energy at the primary metabolism level to compensate for a shortage in food (Casalduero and Muniain, 2008). The exposed area of the parapodia of *Elysia timida* responds to light, ensuring optimum irradiance to be reached by the chloroplasts. Under low light, *E. timida* unfolds the parapodia (Rahat and Monselise, 1979; Monselise and Rahat, 1980). Sacoglossans may regulate light intensity, and by that, control the photosynthesis rate and prevent pigment degradation (Casalduero and Muniain, 2008).

7. Foraminifera

Endosymbiotic algae and chloroplasts are found in 3 orders and 11 families of foraminifera host:

1. Alveolinidae, Amphisteginidae, Calcarinidae, and Numulitidae host diatoms.
2. Soritacea host a variety of different algal types: (a) Peneroplidae host symbiont rhodophytes; (b) Archaiasinae host chlorophytes; and (c) Soritinae host

dinoflagellates, cyanobacteria, and haptophytes (review by Lee (2006). In a single foraminiferal sub-family, *Soritinae*, a great diversity of *Symbiodinium* genotypes (clades C, D, F, G, and H) is found. Based on the molecular clock method of *Symbiodinium* rDNA sequences, the *Symbiodinium* genus originated in early Eocene, and the majority of extant lineages diversified since mid-Miocene, about 15 million years ago (mya) (Pochon and Pawlowski, 2006).

3. Globigerinidae host dinoflagellates and chrysophytes.
4. Candeinidae, Pulleniatinidae, Hastigerinidae, and Globorotaliidae host chrysophytes (review by Lee, 2006).

Peneroplis planatus does not grow if starved. It grows slowly in the dark when fed. It acquires most of its carbon and energy for growth from food and cannot grow solely on carbon compounds that are fixed, transformed, and released by its endosymbiotic algae (Faber and Lee, 1991).

While corals contain about 1–2% organic matter (Erez, 1978), they are much higher in foraminifera. In *Amphistegina lobifera*, the host, the symbiont host organic matter, and the skeleton contain approximately 7%, 20%, and 73% of the total carbon, respectively; in *Amphisorus hemprichii,* the numbers are 5%, 16%, and 79%, respectively. As corals contain the least amount of organic carbon per unit of inorganic (calcareous) carbon when compared with foraminifera, they need to take up fewer nutrients in the form of nitrogen or phosphorous compounds from their surroundings. Translocation from symbionts to *Amphistegina lobifera*, a perforate species, and to the imperforate species *Amphisorus hemprichii* host is sufficient to account for the increase in their biomass (Kuile and Erez, 1991).

8. Sponges

Tropical and temperate sponges (Porifera), which are filter-feeding organisms, harbor photosynthetic symbionts. Hosts include Demospongiae and Calcarea (Diaz, 1996). The symbionts include cyanobacteria, rhodophytes, diatoms, dinoflagellates, chlorophytes, and cryptomonads. Polar sponges are associated with diatoms. Freshwater sponges often contain endosymbiotic microalgae, primarily zoochlorellae (see Wilkinson, 1992; Taylor et al., 2007; Usher, 2008), while the marine sponges harbor diverse and abundant microbial communities (Lee et al., 2001; Taylor et al., 2007).

Based on sequencing analysis, it seems that the symbionts are transferred by a combination of vertical and horizontal symbiont transmissions. Some symbionts are passed down from an ancestral sponge, while others are obtained contemporaneously from seawater (Taylor et al., 2007).

The density of cyanobacteria in the sponge is proportional to the number of sponge cells; the symbiont population is probably controlled by the sponge. The control mechanisms might include sponges that consume excess symbionts, eject symbionts under stress, and use the photosynthetic product by the host sponge

(Wilkinson, 1992). The photosymbionts are restricted to sponge surface cell layers where they are exposed to maximum light (Beer and Ilan, 1998). Phototrophic cyanosponges have characteristics of flattened morphology with a large surface area on which photosynthesis can take place (Wilkinson, 1983), while cyanosponges have a smaller surface area-to-volume ratio. These sponges, which rely on heterotrophic feeding for more than half of their energy requirements, are referred to as mixotrophic (Usher, 2008).

The cyanobacterial symbionts may protect sponges by providing a sunscreen and might also benefit from the possible production of UV-screening substances (e.g., mycosporine-like amino acids (MAAs)) against UV radiation, permitting the holobiont to grow in shallow water (Steindler et al., 2002; Usher, 2008).

The activity of the cell-signal HRF in a sponge with algal symbionts is probably the cause of translocation from the algae to the sponge. *Haliclona cymiformis* HRF stimulates the release of glycerol from *Symbiodinium,* but does not stimulate glycerol release by its own symbionts, red macroalgae, Rhodophyta, *Ceratodictyon spongiosum* (Grant et al., 2006b).

The photosynthate product of the cyanobacteria translocates to the host in the marine association mainly as glycerol, while glucose produced by a chlorella-like green alga was passed to its freshwater sponge. The photosynthetic product of the cyanobacterial symbionts can supply up to 50% of the energy requirements of the host (Wilkinson, 1983). In the Great Barrier Reef, sponges may derive much of their nutrition from photosynthetic symbionts at depths of 15–30 m. Some sponges limit their depth distribution according to the availability of light for photosynthesis (Cheshire and Wilkinson, 1991).

The energy contribution of other photosynthetic associates (diatoms, dinoflagellates, and phototrophic sulfur bacteria) to the sponge is less clear (Taylor et al., 2007). The metabolism of the Mediterranean sponge *Cliona viridis*, associated with dinoflagellates (zooxanthellae), depends on the photosynthetic activity of these symbionts (Schonberg et al., 2005). The growth of *Cliona viridis* is greater under light conditions when compared with those grown under dark conditions (Rosell and Uriz, 1992). It was suggested that during air exposure at low tide, intertidal sponges are unable to filter-feed and may be more dependent on the energy from the autotrophic symbionts (Steindler et al., 2002).

The N cycle in the sponge includes assimilation of particulate organic nitrogen (PON) from seawater, ammonia oxidation, nitrite oxidation, and denitrification (Fig. 5, Taylor et al., 2007). Cyanobacterial symbionts in sponge may contribute to the N budgets of the sponge via atmospheric N_2 fixation (Wilkinson and Fay, 1979; Taylor et al., 2007). This is very important for sponge-growing under oligotrophic conditions. It may explain the fact that on tropical reefs, typically 30–50% (and sometimes 80–90%) of the sponges are cyanosponges (Wilkinson, 1992).

The benefits of symbiosis to cyanobacteria are that it provides an acceptable growing environment. The sponges provide a solid substrate and access to higher levels of ammonium and phosphorus than those occurring in the ocean.

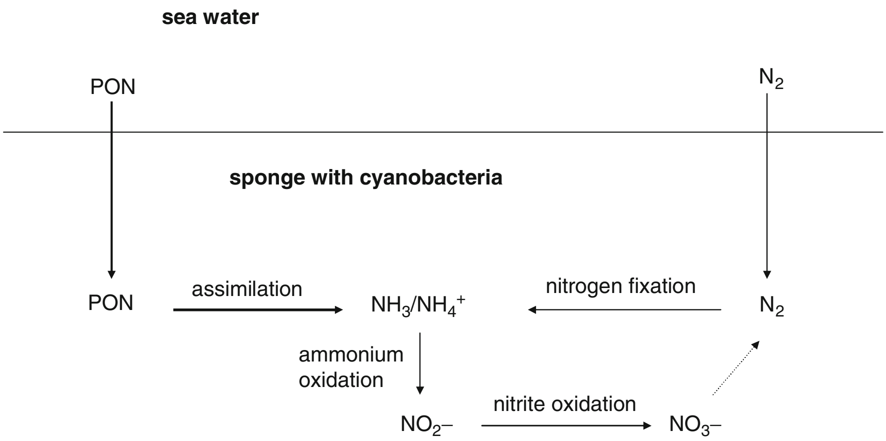

Figure 5. Model of inorganic carbon uptake by sponges. (After Taylor et al., 2007.)

Protection from predation by flagellates and ciliates may be another benefit to the cyanobacteria that are embedded in the host tissue (Usher, 2008).

9. Future

The origin of photosymbiosis is about 200–250 mya (Wood, 1998, 1999; Karako et al., 2002). The genus *Symbiodinium* spp. (clade A) was facilitated by a cooler seasonal global climate during the Eocene period ~50 mya, which promoted regional differences and biodiversity, including the appearance of many modern coral families of scleractinian corals. Clades E, G, and D appeared around 40 mya, and clade B appeared in the early Oligocene ~26 mya, during a warming trend (see Pochon and Pawlowski, 2006). During the last few years, it seems that clade D has had a higher tolerance to thermal stress than clade C, suggesting that corals harboring clade D are more resilient to coral bleaching events, becoming dominant after a bleaching event (Rowan, 2004; Jones et al., 2008). The evolutionary history of *Symbiodinium* suggests that a long-term increase in water temperature may significantly reduce *Symbiodinium* diversity, constituting a serious threat for the survival and diversity of coral-reef ecosystems (Pochon and Pawlowski, 2006).

Symbiosis that is based on trading energy for strategic material is well adapted to tropical oligotrophic water. During these millions of years, the relationship in the association developed through the process of evolution created one of the most impressive biodiverse biological structures of the world, the Great Barrier Reef, which represents our planet even from satellite view.

Since the Industrial Revolution, the increase in ocean temperature and the elevation in the level of pCO_2, which are followed by pH decrease in addition to eutrophication and local pressure, are a major stress and threat to these associations (see in Carpenter et al., 2008; Stambler, 2010). These increases have already led to worldwide damage, bleaching, and death of entire reefs. The extinction risk is now much greater than it was before recent massive bleaching events. Some scientists estimate up to 60% coral mortality globally within the next few decades, and extinction of corals reefs in this century (Hoegh-Guldberg, 1999, 2005; Hoegh-Guldberg et al., 2007; Veron, 2008a, b). We must all do as much as possible to protect these associations, so that corals will be able to adapt to global and local changes.

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THE ROLE OF RHODOLITH BEDS IN THE RECRUITMENT OF INVERTEBRATE SPECIES FROM THE SOUTHWESTERN GULF OF CALIFORNIA, MEXICO

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1. Introduction

Rhodoliths are free-living forms of non-geniculated coralline red algae (Corallinaceae, Rhodophyta) that form extensive beds worldwide over broad latitudinal and depth ranges (Foster, 2001). Synonymous with the maerl beds common in the northeastern Atlantic, rhodolith beds are hard benthic substrates, although mobile, made up of branching crustose coralline thalli. Collectively, they create a fragile biogenic matrix over carbonate sediment deposits thought to be the result of long-term accumulation of dead thalli (Bosence, 1983a). A wide morphological variation of individuals exists and appears to be in response to variation in physical factors (Bosence, 1983b; Steller and Foster, 1995). This variation in morphology and incorporation of whole rhodolith and carbonates into the fossil record have led to their use as paleoindicators of environmental conditions (Foster et al., 1997). Unconsolidated rhodolith deposits have long been harvested for human use as soil amendment in European waters (Blunden et al., 1977, 1981). However, recent studies have shown that beds are highly susceptible to anthropogenic disturbance such as trawling harvesting and reduced water quality (review in Birkett et al., 1998). Slow rhodolith growth (Rivera et al., 2003; Steller, 2003) combined with the negative impacts of burial makes the recovery after disturbance predictably slow. Foster et al. (1997) found rhodolith beds to be very common in the Gulf of California and suggested that there are two main types of beds: wave beds in shallow water (0–12 m) that are influenced by wave action (Steller and Foster, 1995), and current beds in deeper water (10 to >30 m) that are influenced by currents. Both the types, but especially current beds, are also influenced by bioturbation (Marrack, 1999). In order to persist, these algal beds require light, nutrients, and movement from water motion (waves and currents) or bioturbation, which maintain them in an unattached and unburied state (Bosence, 1983a, b; Marrack, 1999).

The structure of individual rhodoliths influences the abundance patterns in the cryptofaunal assemblage. Intact complex thalli, along with high rhodolith densities, are important factors driving this pattern. Complex thalli may provide more space, refuge, and resources through increased interstitial or interbranch space. As a result, rhodolith complexity appears to be a good predictor of abundance and, potentially, for richness. This matrix provides habitat for diverse assemblages of invertebrates and algae (Cabioch, 1968; Keegan, 1974; Bosence, 1983a, b; Steller et al., 2003). This also supports the hypothesis that availability and shape of interstitial cavities is important for the associated crustaceans' assemblage (De Grave, 1999). Variation in physical factors, thought to influence rhodolith morphology (Bosence, 1983a; Steller and Foster, 1995), may therefore directly influence community structure. Thus, we predict that conditions that enhance structural complexity increase the available refuge among the rhodolith branches, and enhance overall species richness and abundance. Rhodolith beds support a rich community of flora and fauna found to be higher in species diversity than soft-sediment benthos alone (Steller et al., 2003). Organisms within a bed can associate with the surface of algal thalli (epi-fauna or flora) within the branches (crypto-fauna or flora) or in the underlying sediments (in-fauna or flora) (Steller et al., 2003). Factors influencing diversity patterns include increased architectural complexity and grain size, reduced sedimentation (Grall and Glemarec, 1997) and seasonal variation (Ballesteros, 1988), and reduced predation.

Bivalves have been shown to be abundant and associated with rhodolith beds and in the NE Atlantic (Hall-Spencer, 1998, 1999). Possibly this is due to larval settlement preferences for coralline, structured or large grain substrates, or refuge from predation. Depth stratification of bivalve species may also be related to variability in the substrate type (Steller, 2003; Kamenos et al., 2004). The high density of bivalves at intermediate bed depths may reflect larval attraction to the structured settlement substrate provided by the rhodoliths or physical conditions found there. In addition, Steller and Foster (1995) found that rhodolith turnover and protection from burial was greater at shallow versus intermediate depths, suggesting that the latter affords reduced sedimentation and water flow favored by surface dwelling bivalves. Increases in summer densities may correspond to winter/spring recruitment periods of many species. It appears that rhodolith beds may positively enhance bivalve populations. However, there is a clear conservation problem between these positive attributes and the degradation resulting from commercial fishing (Hall-Spencer, 1998, 1999; Hall-Spencer and Moore, 2000).

Studies have shown that rhodolith beds support a diverse and dynamic benthic community (Ballesteros, 1988; Grall and Glemarec, 1997). Community descriptions of diversity include common associated species comprising cryptofauna living within interstitial cavities in rhodoliths (Steller, 2003; Hinojosa-Arango and Riosmena-Rodriguez, 2004; Foster et al., 2007). The density of the associated species varied in relation to the size of the rhodolith and the density of the bed (Steller et al., 2003). Recently, Hinojosa-Arango and Riosmena-Rodriguez

(2004) have shown that criptofauna assemblages were organized independently of the main rhodolith species or growth-form S that compose a bed. Steller et al. (2003) also have shown that rhodolith beds are relevant habitat S for scallop recruitment, but little is known about their role for invertebrate recruitment. We hypothesize that, due to different physical settings, the cryptofaunal species will differ in the proportion of juvenile and adult-relative abundance. In addition to the above-mentioned evaluation, seasonal trends will be evaluated as an alternative source of change, as is clear in the bed flora and macrofauna (Steller et al., 2003). Thus, the aims of the present study are to determine if the rhodolith beds are a relevant habitat for cryptofaunal assemblages in the southwestern Gulf of California and if this relationship varies with bed type of season.

2. Sites and Methods for Data Gathering

Rhodoliths were collected in four rhodolith beds (Fig. 1) in central southwestern Gulf of California during winter 1995 and summer 1996. Sampling dates and localities were: wave bed at Isla Coronados (26°06′ N 111°17′04″″ W; 7 m depth) on 17 November 1995 and 2 September 1996; wave bed at Diguet off Isla San Jose (24°53′45″″ N, 110°34′45″″ W; 7 m depth) on 2 February 1996 and 14 July 1996; current bed off Isla San Jose (24°52′36″″N, 110°32′07″″ W; 12 m depth on 4 February 1996 and 16 July 1996; and current bed in Canal de San Lorenzo (24°22′60″″ N, 110°18′41″″ W; 12 m depth) 3 December 1995 and 14 June 1996. All localities are being described in detail by Riosmena-Rodríguez et al. (2010).

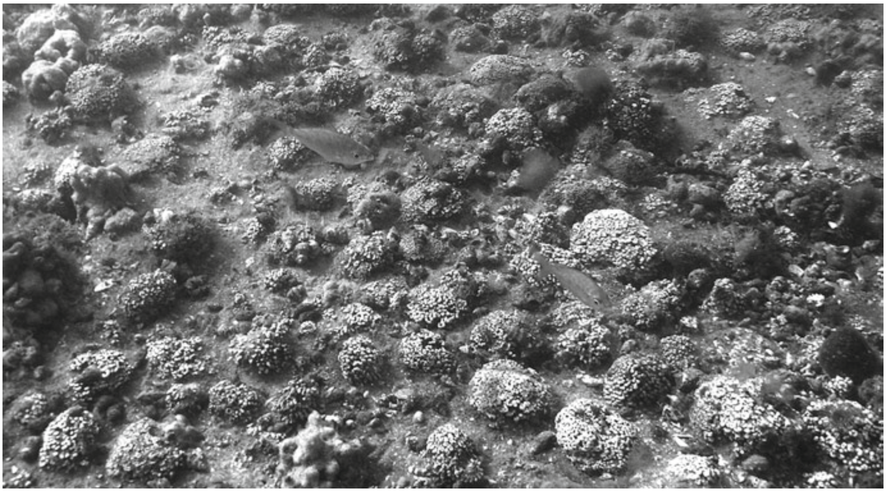

Figure 1. General view of a rhodolith bed in a wave bed.

A similar sampling design was used in each locality and date. Two 20-m transects, at least 50 m apart, were haphazardly positioned within each bed (origin at the boat anchor). The four largest rhodoliths were collected nearest to each 2 m increment along the transect (40 rhodolith/transect). Individual plants were put in separate plastic sealed underwater at the time of sampling. After collection, all materials were fixed in 4% formalin in sea water. At the laboratory, each transect of the 40 rhodoliths forms were examined, and individuals were selected for standard size (3–5 cm dim.) and branch density (4 branch tips/cm^2 at the rhodolith surface). All plants selected had the fruticose growth-form described by Woelkerling et al. (1993). Branch densities were determined as in Steller and Foster (1995). Twenty rhodoliths per site/date were selected and used in the analyses for a total of 160 rhodoliths for the entire study.

Each selected plant was dissected and all animals of >0.3 mm were extracted, segregated into groups, and placed in 70% alcohol. Taxonomic determinations to lowest taxa possible were based on Smith and Carlton (1975; Cnidarians and Amphipods), Harrison and Ellis (1991; Isopods), Sieg and Winn (1978, 1981; Tanaidaceans), Wicksten (1983; Carideans), Salazar-Vallejo et al. (Polychaetes, 1989), Bastida-Zavala (1991; Polychaetes), and Brusca (1980; Echinoderms). Abundance of each species or taxon per rhodolith from each site and date were determined. Only data from Class Turbellaria, Class Polychaeta, Subphyllum Crustacea, Class Asteroidea, Class Ophirouridea, and Class Equinoidea were analyzed because of their abundance and clear trend in adult/juvenile morphology. Normality (Kolmogorov $\alpha = 0.05$) and homoskedasticity (Cochran y Barlett $\alpha = 0.05$) assumptions were met for richness and for abundance data transformed log10. Differences among life stage, location, and season (winter 1995 and summer 1996) were examined with three-way ANOVA (model 1).

3. General Findings

A total of 5,066 organisms were found in 160 rhodoliths sampled, in which 85% of the fauna were possible to identify at least to genus or species level. Overall, 60% of the collected individuals were juvenile (which included recently settled larvae and organisms that are starting to grow) and 40% adults. Our comparison strongly support the idea that most cryptofaunal species found in samples were juveniles but have variations in relation to bed type and season (Figs. 2 and 3). Trends varied in relation to taxonomic group and potentially with richness. Turbellaria and Echinodermata were found with low species number, while crustacean, mollusks, and annelids were found with higher species numbers.

Three species of Turbellaria were determined and all the specimens were new recruit (Figs. 2 and 3). Seven species of Echinoderms were found (Appendix 1) with significant differences between seasons, where only juveniles were observed in winter and a higher proportion of adults were observed in summer in both the beds.

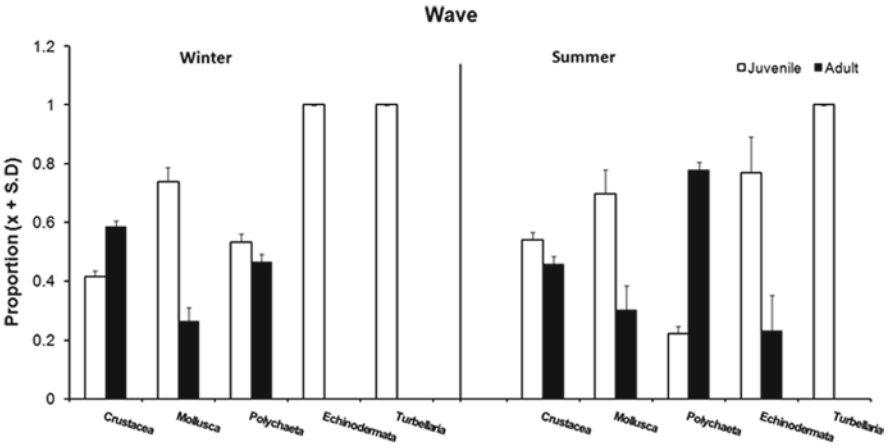

Figure 2. Comparisons in wave beds between the proportion (mean and std) of juvenile and adult invertebrate species.

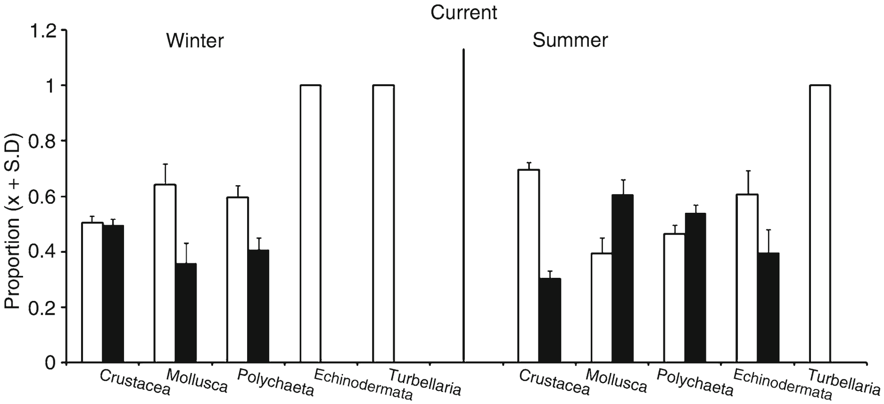

Figure 3. Comparisons in current beds between the proportion (mean and std) of juvenile and adult invertebrate species.

Crustacea, Mollusk, and Annelida were the taxa with higher number of species, wherein we found a tendency in the seasons and/or bed type. We found 21 species of crustaceans, where the juvenile individuals were more abundant than the adults overall (Figs. 2 and 3); this trend is particularly clear in current beds, where we found higher differences among the lifecycle stages (Fig. 4). In the case of wave beds, we found an inverse relationship between seasons with high proportion of adults in winter and high proportion of juveniles in summer. Forty mollusks species were present in the samples but with contrasting patterns. With regard to mollusks juvenile/adult proportion in the current beds, juveniles were

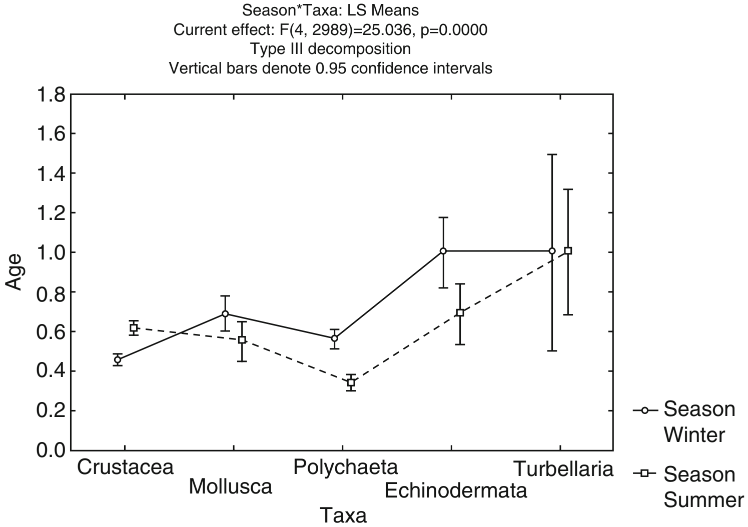

Figure 4. Statistical seasonal comparisons between the major phyla analyzed (age is relative to life span of the animal).

significantly higher in winter, and more adults were found in summer (Fig. 2). In contrast, juveniles in wave beds were significantly higher in both the seasons (Figs. 3 and 4). In Polychaetes, the seasons (summer/winter) have the most consistent differences, with higher juvenile proportion in winter and adults in summer. Differences were also found between the bed type, but this may be an artifact of seasonal variation. We found significant statistical seasonal variation in Crustacea, Polychaetes, and Echinodermata (Fig. 4). However, in mollusks, the significant differences were more related to bed type.

Rhodolith beds are one of the marine habitats, which have been considered for designing marine reserves in the Gulf of California (Sala et al., 2002; Anaya-Reyna et al., 2005) and elsewhere (Birkett et al., 1998). The extensive cover of seafloor by this habitat is a key element for their ecological value (Hetzinger et al., 2006) in relation to the associated biodiversity (Steller et al., 2003; Hinojosa-Arango and Riosmena-Rodriguez, 2004). Our study has shown that rhodolith beds are also the habitat for recruitment of many invertebrate-associated species. Some of them (scallops) are part of the fisheries of the area (Steller, 2003). Also, there are species that are considered in some category of protection (as many species of corals and equinoderms), which are found continuously in the beds (Riosmena-Rodríguez et al., 2010) and started their recruitment as cryptofauna. A similar situation is present in several beds around the world (Hopkins et al., 1991; Birkett et al., 1998) in which rhodolith beds are not only the habitat for adult species but also the recruitment habitat. This situation might enhance the number of species present and their abundance over years. We could not determine mortality among the samples, but it was clear that scallops larvae were present in abundant numbers inside the sea anemone that lives commonly as part of the cryptofauna.

It is well known that the surface of the thalli of coralline algae is a common space for recruitment to many invertebrates (Johnson et al., 1991). This surface is also used as a “signal” to find a hard structure (whether if the hard structure is a free-living species or a species attached to the rocks). In some cases, rhodolith beds might be just the contact point for these larvae, because at a later stage, they will migrate to the surrounding areas (Steller, 2003). The continuous presence of juvenile stages within a rhodolith is strongly related to the trophic structure of each bed (Grall and Glemarec, 1997). This close relationship between scallops and rhodolith as a recruitment habitat explain the extensive mollusk bank that has been a formal fishery (Hall-Spencer, 1998). The impact of fisheries might produce the lack of larvae in the water column and thus inhibit recruitment. An example of this situation can be seen when, according to historical analyses, rhodolith beds were the recruitment habitat for the pearl oyster at the beginning of the twentieth century, but absent since the fisheries collapsed (Steller et al., 2003). A similar situation happened in 1991, when rhodolith beds were heavily impacted by a combination of factors (Steller et al., 2003). These factors were related to high number of fishing permits and lack of critical analysis of the collecting procedures. Most of the fishermen used to dive with Hokka and leather shoes, who crushed and buried rhodoliths making impossible for their yearly recovery and maintained that condition over the last 17 years.

Rhodoliths (maerl) in the Gulf of California urgently need to be considered in the management plan of benthic fisheries (such as scallops, octopus, and trawling fisheries like shrimp). In fact, a management plan for rhodolith beds might also be a great strategy due to their presence in several habitats and depths (Riosmena-Rodríguez et al., 2010, Steller et al. 2009). In addition, their value as monitoring organisms is more than justified based on fisheries needs and the presence of protected species. Similar approaches are currently in place for European waters (Birkett et al., 1998).

4. Summary

The role of rhodolith beds as a recruitment habitat for their associated cryptofauna was evaluated based on seasonal sampling over a series of beds representing two different microhabitats (current and wave beds). Our basic goal was to understand if the cryptofauna was composed mostly by juveniles or adult individuals of each species. Another goal was to understand rhodolith beds as critical habitats for conservation purposes worldwide. We collected 160 rhodoliths and more than 5,000 individuals of 116 species. Turbellaria and Echinodermata are phyla with low number of represented species and mostly juveniles were found with no seasonal variation for the first group and a small variation for the latter. Crustacea, Mollusks, and Annelids were the taxa with higher number of represented species, and a tendency for a particular season and/or bed type was found. Crustaceans were found mostly as juvenile individuals (other than adults) overall in the current beds and with an inverse pattern in wave beds. With regard to Mollusks juvenile/adult proportion in current beds, juveniles were significantly higher in winter, and more adults were found in summer, while in wave beds, juveniles were significantly higher in both the seasons. In Polychaetes, the seasons (summer/winter) have the most consistent differences with higher juvenile proportion in winter and adults in summer. Currently, rhodolith beds are being considered as a relevant habitat in the near-shore areas in the Gulf of California. However, a classification between current and wave beds need to be taken into consideration for protection purposes. Current results have shown the value of rhodolith beds as a recruitment habitat for many species and the urgent need to be considered for fisheries management; there is also a need for a management plan for the habitat itself.

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Biodata of **John J. Lee** author of ***"Fueled by Symbiosis Foraminifera have Evolved to be Giant Complex Protists"***

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FUELED BY SYMBIOSIS, FORAMINIFERA HAVE EVOLVED TO BE GIANT COMPLEX PROTISTS

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1. Introduction

One may wonder in the framework of this book, and in context with the well-balanced chapter by Stambler, why foraminifera have been singled out to be the focus of a separate chapter. The answer is simple. Foraminifera are generally less well known, and they exemplify the power by which symbiosis can drive evolution of a predisposed and malleable group of organisms Lee and Hallock, 1987. As foraminifera are relatively small, when compared with corals and other invertebrates in the same semi- and tropical well-illuminated marine habitats, they are easily overlooked. At times, they form beaches of "living" and "star-sands" (Fig. 1e), and are so abundant that they can be scooped up with a spoon. Snorklers and SCUBA divers can see them as underwater "Christmas tree ornaments" on sea grasses or on macrophyte algae (Fig. 1c). Their tests are composed of $CaCO_3$ and they fossilize well. Testimonial to their abundance in the Tethys Sea are the mountains of fossilized limestone formed from their tests (Fig. 1a, b, d) and quarried to build the Egyptian pyramids. Also intriguing is the fact that different types of algae have driven various lines of foraminifera to evolve tens to hundreds of times larger in size and considerably more complex than their ancestors Hallock, 1985. Modern larger foraminifera are the hosts for a greater variety of symbionts than any other marine group (Lee, 2006). With respect to symbiont type, there is some specificity. Those larger foraminifera that normally host diatoms have never been observed to host dinoflagellates. Similarly, those that host dinoflagellates never host diatoms, chlorophytes, or rhodophytes and so forth.

Transfer of nutrients between symbiotic partners has been probed a number of times. Feeding on algae and bacteria seems to be the nutritional major pathway (Faber and Lee, 1991; Lee and Bock, 1976; ter Kule et al., 1987), although there is evidence from one laboratory (Röttger, 1972) that starved cultures of one foraminifer *Heterostegina depressa* that thrived in the light relying only on their algal photosynthates. The release of photosynthetates from the symbionts to the host has been demonstrated several times (Kremer et al., 1980; Lee et al., 1984). As mentioned later (Section 3.5), the addition of sterile host homogenate to axenic cultures of the symbionts stimulates the release of photosynthates. Morphological evidence suggests that the cellular sheath fibers of the red algal symbiont of *Peneroplis* (Section 2.3) are constantly digested by the host. Most recently, TEMs of the new species of

Figure 1. (**a**) Mountain of fossil numulitic foraminifera at Taba, Egypt; (**b**) close up of a weathered edge of the limestone showing profiles of numulites; (**c**) seaweed at Taba (15 m depth) showing that the larger foraminifera are abundant today on the sea floor and easily seen by a SCUBA diver. The larger white discs are *Amphisauris hemprichii* and the smaller oblate spheres are *Amphistegina spp.*; (**d**) pyramid at Giza, Egypt, built of numulitic limestone; (**e**) "Star sand" (*Baculogypsina*) on Tangke Beach, Sipan (Photo by Alan Davis) (scale = 4 mm); (**f**) and (**g**) two modern numulitic foraminifera; both are hosts of diatom symbionts, (**f**) *Operculina ammonoides* (scale = 0.5 mm) and (**g**) *Heterostegina depressa* (scale = 0.5 mm).

Marginopora suggest that apotosis or autolysis seems to play a role in the transfer of nutrients from the symbiont in that host (Fig. 10a–c). Division of the symbionts is often observed (Fig. 9c, d), making the speculation possible, but the correlation between rates of apotosis and new cell formation needs to be carefully studied.

2. Symbiotic Algae

2.1. DIATOMS

Although diatoms are abundant in the plankton and the photic zone of the benthos of every coastal environment and are a major food source for many different types of organisms, their association as symbionts is uncommon. This in itself is extraordinary, given the fact that many different laterally transferred symbiotic relationships have evolved from smaller organisms that have been taken in by herbivores as food, but which have escaped digestive processes. Given the breadth of their consumers and the frequency of their contacts, one might expect greater numbers of associations of diatoms with marine invertebrates or other protists. On the other hand, a number of different and distantly related families of foraminifera, Alveolinidae, Calcarinidae (Figs. 1e, 2f, and 4a–d), Amphisteginidae Figs. 2f and 5a–e), and Numalitidae (Fig. 1f, g) are hosts for endosymbiotic diatoms, and through time, have evolved in size and complexity to become part of the collective group known as larger foraminifera. Another family of foraminifera, Elphidiidae (Fig. 3a–e), has members that sequester photosynthetically active plastids from diatoms. The diatoms involved in symbiotic relationships are taxonomically quite diverse. They share one common characteristic. On their surfaces,

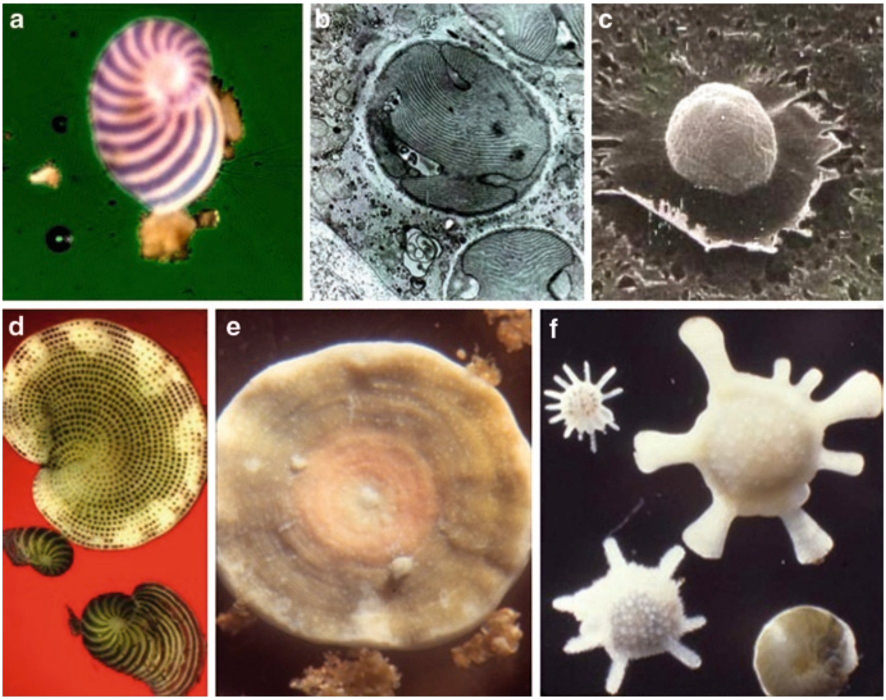

Figure 2. (**a**) *Peneroplis*, a red algal symbiont-bearing foraminifer (scale = 0.5 mm); (**b**) TEM of *Phorphridium puperum*, the red algal symbiont *in hospite* in *Peneroplis.* Note the less dense cytoplasm surrounding the symbiont (scale = 1 μm). There is no symbiosome membrane. Arrow points to golgi apparatus of symbiont; (**c**) a cultured *Phorphridium puperum* on the surface of a filter (scale = 1 μm). The fried-egg appearance is due to the jelly-like fibrillar cellular envelope being sucked off the external cell surface; (**d**) archaiasins, green algal –bearing hosts; (**e**) a disc-shaped soritid showing target-like zonation. Symbionts are densely packed in the middle chamberlets (scale = 0.5 mm). They are less dense in the outermost chamberlets and in the juvenile chambers; (**f**) Three “star sand” species, *Calcarina defrancii*, *Calcarina hispida*, *Calcarina gaudichaudii*, and the “living sand” *Amphistegina lobifera* (scale = 1 mm).

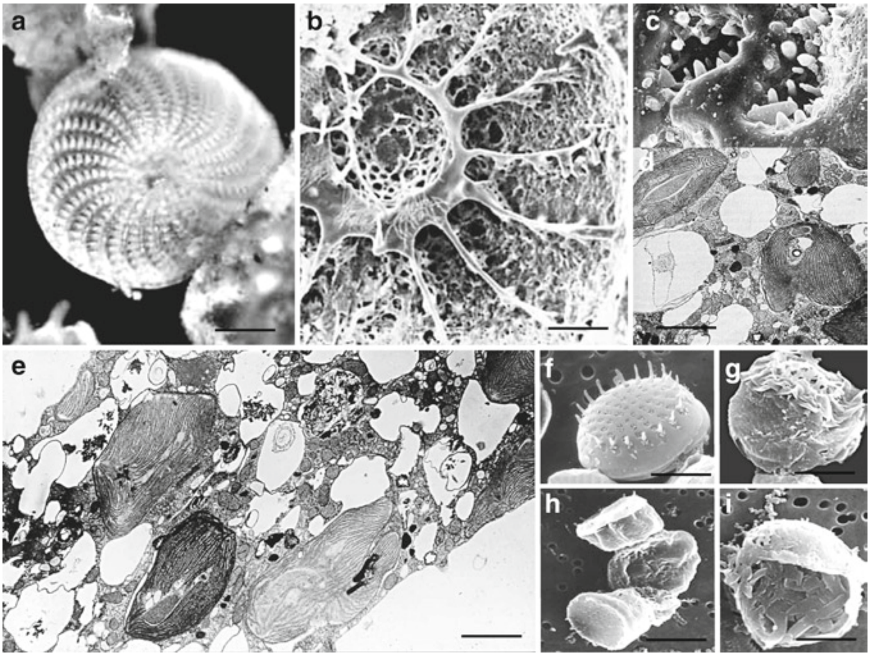

Figure 3. (**a**) *Elphidium* showing sutural fossae (scale = 200 μm); (**b**) SEM of the inside of *Elphidium clavatum* showing canal system; (**c**) diatom trapped by the denticles lining the fossae (scale = 20 μm); (**d**) TEM of diatom plastids *in hospite*; (**e**) TEM of typical chamberlet of *Elphidium clavatum* showing plastids in various states. There is more than one plastid in the symbiosome vacuole to the lower right (scale = 3 μm); (**f**) SEM of the frustule of *Nannofrustulum shiloi* from an axenic culture (scale = 2.5 μm); (**g**–**i**) Cells from an axenic culture of *Nanofrustulum shiloi* treated with host homogenate (scale = 2.5 μm) (see text).

they possess a 104-kDa glycoprotein that is absent in all non-symbiotic species. Based on isolation techniques, over 3,000 diatom hosts have been examined. Only a small number of different diatom species (≈25) have been identified as endosymbionts. Six species, *Nitzschia frustulum var. symbiotica*, *Nitzschia panduriformis var continua*, *Nitzschia laevis*, *Nanofrustulum shiloi*, *Amphora tenerrima,* and *Amphora roettgerii* accounted for 75% of all the isolated symbionts. Usually, there is only a single species of endosymbiotic diatom in each foraminifer, but sometimes, two or three species have been recovered from the same host (summarized and reviewed in Lee and Correia, 2005). Hosts do not seem to be finical with respect to the symbionts that they harbor. The same host species can harbor many different species of diatoms. The latter observation is based on opportunistic sampling and taunts deeper investigation. Systematic sampling of the same host in different seasons, over long transits, along environmental gradients is needed to clarify our knowledge of the adaptive range of foraminifera and the diatom symbionts that they host.

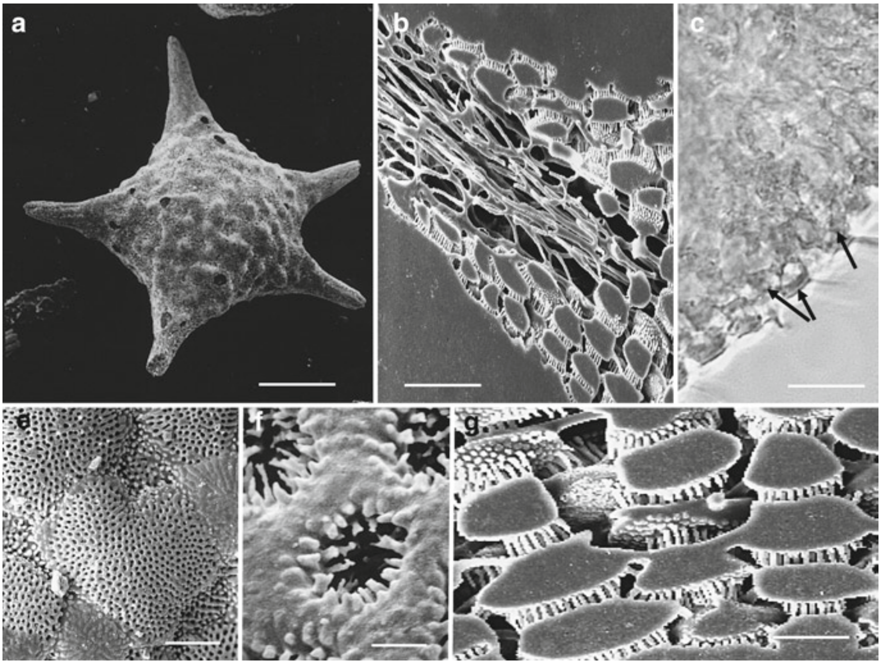

Figure 4. (**a**) SEM of *Baculogypsina sphaerulata* (scale = 0.5 mm; (**b**) Hottinger cast of a polished section midway through a spine showing spinal canals and peripheral chamberlets. The gray areas are where seawater or cytoplasm were in life; the dark spaces are where the shell was before it was dissolved by EDTA or acetic acid (scale = 200 μm); (**c**) Phase-contrast view of a histological section at the periphery of a chamberlet. Arrows point to individual symbionts. Straw-like structures are pore liners (scale = 20 μm; (**d**) SEM of blisters on the surface of *B sphaerulata* showing pores (scale = 200 μm); (**e**) Enlargement showing the pore with its denticles (scale = 5 μm; (**f**) SEM of Hottinger cast of a polished-section chamberlets at the periphery of the cell showing connecting tubules (scale = 50 μm).

In sharp contrast to our knowledge of *Symbiodinium* from various hosts, there is a dearth of information about variation within the species of diatoms involved in symbiosis. Only the most common endosymbiotic diatom (isolated in ≈30% of the host samples), *Nitzschia frustulum var. symbiotica*, has been studied in this respect. It is morphologically quite variable (Lee et al., 2001). One study of four endosymbiotic diatoms in culture suggested differences in their optimal irradiance (Lee et al., 1982). *Nannofrustulum shiloi* and Nitzschia laevis isolated from Amphistegina lessonii grew best in the highest light level in which they were tested (312 μW cm^{-2}). The two endosymbiotic diatoms isolated from a shade or deep-dwelling host species, *Heterostegina depressa*, were photoinhibited at high light intensities and grew best at the lowest light level tested (19 μW cm^{-2}).

One might formulate the following logical hypothesis: as larger foraminifera actively feed on diatoms, and as they can host a variety of different species, is it probable that the symbionts hosted are a reflection of the most abundant species

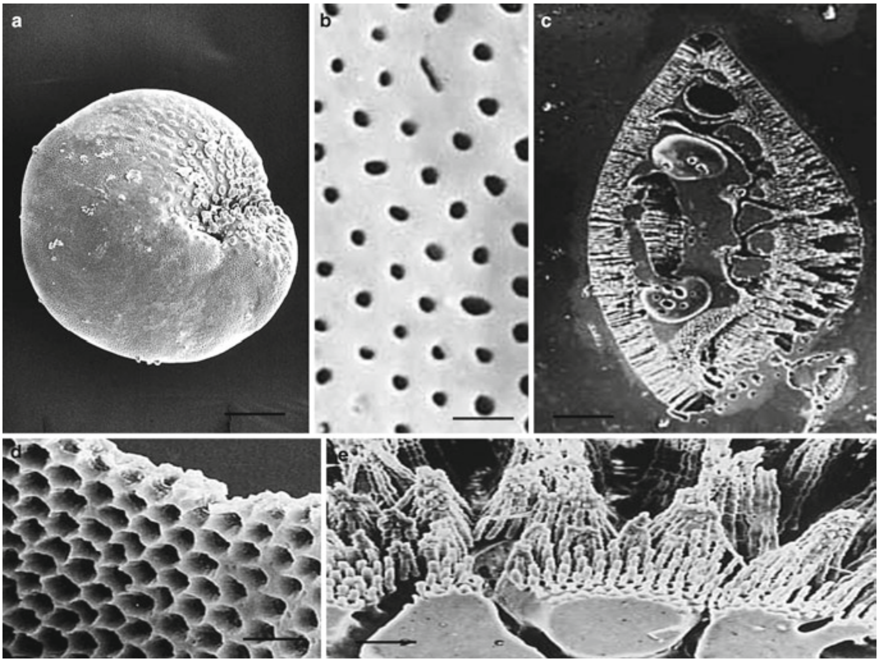

Figure 5. (**a**) SEM of umbilical side of *Amphistagina lessonii* from Eilat, Israel (scale = 0.5 mm); (**b**) Enlargement of the surface showing the pores (scale = 20 μm); (**c**) SEM of a Hottinger cast of a polished section of *A. lessonii* showing pore canals leading to each symbiont in a chamberlet (scale = 200 μm); (**d**) SEM of the interior of a test showing the expanded pore rims. An individual diatom fits into each pore rim (scale = 20 μm). Transfer of nutrients from seawater in the pore canal through the host cell membrane and through the symbiosome membrane to the symbiont probably takes place; (**e**) SEM enlargement of the surface of three chamberlets showing greater detail of the arrangement of the pore rims and pore canals at the cell periphery (scale = 50 μm).

available in the community? Observations from systematic searches in the SEM of *Halophila* leaflets collected from habitats in the Gulf of Eilat with abundant populations of diatom-bearing hosts falsified this hypothesis. Diatoms found as endosymbionts were rarely encountered in the habitat of the foraminifera. They are starving propagules when released from their hosts to the environment. This finding is in accordance with nutritional studies of axenic symbionts in batch culture. They grow best in cultures with N and P levels that are 1–2 orders of magnitude higher than that they encounter in the Gulf of Eilat.

Many other questions arise. Foraminifera have biphasic lifecycles, one phase of which reproduces asexually, so that the symbionts are acquired by direct vertical transfer from parent to offspring. Does that make the hosts more vulnerable to environmental stress or can established symbionts be replaced by other species? Do symbiont species compete with each other for niche space within a host? Can environmental factors affect the competitive survivability of one endosymbiont species over another in a particular host? We speculated that the species composition of symbionts would change, given the opportunity to do so, because different symbionts vary in their reproductive rates under different environmental conditions giving them a competitive edge in filling the symbiont niche in their host. While not investigated very deeply, two experiments probed this hypothesis (Lee et al., 1983, 1986). *Amphistegina lessonii* were incubated in sea water containing DCMU (3-(3,*N*-1,4-dichlorophenyl)-1,1 dimethyl urea), until they were bleached (rendered nearly aposymbiotic). They were then transferred to modified tissue culture flasks with windows cut in their sides, which were covered with durable filters. The filters had 3-μm pores so that the test algae and the foraminifera were retained, while at the same time, seawater freely passed through. Except for the control flask, the foraminifera were incubated with mixtures of 3 different types of algae: 1) diatoms species previously isolated as symbionts; 2) freeliving diatom species; 3) *Chlamydomonas provasoli*, a green symbiont isolated from another larger foraminifer, *Cyclorbiculina compressa.* Replicate flasks were incubated at either 10 or 20 m seaward of the Steinetz Marine Biological Laboratory. After several weeks of incubation, the experiment was terminated and the hosts and their symbionts were examined. None of the free-living diatoms or *C. provasoli* were recovered. The control cultures "rebrowned," indicating that their original symbionts, *Nitzschia panduriformis* and/or *Nanofrustulum shiloi*, divided and refilled the host symbiont niches. However, in the experimental flasks, *Nitzschia laevis* or *Nitzschia valdestriata* replaced them at 20-m depth. In a second experiment, the hosts initially had *Nitzschia laevis* as their endosymbiotic algae. They were less easily displaced than the *Nitzschia panduriformis* in the "rebrowning" process after aposymbiotic stress. While the experiments showed that replacement is possible, they really only tantalize further investigation. Is there an adaptive value to symbiont selection (or persistence)? Much more detailed studies are eagerly awaited.

Diatom plastids sequestered by species of Elphidiidae are photosynthetically active as they are in their natural state (Lee et al., 1988; Lopez, 1979). Studies of this symbiotic phenomenon by Correia and Lee (2000) showed that in culture, each *Elphidium excavatum* retained ≈3.7×10^4 plastids. This number is slightly higher than the number that Lopez (1979) found for fresh specimens of *Elphidium williamsonii* (9.7×10^3), *E. excavatum* (1.2×10^3), and *Haynesina germanica* (5.2×10^3) collected from the shallow waters of Limfjorden, Denmark. Lee and Lee (1990) noted that lower numbers of plastids were sequestered by *Elphidium crispum* collected near Drake's Island in Plymouth Harbor, England. Fine structural studies and photosynthetic pigment analyses suggest that most plastids are in a good state of preservation and are surrounded by host vacuoles (Fig. 3c, d) (Correia and Lee, 2002a; Knight and Mantoura, 1985; Lee et al., 1988; Lopez, 1979). Feeding experiments agreed with fine structural and pigment analysis. Only diatom plastids were retained by *E. excavatum* (Correia and Lee, 2000). Experiments with starved *E excavatum* in the laboratory indicated that plastids decreased over time. The number of plastids remaining in the cytoplasm of the foraminifer was higher for foraminifera that were incubated in a 12-h day/

night cycle than it was for those incubated in complete darkness. The half-life of the plastids of *Amphora coffeformis* was estimated to be ≈9 weeks (Correia and Lee, 2002b).

2.2. DINOFLAGELLATES

It seems strange that only recently we have begun to understand the diversity of the dinoflagellate symbionts in the family Soriticiidae because they were the first symbionts of the larger foraminifera to be described in detail (Doyle and Doyle, 1940). However, in retrospect, it is understandable. The symbionts are small (7–15 μm) and looked very similar *in hospite.* Until recently, cultural methods were problematic and the lifecycle stages could not be studied in detail. Beginning in the mid-1970s, Trench and his students (Chang and Trench, 1982; Iglesias-Prieto et al., 1992; Leutenegger, 1977; Schoenberg and Trench, 1980a, b, c) began to accumulate evidence of the diversity among the zooxanthellae belonging to the *Symbiodinium* species complex. Emphasis on morphological, physiological, and behavioral differences quickly gave way to molecular methods as soon as they became available. Molecular phylogenies based on nrDNA recognize between four and ten distinct clades of *Symbiodinium* (Baker, 2003). From the beginning (Langer and Lipps, 1995; Lee et al., 1995), it was clear that the symbionts from foraminifera and those from corals, other invertebrates, and ciliates were related to each other (reviewed in Baker, 2003; Pochon et al., 2001, 2006). Foraminiferan lineages fall mainly in *Symbiodinium* clades F, G, and H (Pochon et al., 2006). Some molecular evidence has been gathered to suggest that there may be host–symbiont specificity in soritid foraminifera (Garcia-Cuetos et al., 2005; Pochon et al., 2004). Recently, studies have returned to looking at other characters that might be used to define specific epithets for the symbionts (Lee et al., manuscript submitted). The isolates from soritiecean foraminifera are quite different from each other and already-named species. Starting with characters that have been used to distinguish species of *Symbiodinium* and other dinoflagellates, there was an examination of the characteristics of 16 different isolates of endosymbionts from soriticean foraminifera. The overall size was a potential character, as where the lifecycle behavior, settling pattern of zoospores, characteristics of the cell surface, chromosome numbers, nuclear to cell volume ratio, plastid architecture, presence or absence of membranous bodies, nucleoli or a nucleomorph, and phylotypes. Of doubtful use were characteristics of the accumulation body, oil droplets, oxylate crystals, number of pyrenoid stalks, and tabulation of surface plates. Very briefly, the symbionts of soritids fall into two morphological groups: (1) those that possess reticulate plastids similar to those of the type species of *Symbiodinium microadriaticum* from *Casseopea frondosa* (Fig. 11f) and (2) those that have petal-like plastids (Fig. 11c, d). Evidence at this point is fragmented, but it seems that all the species described so far (from corals and invertebrates) have reticulate plastids. The *Symbiodinium* with petal-like plastids have only been found in some soritid hosts. TEM studies of a petal-like plastid bearing zooxanthella *in hospite* in a new species of *Marginopora* from the Heron-Wistori Channel (GBR. Australia) revealed that this alga had a prominent nucleolus, membranous vesicles, and a nucleomorph in the plastids (Fig. 11a, b, h) (Lee et al., manuscript submitted). None of these structures have been observed so far in other *Symbiodinium* spp *in hospite* or *in vitro.* The TEM clearly shows that *in hospite,* the new species of *Marginopora* from the Heron-Wistori Channel does not have plates in its alveoli (Fig. 11g). Clearly, the data obtained so far on species diversity among the soritid symbionts are quite incomplete, but they provide promise that effort will yield meaningful data. *Amphidinium* spp. have been isolated from soritids, but the low frequency of their occurrence suggests they are only minor players in foraminiferal symbioses (Lee et al., 1997).

2.3. CHLOROPHYTES, CYANOBACTERIA, AND RHODOPHYTES

We know the least about these symbionts. Little work beyond isolation, identification, and fine structure has been done on the symbionts or holobionts. Our knowledge of the fine structure of the zoochlorellae from archaiasines comes from a collection of *Archaias angulatus* and *Cyclorbiculina compressa* from Key Largo, Florida (Fig. 2a) (Lee and Zucker, 1969; Lee et al., 1974, 1979; Müller-Merz and Lee, 1976). *Chlamydomonas hedleyi* was described as the symbiont from the former, and *C. provasoli* was described from the latter. In TEM sections, they are easily distinguished from each other by the structure of their pyrenoids. Molecular analysis later expanded the identities of the symbionts from all five extant genera of the archaiasines. They belong to the *Chlamydomonas eugametos* lineage, which cluster together suggesting a common ancestor (Pawlowski et al., 2001). The sequence divergence suggests that there may be more species than the two already described, but this remains to be investigated.

A unicellular rhodophyte, *Porphyridium purpurum*, has been identified as the endosymbiont of a soritine family, whose members host red symbiotic algae (Hawkins and Lee, 1990; Lee, 1990). The fine structure of the symbionts was compared with a unicellular rhodophyte, *Porphyridium purpurum*, which has been identified as the endosymbiont of a soritine family whose members host the red symbiotic algae (Hawkins and Lee, 1990; Lee, 1990). The fine structure of the symbionts was compared with a reference strain (UTEX 161) grown in the same medium and conditions, and on the basis of a morphospecies concept, it was considered that they were conspecific. Apart from the fact that the symbiosis with red algae is unusual, and perhaps unique to only one family of foraminifera, symbiont's biology is interesting in several respects. *In hospite,* the symbionts are not surrounded by a second or host-derived symbiosome membrane; they lie free in the cytoplasm of their host. This could be interpreted as an advanced symbiosis on the pathway to becoming a cellular organelle. However, as the symbiont can be grown in culture, it would seem that the symbiont has not transferred many or any of its genes to its host. In culture, *Porphyridium purpurum* has a very thick fibrous sheath, but *in hospite* it does not (Fig. 2b, c). The golgi are quite prominent *in hospite* suggesting that the sheath fibers are being secreted, but sheath-fiber digestion may be an important pathway for transferring energy from symbionts to their hosts.

Red cyanobacteria are common in some populations of soritids. They are not the only symbiont, but they are so abundant that in spite of the presence of *Symbiodinium* they give the holobiont the appearance of red bands. Rarely, the holobiont is totally red (See cover of *Symbiosis* 42 (2006). Red cyanobacteria are common in some populations of soritids. They are not the only symbiont, but they are so abundant that in spite of the presence of *Symbiodinium,* they give the holobiont the appearance of red bands. Most of the population of *Marginopora vertebralis* directly seaward of the Lizard Island Station (GBR) characteristically have a red medial band of reddish chamberlets (See cover of *Symbiosis* 42 (Lee, 2006). Rarely the holobiont is totally red. Illustrated on the same cover is a red *Amphisorus hemprichii* from the Gulf of Aqaba-Eilat. The cyanobacteria were illustrated (Lee et al., 1979) and isolated in culture. However, the cultures survived only a few transfers, challenging future investigators to find conditions that will support sustainable cultures. It seemed logical to see if the cyanobacteria fixed N in the holobiont association. An attempt using the acetelyene → ~~e~~thylene method was unsuccessful (J.J. Lee and J.B. Waterbery, unpublished, 2000). A probe for *nif* genes might answer the question.

A haptophyte was first observed in the histological sections of *Marginopora vertebralis* collected seaward of the Marine Station on Lizard Island, GBR, Australia. It is present in small numbers (~20) in almost every host from this site. The symbiont has been isolated in culture and appears to be a vegetative phase of a *Pleurochrysis* sp. A quantitative morphological approach was used to study the formation of the scales in the golgi apparatus and their transport to the surface of the cells (Hawkins and Lee, 2001). Filamentous actin was shown to be involved in the polarized secretion of the scales (Hawkins et al., 2003).

2.4. PLANKTONIC FORAMINIFERA

While this review is focused on larger foraminifera, one cannot fail to mention algal symbioses in many of the planktonic foraminifera. Members of the planktonic family Globigerinidae host dinoflagellates and chrysophytes (Anderson and Be, 1976; Faber et al., 1988, 1989; Spiro, 1987). Members of four other planktonic families, Candeinidae, Pulleniatinidae, Hastigerinidae, Globorotaliidae, are also the hosts for chrysophytes (Gastrich, 1988). Most interesting is the fact that that they expose their algal symbionts during the day on their very long spines and withdraw them in the evening. Another interesting facet of their symbiosis is that they consume their symbionts just before producing gametes.

3. Factors That Seem To Underlie the Predisposition of Foraminifera To Be Good Habitats for Endosymbiotic Algae

3.1. LIFECYCLE

Foraminifera have lifecycles that classically include a sexually reproducing haploid phase and one or more asexually reproducing phases. Asexual reproduction dominates the lifecycles of larger foraminifera and assures vertical transmission of the symbionts to progeny (Fig. 8a) (e.g., Lee et al., 2009; Pochon et al., 2006). Sexual reproduction in these huge protists is suspected to take place annually or bi-annually allowing potential changes to more adaptive symbionts during horizontal acquisition of symbionts by juveniles.

3.2. DECOUPLING OF KARYOKINESIS WITH GROWTH

In most eukaryotic groups, the cell cycle couples karyokinesis with growth. However, this is not the case of the asexual phase in foraminifera. This phase is always multinucleate and the signals for nuclear division are correlated with growth in volume instead of cytokinesis. This uncoupling and the signals for replication of cellular organelles associated with growth in cytoplasmic volume coupled with multicameral organization underpin the ability of the cells to reach large sizes. The nuclei of the asexual phase tend to be distributed more densely in the innermost chambers. In those foraminifera that are heterocaryotic, the somatic (macro) nuclei in the outer chambers degenerate during multiple fission, while the generative nuclei in the inner chambers contribute to the next generation. In the sexual phase, the cells are uninucleate until the formation of gametes begins.

3.3. MULTICAMERAL ORGANIZATION AND COMPARTMENTALIZATION OF CELLULAR ACTIVITIES

If karyokinesis is decoupled in a protist group with shells, there are two common growth modes: (1) abandon the shell and produce (molt) or find a new one; or (2) add new living space to the preexisting test. With respect to the latter, an open growth pattern is followed by the cup-shaped tintinnid ciliates. In contrast, a multicameral pattern of growth is followed by the foraminifera. Rhizopodial activities outside the aperture of the shell form the next chamber wall external to the previous one, thereby compartmentalizing the cytoplasm as the shell grows. The old aperture becomes the foramen from one chamber to the next. The chamber-wise growth of foraminifera produces a separation of the cytoplasm into functional and controllable units of appropriate volumes. This provides the cell body with a permanent compartmentalization and duplicate organelles that keep units of protoplasm within the range of "ordinary" cell size and has allowed some of these larger adult foraminifera to reach sizes (up to 15 cm discoidal diameter) corresponding to multicellular organisms (e.g., meiofauna in the same habitat). Cytoplasm freely flows from one chamber to the next. In some larger foraminifera, in-folding of the cell wall further divides cytoplasmic units into chamberlets. Even in the simplest multi-chambered foraminifer, cellular activities are compartmentalized. Capture of food and formation of phagolysosomal vacuoles occur in the external granuloreticulopodial network (Faber and Lee, 1991; Lee et al., 1991). If food vacuoles are brought into the test through an aperture, they enter the youngest outermost chamber which itself is more vacuolated than the inner chambers. Thus, lysosomal activity is regionalized and should algae escape from this region to an inner one, they are relatively safe from the host's digestive resources.

3.4. LOOSENESS OF FIT IN SOME OF THE ASSOCIATIONS

As mentioned in Section 2.1, the diatom-bearing hosts are not finical with respect to the symbionts that they host. The same may be true for some of the soritids, although as mentioned in Section 2.2, some molecular evidence suggests that there may be host–symbiont specificity (Garcia-Cuetos et al., 2005; Pochon et al., 2004). This has not been probed in other LF groups. Diversity in acceptable symbiotic partners certainly is a positive factor to foster survival and growth in changing environments.

3.5. BUILT-IN SYMBIONT ACCEPTING METABOLIC/ PHYSIOLOGICAL MOTIFS

3.5.1. Symbiont Surface Envelope Alterations

There are only indirect indications that these motifs exist. But they must. Irrespective of the type of the symbiotic algae, the cell envelopes of all endosy mbiotic algae are modified *in hospite* in all of the lineages of algal-bearing foraminifera. This is a serious problem with respect to the diatom-bearing forms, because diatoms are diagnosed on the basis of the characteristics of their frustules. Fortunately, frustules were formed when the first symbionts were isolated and cultivated. This was probed with respect to *Amphistegina lobifera*, a diatom-bearing LF. Cells of this LF were crushed, ground, and homogenized. After being sterile-filtered, the homogenate was added to logarithmically growing axenic cultures of *Nanofrustulum shiloi* and *Amphora tennerrima*. The algae continued to grow and divide, but they ceased to form frustule elements (Fig. 3f–i). The frustule elements that were already present were not resorbed, but the new cells were protoplasts (Lee et al., 1984). The homogenate also stimulated the cells in culture to release as much as 190–9000% more ^{14}C-labeled photosynthetates into the medium. Similar host homogenate stimulation was noted in experiments on zooxanthellae of cnidarians by Muscatine (1967) and Sutton and Hoegh Guldberg (1990).

3.5.2. Friend or Food

There must also be several signaling motifs present in foraminifera that underlie the common development of symbiosis in so many different and unrelated groups of foraminifera. This topic has barely been explored (Chai and Lee, 1999a, b, 2000; Lee and Reyes, 2006). The frustrule fraction from homogenized growing axenic cultures of 11 endosymbiotic species and five non-symbiotic diatom species were compared by immunoblotting them with polyvalent sera developed in rabbits against either.

Nanofrustulum shiloi, *Nitzschia frustulum*, *Nitzschia panduriformis*, or *Amphora tenerrima*. A 104-kDa glycoprotein (CSSA, Common Symbiont Surface Antigen) was found on the surfaces of all the symbiotic species tested, and was absent in the non-symbiotic species tested (Chai and Lee, 1999a, 2000). Blocking this antigen with antibody caused a loss of the ability of the diatom to bypass digestion and be drawn into the test to become an endosymbiont within the foraminifera. Using immunocytochemical and fine structural techniques, they found that receptors for the CSSA were abundant on the pseudopodia, making initial contact with the diatoms and on the primary organic lining of the test. Thus, it is clear that the initial recognition between the host foraminifer and the potential symbiotic diatoms is mediated by a cell signaling system involving molecules on the surfaces of diatoms and the pseudopods of the foraminifera. Soon after contact, the symbiotic diatom is phagocytosed and subsequently brought into the interior of the foram's test away from the active digestive processes (Chai and Lee, 1999b, 2000).

The CSSA is produced by the diatom even after it has lost its normal cell envelope, and it seems necessary to maintain the association even after the association is established (Chai and Lee, 2000). We recently tested the diatom polyvalent antisera with the CSSA against the soritid *Symbiodinium* strains in our culture library, and found that the antiserum did not have any affinity for the dinoflagellates cell envelopes (Lee and Reyes, 2006). One must conclude from this evidence that a different signaling molecule and receptor system is involved in the *Symbiodinium*-soritine symbiosis. Using both RFLP analysis and SSU rDNA sequencing, Garcia-Cuetos et al. (2005) distinguished 22 phylotypes of Soritinae, which were associated with 3 clades and 5 subclades of *Symbiodinium*. Fourteen of the twenty-two soritine phylotypes showed strict symbiont specificity. Only one of the soritine phylotypes was found to be the host for more than two groups of *Symbiodinium*. This may suggest that as the symbionts and hosts have a finical relationship, there may not be some common antigen on the surfaces of the various clades of *Symbiodinium* analogous to the CSSA found on the surfaces of the endosymbiotic diatoms. As the soritine hosts are also known to harbor secondary symbionts belonging to two widely divergent groups (Prymnesiophytes and Cyanobacteria) in addition to dinoflagellates (Lee et al., 1997), symbiont recognition, establishment, and maintenance is likely to be multi-component process. Perhaps, some common exported metabolite(s) is a component of the maintenance process.

3.5.3. Morphological Changes in the Host Cells

Another currently elusive motif that must be present underlies the gross morphological adaptations that each of the families has undergone in response to symbiosis with algae. In general, each family, in its own way has become larger, flatter, more compartmentalized, and has developed some system that facilitates nutrient exchange with the symbionts and the external environment (Hallock et al., 1986; Hottinger, 1978; Leutenegger, 1984).

(i) *Discoid shells – the soritine lineage*. During the Tertiary, the discoidal shells developed in the soritids. The newly discovered species of *Marginopora* is an example of how complex these annularly growing discs with over-crossed stolon systems have evolved (compare Figs. 6 through 8). *Marginopora* tend to be attached to substrates and they have evolved a thicker aperatural face. Apertures have been multiplied by thickening the periphery of the disc and by refolding its internal margins (Fig. 8c). Presumably, this increases the number

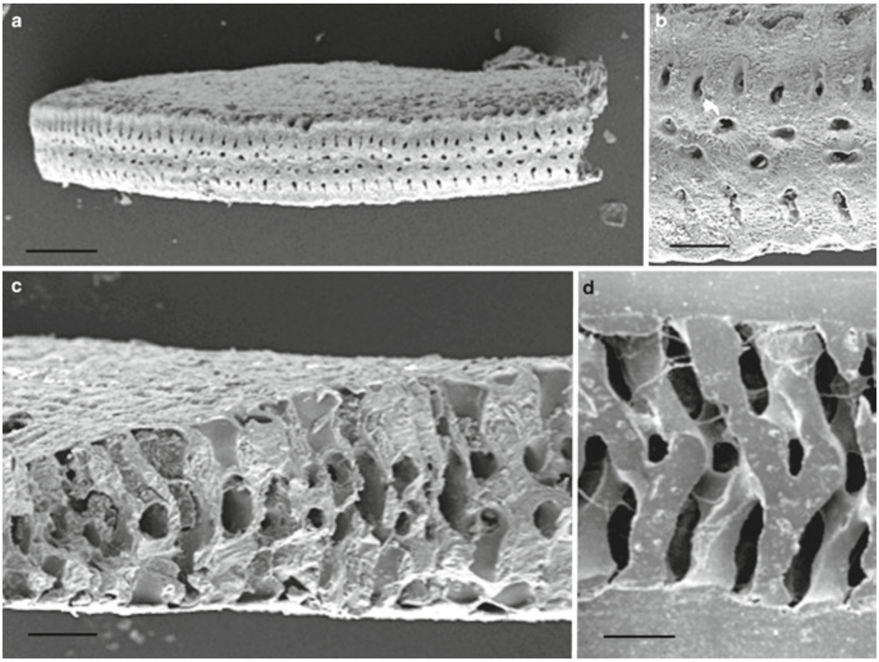

Figure 6. (**a**) SEM of the edge of the soritid foraminifer, *Amphisorus hemprichii*, from Eilat, Israel (scale = 0.5 mm); (**b**) higher magnification, same as (**a**), showing the elongate peripheral apertures and the more oval central ones. Note the slightly raised apertural lips (scale = 30 μm); (**c**) SEM of dissected shell showing central annular canal, chamberlets, and interconnections between chamberlets and canals (scale = 50 μm); (**d**) SEM of Hottinger cast showing the same as (**c**), but emphasizing the continuity of the subdivided protoplasm. The gray areas are where seawater or cytoplasm were in life; the dark spaces are where the shell was before it was dissolved by EDTA or acetic acid (scale = 30 μm).

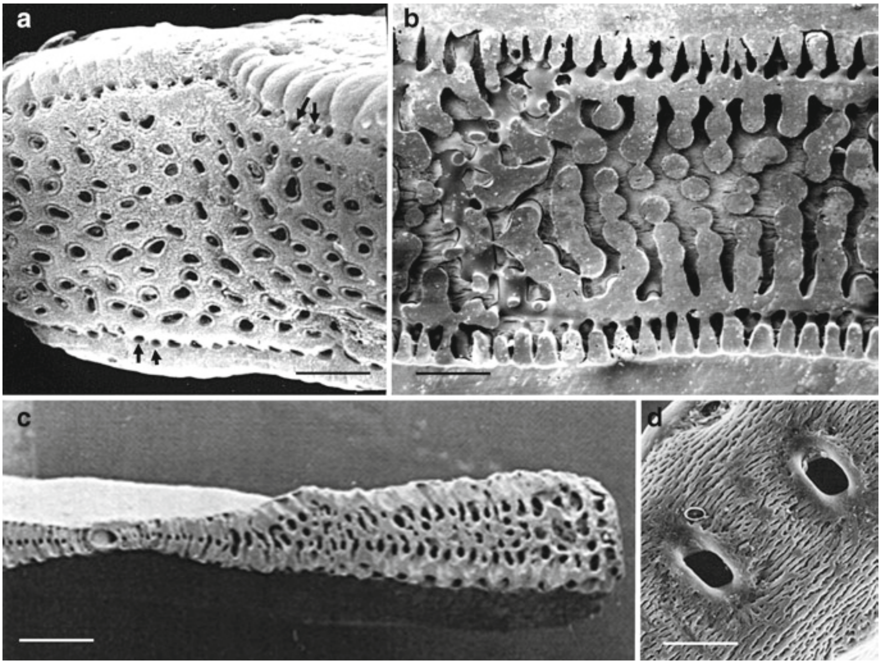

Figure 7. (**a**)–(**c**) are edge views of the soritid, *Marginopora vertebralis*, from Lizard Island, GBR, Australia. (**a**) SEM of external surface. Note: diagnostic marginal apertures (*arrows*) and peripheral raised chamberlets (ch) (scale = 30 µm); (**b**) SEM of Hottinger cast showing the diagnostic marginal annular canal associated with the marginal apertures (*arrows*) and peripheral raised chamberlets (ch). The internal canals and their various interconnections with each other are also evident (scale = 50 µm); (**c**) SEM of lower magnification of a Hottinger cast showing the addition of annular canals and cell complexity as the foraminifer (scale = 50 µm); (**d**) SEM of an external view of the apertures of *Sorites marginalis*, from Key Largo, Florida. This foraminifer is the smallest and simplest of the extant soritids (scale = 20 µm).

of protoplasmic streams interacting with the internal chamberlet system. The more mature specimens lose their juvenile apparatus (Fig. 8a, d). In the SEM, apertures are observed in the central disc face suggesting that pseudopodia may emerge from this region as well.

(ii) *Discoid shells – the numulitid lineage.* Numulitids, as the name implies, are coin-like in shape. One might think that with a shape that maximizes surface area and exposure of symbionts to light, there might be little other internal or external adaptations for symbiosis. However, in fact, they have very complex internal architecture. Externally, the tests of some of them reflect the depth of their ranges. For example, the test of *Assilina ammonoides* becomes thinner with depth. Interseptal blister-like pustules, which may act as light-condensing lenses, gradually inflate in size as the water depth increases. All numulitids have a marginal cord and canal system. They have complex

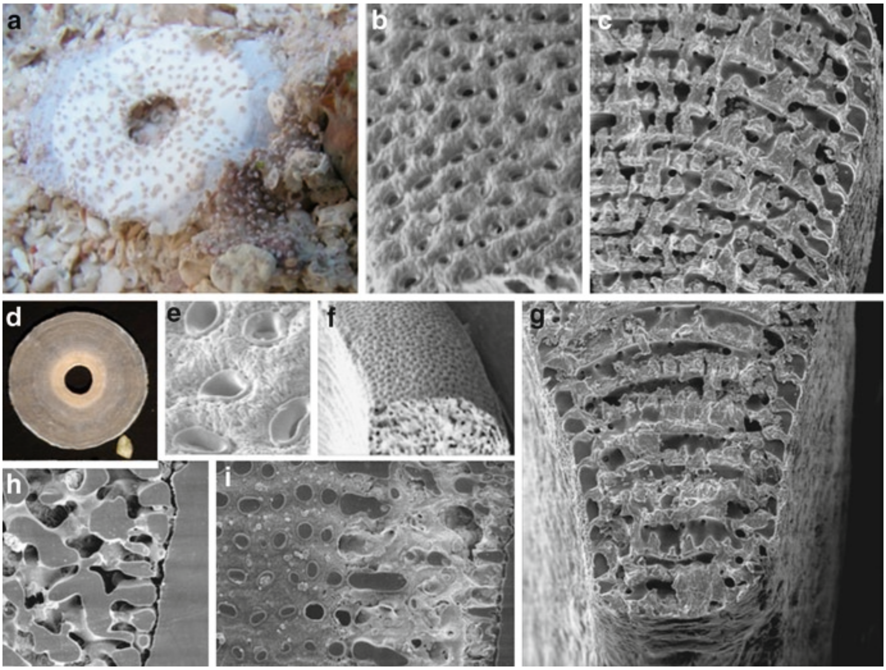

Figure 8. All are illustrations of a new species (yet to be described) of *Marginopora* from the channel between Heron and Wistori Islands, GBR, Australia; (**a**) a foraminifer that has just undergone asexual reproduction; (**b**), (**e**), and (**f**) (at different magnifications) showing that the disc edge is rippled, similar in feeling to some coins; (**d**) the whole organism (scale = 0.5 mm); (**e**) shows that the apertures are close to circular and have raised lips; (**c**) and (**g**) shows SEM of dissected shell illustrating the diagnostic peripheral raised chamberlets (ch), marginal annular canal associated with the marginal apertures (*arrows*), and chamberlets and interconnections between chamberlets and canals (scales = 200 μm); (**d**) Light micrograph showing the annular uneven distribution of symbionts. There are fewer symbionts in the inner or oldest part of the cell; (**h**) SEM of Hottinger cast showing the diagnostic marginal annular canal associated with the marginal apertures (*arrows*) and peripheral raised chamberlets (ch). The internal canals and their various interconnections with each other are also evident (scale = 50 μm); (**i**) SEM of Hottinger cast through a section that emphasizes the individuality of the successive internal annular canals (scale = 50 μm).

intraseptal canal systems that interconnect with each other with tubes and stolons as well as the spiral canals in the umbilicus from which the pseudopods emerge (Hottinger and Dreher, 1974). As in the amphistegines, there are pores in the surface of the test that have tubes that pierce the shell and reach the cell membrane of the chamberlets.

(iii) *Cone-shaped amphistiginid lineage*. Distinct from the soritids like *Marginopora* that are sedentary as adults, the amphisteginids are foragers. The latter have evolved a cone within a cone shape. The single aperture to the test is in the directrix of the circular cone, so that the pseudopodia that emerge bind the

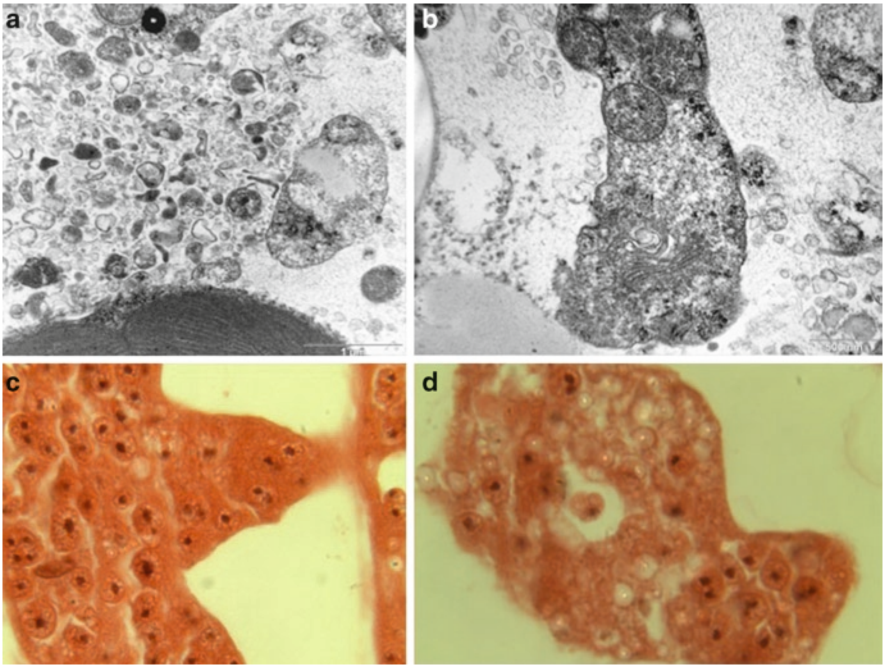

Figure 9. (**a**) and (**b**) are TEMs of different regions of the new *Marginopora* species showing the contrast in cytoplasmic density and inclusions between the middle (**a**) and outer parts (**b**) of the cell. (**a**) Symbiont labeled sy. (**b**) An internal membrane bounded pseudopodial extension with mitochondria (m), a golgi apparatus (*arrow*), and secreted electron dense granules (dg); (**c**) and (**d**) Hemotoxylin and eosin histological sections of a specimen sacrificed just after dawn, showing many symbiotic dinoflagellates. The nuclei are stained black. If you look carefully, you can see the unstained membranous vacuoles surrounding the nucleus. A macronucleus (*arrow*) is seen in (**c**). Note: several symbionts have recently divided.

organism to the substrate and pull the test along as it searches for food. The symbiotic diatoms are cortical on the vertex surface of the cone. In between the last whorl of chambers, there are stellar blind supplementary chamberlets. The blind diverticula of *Amphistegina lobifera* (see Hottinger, 2000; his text Fig. 11) reminds one of the gut diverticula in sea slugs. The cell membrane surface of each chamber and chamberlet is bubbly (Fig. 5e). The inside of each bubble is cup-shaped (Fig. 5d) and contains an individual symbiotic alga. The cup is formed as an internal rim around a pore tube that runs through the shell (Fig. 5e) and presumably brings fresh seawater near to each symbiont (Leutenegger and Hansen, 1979).

(iv) *The alveolinid fusiform lineage*. Fusiform lineages have arisen twice in the history of LF; the fusulinids in the Pennsylvanian and the Cretaceous-Cenozoic aveolinids. *Alveolinella* has been observed to burrow in the upper layer of sediment and it has been suggested that this shape aids this behavior (Lipps and

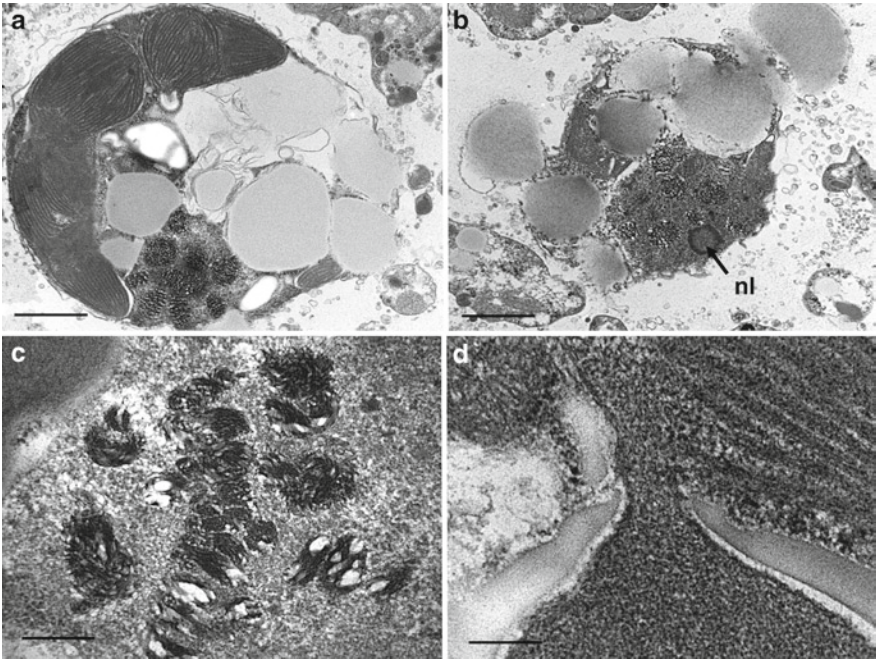

Figure 10. All figures are TEMs from the new species of *Marginopora.* (**a**)–(**c**) symbionts apparently at some stage of autolysis. Note: release of some product (lipid?) from symbiont cells into host; condensed symbiont chromosomes; nucleolus in (**b**) (*arrow*) appears normal. (**a**) and (**b**) scales = 1 µm; (**c**) scale = 200 nm; (**d**) pyrenoid stalk of symbiont showing no intrusion of thylakoids (scale = 200 nm).

Severin, 1986), a hypothesis that remains untested. Hottinger (1984) suggested that *Alveolinella* may hide under the shallow layers of coral sand as a means to regulate its irradiance, another hypothesis that remains to be probed. Alveolinid tests have a streptospiral involute coiling architecture. Tubiform chambers about half a whorl are flattened to become broad sheet-like spaces. These later become subdivided (Reichel, 1936, 1937). The apertures line up in a broad equatorial face and many additional polar apertures. Presumably most of the feeding activity is by pseudopodia emerging from the polar apertures. Internally, there is a winding narrow coil of protoplasm called acolumella that is formed by the chamber septa that meet at the shell pores.

(v) *The calarinid "star-sand" lineage*. The complexity of the canal systems in the calcarinids (e.g., Fig. 4) is almost unbelievable and has attracted research almost from the beginning of micropaleontology (e.g., Carpenter, 1862; Hofker, 1927). Hottinger and Leutenegger (1980) have detailed the comparative anatomy and taxonomy of this group. Streams of pseudopods emerge from the spines of the stars, which themselves are caniculate. Internally, the spinal canals are fed from enveloping canals at the distal dorsal end of the chambers. The chambers

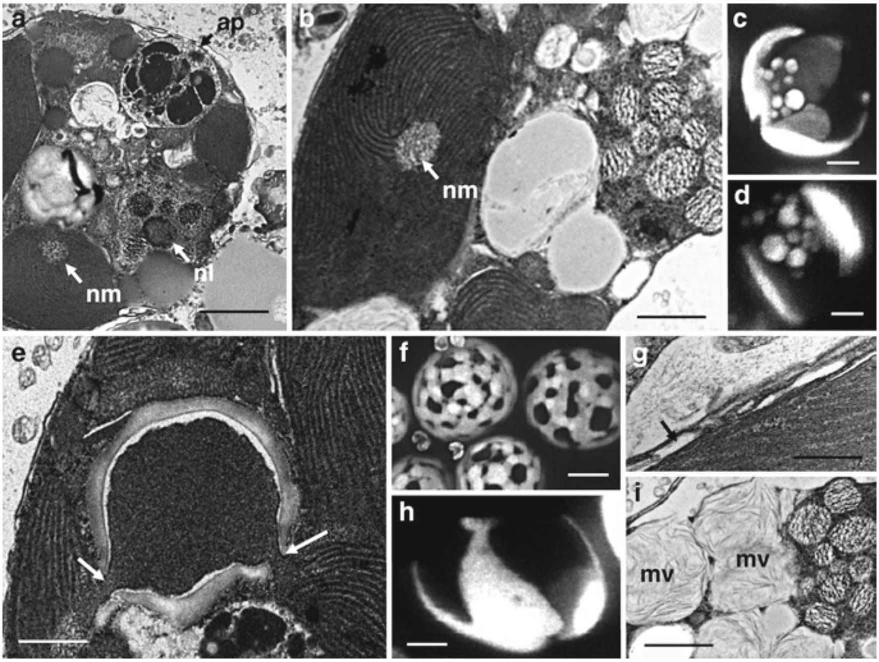

Figure 11. (**a**), (**b**), (**e**), (**g**), and (**i**) are TEMs of the symbionts *in hospite* in the new species of *Marginopora*.; (**c**), (**d**), (**f**), and (**h**) are confocal micrographs of autofluorescent plastids and membranous vacuoles in *Symbiodinium* spp from cultures. (a) Section showing plastid lobes (p), nucleomorph (*nm arrow*), nucleolus (*nl arrow*), and residual (or accumulation body) (*rb arrow*) (scale = 1 µm); (**b**) section showing the filamentous nature of the plastid nucleomorph in context with other organelles. Membranous vacuoles (mv) are seen at three edges of the micrograph (scale = 1 µm); (**c**) view of the petal-like plastids and membranous vacuoles in an isolate from a *Marginopora vertebralis* collected in Hawaii; (**d**) view of the petal-like plastids and membranous vacuoles in an isolate from a *Marginopora vertebralis* collected in Ritidium Bay, Okinawa, Japan; (**e**) section showing the two stalks of the symbiont's pyrenoid (*arrows*) (scale = 200 nm); (**f**) view of the reticulate plastids in an isolate from a *Marginopora vertebralis* collected near Bird Island, Lizard Island Group, GBR, Australia; (**g**) section through the cell envelope of the symbiont showing that the alveolar sacs are empty and devoid of plates (*arrow*) (scale = 200 nm); (**h**) section showing nucleus (n) and closely apposed membranous vacuoles (mv) (scale = 200 nm).

are connected with each other by multiple parallel stolons in alternating rows formed when previous structures are resorbed as new chambers are added. There are pores and canals through the shell (Fig. 5b–f) that probably allow nutrient exchange with the interior, but this remains to be tested experimentally. A recent cytological study of *Baculogypsina sphaerulata* by Hyams-Kaphzan and Lee (2009) found that the symbionts are widely distributed in most of the chamberlets, especially in the umbilical and central parts of the test. In the proloculus and inner chamberlets, the symbionts were somewhat less abundant. The canal liners and the expanded internal pore domes were empty of individual symbionts. This is in contrast with Amphistegina, where there is a special relationship between the symbionts and the pores.

(vi) *The achaiasine "green sand" and peneroplid "red sand" lineages*. These lineages have planispiral-involute architecture modified by the elongation of the peripheral face apertures. As peneroplids increase in size, they may form crosier-like planispiral-evolute chambers with an ever-enlarging apertural peripheral face (Fig. 2a).

4. Conclusions and Acknowledgments

This contribution was written while the author was a visiting scholar at the Eilat campus of Ben Gurion University and a research associate at the IOLR National Center of Mariculture in Eilat. The fabric of this cellular evolution thesis has been woven from many threads expressed previously in the literature by this author and others, but the discovery of the complex new species of *Marginopora* from the Heron-Wistori Channel gave the thesis a new modern evolutionary perspective. I am indebted to Libbie Hyman of the American Museum of Natural History, who, early in my scientific career, discussed the foraminiferal research going on in my lab on an almost weekly basis. Although her idea that "Protozoa" were acellular (Hyman, 1940) may seem quaint when not viewed in the context of her time, it is, when rephrased in contemporary terms, quite a valid point of view. I am also indebted to discussions with Norman Newell, also at the American Museum of Natural History, who was always interested in the ecological bases of paleontological observations. He was particularly fascinated by incidents in the fossil record, where the descendants of ordinary foraminifera suddenly evolved to tens or a hundred fold the size of their ancestors (Newell, 1949). Lastly, I acknowledge the contributions of many others who were involved in the discussions of the theme at the Lizard Island Workshop on the Biology of Foraminifera (Hottinger, 2000; Lee and Hallock, 2000).

Without the advantage of tissue organization and specialized organ functions, the LF have nonetheless produced remarkably advanced cytoplasmic and morphological adaptations, mimicking, in some cases, the specialized functions of organ-forming metazoa. In brief, driven by symbiosis with algae, the LF have probably reached an apex in evolutionary advancement for unicells. The mechanisms, which underlay these morphological adaptations, are not clear at all, but that they have occurred is a fact. We can speculate on the possible usefulness of each of the holobiont morphotypes, but all remains untested; a great challenge for future research.

5. Acknowledgment

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PART 7: CARNIVOROUS PLANTS

Adamec
Rice

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ECOPHYSIOLOGICAL LOOK AT PLANT CARNIVORY

Why Are Plants Carnivorous?

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1. Introduction

About 650 species of vascular carnivorous (Latin: carnis – flesh, vorare – to swallow) plants occur throughout the world (e.g., Rice, 2006) out of the total of about 300,000 species of vascular plants. Carnivorous plants belong to 15–18 genera of 8–9 botanical families and five orders (Givnish, 1989; Juniper et al., 1989; Müller et al., 2004; Heubl et al., 2006; Porembski and Barthlott, 2006; Studnička, 2006). Owing to many remarkable and striking morphological, anatomical, physiological, and ecological features, carnivorous plants have always attracted considerable interest of both researchers and gardeners. Nevertheless, the degree and extent of knowledge of the main disciplines studying this particular ecological functional plant group have always considerably lagged behind the study of noncarnivorous plants. However, similar to the dynamically growing knowledge of noncarnivorous plants, the study of carnivorous plants has developed very rapidly and progressively within the last decade, mainly due to the use of modern molecular taxonomic approaches. Also, modern ecophysiological research of carnivorous plants has progressed considerably within the last decade and has elucidated most of the particulars of carnivorous plants. Thus, we are increasingly more able to discuss to what extent carnivorous plants are unique from or common to "normal" noncarnivorous plants.

The aim of this paper is to classify and review the recent experimental results and concepts concerning plant carnivory from an ecophysiological point of view, with an emphasis on mineral nutrition, growth characteristics, and comparison of aquatic and terrestrial carnivorous plants. The latter two subjects have often been neglected in previous reviews (cf. Juniper et al., 1989). The present review is focused on mineral nutrition, as it is believed that mineral nutrition represents the key processes and the main benefit of carnivory for these plants (Adamec, 1997a; Ellison and Gotelli, 2001). A new model of "nutritional" cost–benefit relationships is presented. However, there are several other remarkable ecological phenomena associated with carnivory, e.g., prey–pollinator conflict (Zamora, 1999), prey attraction (Givnish, 1989), prey selectivity (Harms, 1999), competition between carnivorous and noncarnivorous plants (Brewer, 1999a, b), and relationships within inquiline communities in pitcher traps (Gray et al., 2006). Most of these phenomena were thoroughly reviewed by Ellison et al. (2003) and will not be mentioned in this study.

The present review follows from previous review publications in this field. Undoubtedly, Darwin (1875) was the first who summarized multilateral research on carnivorous plants, even though the main focus of his book was aimed at his studying the irritability of *Drosera* tentacles. He was the first to prove digestion of prey and to reveal that carnivorous plants showed enhanced growth if fed on insects and/or animal proteins. Darwin's book greatly influenced and inspired several generations of botanists and physiologists studying carnivorous plants. About 70 years after Darwin, the knowledge of carnivorous plants, based on literature items, were comprehensively reviewed in a monograph by Lloyd (1942). Physiological investigations on carnivorous plants, focusing on mineral and organic nutrition, trap excitation and movement, and digestive enzyme secretion were reviewed by Lüttge (1983). Evolution and ecological cost–benefit relationships of carnivorous plants were discussed thoroughly by Givnish (1989) in his review. Carnivorous plant biology, with an emphasis on cytology, anatomy, biochemistry, and physiology, was reviewed in detail in an excellent monograph by Juniper et al. (1989). This review includes all literature sources published before 1987–1988, and serves as a reference list of literature. In the decade after this monograph appeared, the mineral nutrition of carnivorous plants has been studied intensively. The subjects of mineral and organic nutrition of carnivorous plants as key ecophysiological processes associated with carnivory were classified and thoroughly reviewed by Adamec (1997a), who separately analyzed processes in field- and greenhouse-grown plants, and also in terrestrial and aquatic carnivorous plants. Modern trends in studying carnivorous plants with an emphasis on phylogenetic diversity and cost–benefit relationships were reviewed in a well-arranged way by Ellison and Gotelli (2001). Selected ecological phenomena and processes associated with carnivory were reviewed in detail by Ellison et al. (2003). Proceedings of a special session on "Biology of Carnivorous Plants," at the International Botanical Congress held in Vienna, Austria, in 2005, were published in a special issue of Plant Biology 8(6) in 2006 (for comments, see Porembski and Barthlott, 2006). In this special issue, Ellison (2006) reviewed ecophysiological subjects of nutrient limitations in carnivorous plants and identified modern directions for this research. Finally, Guisande et al. (2007) have recently published a detailed review on the bladderwort (*Utricularia*) genus, which also includes some ecophysiological points.

2. Plant Carnivorous Syndrome

All plants considered carnivorous have to fulfill several criteria to separate them from other ecological plant groups (e.g., saprophytes). Nevertheless, due to a great diversity of ecological and functional plant traits, these criteria are still partly ambiguous (cf. Juniper et al., 1989; Adamec, 1997a). Thus, what is really *crucial* for a working definition of "plant carnivory"? Considering that the main ecophysiological benefit and consequence of carnivory is the uptake of growth-limiting mineral nutrients from prey, the criteria for the carnivorous syndrome (i.e., cluster of characters) may be as follows: (a) capturing or trapping prey in specialized traps, (b) absorption of metabolites (nutrients) from killed prey, and (c) utilization of these metabolites for plant growth and development (Lloyd, 1942; Givnish, 1989; Juniper et al., 1989; Adamec, 1997a). As all plants are able to absorb organic substances from soil (e.g., from dead animals), the criterion of capturing prey in traps, which actively kill prey, separates carnivorous from saprophytic plants. Moreover, Juniper et al. (1989) and many later authors state two other criteria, such as prey attraction and digestion. However, on the basis of recent knowledge of this issue, it is possible to conclude that these additional criteria are not indispensable for functioning of carnivorous plants. First, the ability to attract prey has only been studied and confirmed in a part of carnivorous plants yet, and it is not clear whether or not it occurs in very abundant genera of carnivorous plants, such as *Utricularia* and *Genlisea* (Givnish, 1989; Guisande et al., 2007; Płachno et al., 2008, unpubl.). Moreover, the study on north European *Pinguicula* species (Karlsson et al., 1987) did not reveal prey attraction in *P. alpina*, in contrast to other species, without the plant being limited by prey capture. Second, it is generally accepted that carnivorous plants can also digest prey without secreting their own hydrolytic digestive enzymes in traps, relying only on enzyme secretion by trap commensals (e.g., Givnish, 1989; Jaffe et al., 1992; Butler et al., 2008). Thus, these two additional criteria – prey attraction and digestion – may rather be considered technical details that can only improve the efficiency of carnivory, but are not indispensable for carnivory as such. In an analogy with parasitic plants (holoparasites and hemiparasites), Joel (2002) proposed the term "holocarnivory" for carnivorous plants secreting their own digestive enzymes (e.g., *Dionaea*, *Drosera*, *Drosophyllum*, *Pinguicula*, *Nepenthes*) and "hemicarnivory" for those plants which do not (e.g., *Brocchinia, Roridula*).

However, the diversity of ecological relationships concerning prey digestion is evidently wider, and an additional classification can be based on the way by which carnivorous plants gain nutrients from prey, regardless of secretion of own enzymes. All carnivorous plants, except *Roridula,* can gain nutrients from prey carcasses more-or-less *directly,* and such a type of carnivory can be termed as "direct." Two *Roridula* species, however, capture prolific prey, but they usually do not digest it. The captured prey are grazed by kleptoparasitic hemipteran bugs *Pameridea* which are found only on the *Roridula* plants and which defecate on its surface; the plants absorb nutrients through specialized cuticular gaps (Ellis and Midgley, 1996; Midgley and Stock, 1998; Anderson and Midgley, 2003; Anderson, 2005). Thus, mineral nutrients from prey are gained *indirectly*, through excrements of the bugs as mediator, and this type of carnivory can be termed as "indirect."

By discussing the carnivorous syndrome, one can make a physiological look at plant carnivory and question to what extent carnivorous plants are physiologically unique within the plant kingdom. In line with Juniper et al. (1989, p. 10–11), it is possible to point out five physiological key processes that are typical and common for plant carnivory: (a) rapid movements of traps; (b) their electrophysiological regulation; (c) hydrolytic enzyme secretion; (d) foliar uptake of nutrients; and (e) stimulation of root nutrient uptake by foliar nutrient uptake. Yet, *all* these *individual* processes can also occur in noncarnivorous plants and, therefore, they are not confined only to carnivorous plants and are not unique in this plant group. In carnivorous plants, however, they occur very often and together, forming a coordinated functional unit within which one process is firmly coupled with another one.

3. Ecological Characteristics of Terrestrial Carnivorous Plants and Their Habitats

The majority of terrestrial carnivorous plants grow in bog and fen soils in which they encounter persistent unfavorable conditions. The soils are usually wet or waterlogged, at least during the growing period. The only exception may be *Drosophyllum lusitanicum,* growing in dry sandy or rocky soils (Adlassnig et al., 2006), or hemicarnivorous epiphytes such as *Catopsis berteroniana*. The soils are mostly acidic (pH 3–6; e.g., Roberts and Oosting, 1958; Chandler and Anderson, 1976; Juniper et al., 1989, p. 21–22), but some are neutral or slightly basic (e.g., Schwintzer, 1978). They usually contain a high proportion of slowly decomposing organic matter (plant remnants). Owing to waterlogging, the soils are partly (hypoxia) or entirely (anoxia) deprived of oxygen. Moreover, changing of anaerobic and aerobic conditions is also harmful (postanoxic injury; Crawford, 1989, p. 105–129). In wet soils, decomposition of organic matter may lead to a high concentration of toxic H_2S (or S^{2-}) and a low redox potential. When redox potentials are low, iron and manganese may solubilize and become toxic to plant roots, while some other microelements may become unavailable to plants (Crawford, 1989).

It is presumably the very low level of macronutrients available to plants, which is the primary unfavorable ecological factor in these soils, which is overcome by carnivory (Lüttge, 1983; Juniper et al., 1989). However, there is a tremendous difference between the *available* and *total* macronutrient content in most bog and fen soils. For example, Roberts and Oosting (1958) reported very low available nutrient content in bog soils with *Dionaea* in North Carolina (in mg kg^{-1} dry weight, DW): NH_4^+, 2; PO_4, less than 2; K, 2; Mg, 1; and Fe, 1. There was a complete lack of detectable NO_3^-, Ca, and Mn. However, the available nutrient content in more fertile fen soils can be one to two orders of magnitude higher (e.g., Schwintzer, 1978; Aldenius et al., 1983). In contrast, the following total N and P contents were found in bog soils inhabited by four Australian and New Zealand *Drosera* species (in g kg^{-1} DW): N, 0.46–2.5 and P, 0.09–1.9 (data summarized by Chandler and Anderson, 1976).

Normal functioning of carnivorous plant roots (uptake of nutrients and water) is dampened by low nutrient availability in soils, and this stress factor is greatly *amplified* by waterlogged and anoxic soils. Therefore, carnivory of most terrestrial carnivorous plants can be explained as an adaptation to *all* these stress factors. The extent of adaptation of carnivorous plant roots to waterlogging alone has not yet been studied. Nevertheless, according to initial studies, roots rely on aeration diffusive mechanism in roots supported by exodermal diffusive barriers (Adamec, 2005; Adamec et al., 2006).

Terrestrial carnivorous plants have adapted to these unfavorable factors as typical stress-strategists by growing slowly (see below). They do not require a high supply rate of mineral nutrients from soils, as they are able to store nutrients in their organs and reutilize them efficiently (Dixon et al., 1980; Adamec, 1997a, 2002). A weakly developed root system is a common characteristic of most carnivorous plants (Lüttge, 1983; Juniper et al., 1989, p. 21–22; Adamec, 1997a). The root:total biomass ratio ranges from only 3.4% to 23% in various carnivorous plants (Karlsson and Carlsson, 1984; Adamec et al., 1992; Karlsson and Pate, 1992; Adamec, 1997a, 2002). Roots are usually short, weakly branched, and able to tolerate anoxia and related phenomena (H_2S) in wet soils. They are able to regenerate easily. Generally, even in spite of an absence of any study on the uptake of mineral ions by roots of carnivorous plants, it may be concluded for several reasons that the capacity of carnivorous plant roots for mineral nutrient uptake is limited and compensated by nutrient uptake from prey. Yet Adamec (2005) has recently studied the ecophysiological characteristics of carnivorous plant roots and has found that their aerobic respiration rate and water exudation rate per unit biomass is comparable with those reported in roots of noncarnivorous plants in the literature, or even higher. Thus, roots of carnivorous plants are physiologically very active per unit biomass and well adapted to endure soil anoxia.

4. Animals as Prey for Plants: What an Advantage?

Considering possible advantages of carnivorous plants to capture animals as prey, which could be substantial for the evolutionary ecology of carnivorous plants, at least two main aspects can be taken into account. One of the aspects can be ecological: as most carnivorous plant species are able to capture relatively small prey items (relative to plant or trap size, Karlsson et al., 1987; Givnish, 1989; Harms, 1999), which are abundant at sites (e.g., ants, small flies, mosquitoes, crustaceans, etc.), this fact ensures the relatively reliable catch of prey over a time, though there are very great differences in prey capture effectiveness (over 10 times) between individual plants even at the same microsite (Karlsson et al., 1987, 1994; Thum, 1989a, b). Moreover, many potential prey taxa are adapted to visiting plants for food. Thus, almost all carnivorous plants are able to capture at least some prey within a given time period.

The second aspect can be nutritional: when compared with plant-tissue nutrient content (i.e., nutrient amount per unit dry biomass, DW) animals as prey represent a relatively rich source of some macrobiogenic mineral nutrients, and it is possible to consider this relationship as the main benefit that predetermined the evolution of plant carnivory from the very beginning. As stated earlier, typical wet, peaty, or sandy soils inhabited by carnivorous plants have a very low available N, P, and K content, but the tissue N and P content in prey carcasses is commonly about 5–10 times higher than that in carnivorous plant organs (see below), while the K content is comparable. Thus, it is possible to assume that it was N and P uptake from animal prey that represented the main benefit and evolutionary advantage of carnivorous plants to which these plants have adapted from the beginning of their evolution. The following total nutrient content was found in terrestrial insects or aquatic crustacean zooplankton (g kg^{-1} DW): N, 99–121; P, 6–14.7; K, 1.5–31.8; Ca, 1–44; and Mg, 0.94 (Reichle et al., 1969; Watson et al., 1982; Wærvågen et al., 2002; DeMott et al., 2004; Woods et al., 2004). However, a part of insect nutrients is not available to carnivorous plants (Dixon et al., 1980; Adamec, 2002).

5. Mineral Nutrition of Carnivorous Plants – General Principles

The term mineral nutrition of plants includes processes of mineral nutrient uptake by plants from the ambient medium, nutrient translocation within the plant, incorporation of mineral nutrients to plant metabolism and physiological functions, and release from primary physiological functions and entry of secondary ones. Our knowledge of mineral nutrition of carnivorous plants can still be considered to be fragmentary, as it is confined to about 50 species and less than 75 studies since the 1950s.

Although growing in mineral-poor habitats, both terrestrial and aquatic carnivorous plants have nearly the same composition of macroelements as noncarnivorous wetland and aquatic plants (Adamec, 1997a; Ellison, 2006; cf. Dykyjová, 1979). However, terrestrial carnivorous species have considerably lower foliar tissue content of macroelements than aquatic ones. Ellison (2006) has recently reviewed the literature data on foliar nutrient content in terrestrial carnivorous plants to be on an average 1.26% of DW for N (quartiles 0.9% and 1.9%), 0.094% of DW for P (quartiles 0.07% and 0.16%), and 0.75% of DW for K (quartiles 0.50% and 1.0%), and has compared them with those for noncarnivorous plants. According to his review, the mean tissue N, P, and K contents in terrestrial carnivorous plant leaves are generally lower than those in the leaves of noncarnivorous, usually mesophytic plants (mean N, 1.8% of DW; P, 0.105%; K, 1.9%), but great overlapping occurs, especially for N and P. After the foliar tissue N, P, and K content, out of all functional plant groups reviewed, terrestrial carnivorous plants mostly resembled the groups of evergreen trees and shrubs. In terrestrial carnivorous plant leaves, the mean values could lie within 0.1–0.3% of DW for Ca and 0.2–0.3% for Mg (Adamec, 1997a, 2002). On comparison, the mean values in aquatic carnivorous plant leaves/shoots could lie within 1.5–2.5% of DW for N, 0.20–0.35% for P, 2.0–3.0% for K, 0.3–0.8% for Ca, and 0.2–0.4% for Mg (Adamec, 1997a, 2000, 2008b). However, three critical comments should be added to the issue of interpretation of tissue nutrient content in carnivorous plants. It is obvious that prey captured in traps of aquatic carnivorous plants were also included in the tissue nutrient contents (overestimated P and Ca content; see Adamec, 1997a). As leaf or shoot nutrient contents depend markedly on the leaf/shoot age (senescence) or position on the carnivorous plant (Adamec, 1997a, 2000, 2002, 2008b), it must be always clear what was the age (or position) of the organ analyzed. Furthermore, it has commonly been demonstrated in various carnivorous plant species that tissue nutrient content can remain unaffected or be even significantly lower after feeding on prey or soil fertilization, as a result of more rapid growth (Adamec, 1997a, 2000, 2002, 2008a). That is why tissue nutrient content alone is an unreliable measure of nutrient uptake by carnivorous plants, its interpretation value is limited, and plant growth rate should also be considered in relevant studies (Adamec, 2008a; Farnsworth and Ellison, 2008). Therefore, due to these objections, it is reasonable to also determine the nutrient stoichiometry of carnivorous plants to consider the relative nutrient limitations of plant growth (Ellison, 2006). On the basis of this approach, the latter author could demonstrate a *co*-limitation of the growth of carnivorous plants in the field or natural soils by N + P or N + P + K, rather than *only* by N, P, or K.

The most extensive process of mineral nutrition is photosynthetic fixation of CO_2 by leaves. All carnivorous plants are green and able to fix CO_2 (autotrophy) although the growth of some species (mainly aquatic) is partly dependent on organic carbon uptake from prey (facultative heterotrophy; see Lüttge, 1983; Adamec, 1997a). Many carnivorous plants of all taxonomic groups fix CO_2 according to the C_3 scheme of the Calvin cycle (Lüttge, 1983), but anatomical evidence in favor of the C_4 type has been given in six Mexican succulent *Pinguicula* species (Studnička, 1991). Generally, the characteristic for photosynthesis is that the maximum net photosynthetic rate per unit DW or leaf area (P_{max}) of leaves of terrestrial carnivorous plants is about 2–5 times (mean about 3 times) lower than that of other noncarnivorous plants (for the review see Ellison, 2006). Such a low photosynthetic rate presumably reflects the relatively low growth rate of terrestrial carnivorous plants as typical S-strategists (see below). Generally, low P_{max} values in terrestrial carnivorous plants are further supported by their very low values of photosynthetic nutrient use efficiency for N and P. As reviewed by Ellison (2006), these mean values for carnivorous plants are about three times lower for N and about two times lower for P than those for terrestrial noncarnivorous plants. The relationship between carnivorous plants' photosynthetic performance and carnivory is, however, complex and ambiguous (Juniper et al., 1989). First, P_{max} of traps is usually lower than that of other noncarnivorous leaves of the same plants, i.e., pitchers *vs.* phyllodia (Givnish et al., 1984; Ellison and Gotelli, 2002). Second, the photosynthetic effect of prey addition is quite different in different carnivorous plant species (cf. Méndez and Karlsson, 1999; Ellison and Gotelli, 2002; Ellison and Farnsworth, 2005; Wakefield et al., 2005; Farnsworth and Ellison, 2008); the same characteristics also hold for aquatic carnivorous species (Adamec, 2008a). Nevertheless, at least in most *Sarracenia* species and in *Darlingtonia californica*, prey addition does significantly increase P_{max}, *sensu* the prediction by Givnish et al. (1984).

6. Mineral Nutrition of Carnivorous Plants: Mineral Nutrient Economy

The three principal processes of mineral nutrition determine the mineral nutrient budget in terrestrial carnivorous plants: foliar nutrient uptake from prey and root nutrient uptake from the soil, mineral nutrient reutilization from senescing shoots (mineral nutrient economy), and stimulation of root nutrient uptake by foliar nutrient uptake.

According to the detailed review by Adamec (1997a) based on numerous experimental data, uptake of the following mineral nutrients from prey carcasses or mineral nutrient solution by traps of various species of terrestrial (or partly also aquatic) carnivorous plants has been proven so far: N, P, K, Na, Ca, Mg, and S. Yet the importance of a given macronutrient taken up from prey for a plant ecophysiological role depends on its uptake efficiency from prey carcasses. So far, only three studies have determined the nutrient uptake efficiency from model insect prey in terrestrial carnivorous plants, and the greatest attention has been focused on N. In greenhouse-grown *D. erythrorhiza* fed on fruit flies (*Drosophila*), Dixon et al. (1980) found that 76% of the initial total N, having been contained in flies, had been taken up by the leaves. Obviously, a good deal of N in the spent flies was present in unavailable chitinous skeletons. However, much lesser efficiency (39–51%) of N uptake from fruit flies was estimated in three north European *Pinguicula* species and *D. rotundifolia* in a greenhouse experiment (Hanslin and Karlsson, 1996); in field-grown plants, the efficiency was only 29–41%. Adamec (2002) compared the uptake efficiency from fruit flies and mosquitoes in greenhouse-grown *D. capillaris* and *D. capensis*. The uptake of N, P, K, and Mg was relatively efficient (43–62% N, 61–97% P, 60–96% K, 57–92% Mg), while that of Ca was not and depended greatly on tissue Ca content in the insects. Similar values of uptake efficiency from fruit flies (56–65% N, 59–67% P) indirectly follow from the greenhouse growth experiment in *D. closterostigma* (Karlsson and Pate, 1992). Thus, the uptake efficiency of P, K, and Mg from prey can be much greater than that of N, but the true field-based values are still unknown. Contrary to certain knowledge of the efficiency of mineral nutrient uptake from prey by traps, there are still virtually *no* published data on the uptake affinity and capacity of roots of carnivorous plants for mineral ions, to be compared with those in noncarnivorous plants. It is possible to expect, however, that the root uptake affinity will be relatively high, while the uptake capacity (due to slow growth) will be very low.

A further typical ecophysiological characteristic of terrestrial carnivorous plants is their extraordinarily good mineral nutrient economy, such as very efficient reutilization (i.e., recycling) of N, P, and K from senescing leaves/shoots. Reutilization efficiency in various terrestrial carnivorous species was found to be 56–99% for N, 51–98% for P, and 41–99% for K (for details see Adamec, 1997a, 2002). Like in typical noncarnivorous plants, less efficient Mg reutilization and zero or even negative Ca reutilization were usually found in carnivorous plants. Mean reutilization efficiencies of N (70–75%) and P (75–80%) in carnivorous plant leaves or shoots are by 20–25 percentage points greater than those found in noncarnivorous bog or fen plants which usually grow at the same microsites (Adamec, 2002; cf. Aerts et al., 1999). This comparison shows that terrestrial carnivorous plants, in spite of their ability to take up needed nutrients from prey, make a great physiological effort to minimize mineral nutrient losses (of N, P, K) from senescing organs.

One of the typical and fascinating ecophysiological peculiarities of mineral nutrition in terrestrial carnivorous plants is a marked stimulation of root nutrition by foliar uptake of mineral nutrients from prey. The stimulation was repeatedly confirmed in about ten terrestrial carnivorous species under greenhouse or field conditions within the last 25 years (e.g., Hanslin and Karlsson, 1996; Adamec, 1997a, 2002). Presumably, this represents one of the most important ecophysiological adaptations of carnivorous plants. Generally, in various growth experiments, carnivorous plants fed on insects or mineral nutrient solutions grew rapidly and accumulated much more mineral nutrients in their total produced biomass (about 1.6–27 times more for N, P, K, Ca, and Mg when compared with unfed control plants) than they could take up theoretically from the limited foliar nutrient supply. Thus, stimulation of absorptive activity of roots is the essence of the very high efficiency of foliar nutrition for carnivorous plant growth. Only mineral, but not organic substances, caused this phenomenon. This means that mineral substances taken up by leaves from prey stimulated, in an unknown way, the activity of roots which then took up the amount of nutrients needed for increased growth from mineral-poor soil. It is possible to assume that the extent of this stimulation will be several times greater for K, Ca, and Mg uptake than that for N and P under natural conditions, as prey are a rather poor source of these metallic cations. Hanslin and Karlsson (1996) proved in some carnivorous species in the field that the stimulatory effect on roots was of a quantitative nature, dependent on the amount of prey.

The essence of the stimulation of root uptake in carnivorous plants has not yet been explained. Adamec (2002) tried to explain this effect in three *Drosera* species. Slightly greater root lengths could only explain about 17% of the uptake stimulation, with the higher theoretical uptake rate of roots per unit root biomass being only about 15–30%, but the greater root biomass could explain 70–85% of the effect. Metabolic root activity (as aerobic respiration), however, was unchanged. Moreover, the stimulatory effect on the roots was related to tissue mineral nutrient content in neither roots nor shoots. Although the total root biomass of the fed plants was markedly greater than that in unfed controls, the proportion of root biomass to the total biomass of fed plants mildly decreased, according to a theory. A crucial question is what are the mineral nutrients taken up by leaves from prey, which can stimulate root nutrient uptake in carnivorous plants? It could be phosphate alone (Karlsson and Carlsson, 1984), but the role of other nutrients (especially N) is as yet unknown.

7. Growth Effects of Carnivory

7.1. GREENHOUSE CONDITIONS

Many principal pieces of knowledge of mineral nutrition of terrestrial carnivorous plants have been obtained in greenhouse growth experiments (Adamec, 1997a). As these experiments represent a considerable simplification of true natural conditions (e.g., lack of competition, mortality, and rain), the results reflect the *potential* physiological abilities of carnivorous plants to take up mineral nutrients by leaves from prey or roots from soil, rather than the ecological importance of carnivory. It has been found in all terrestrial carnivorous species studied that they can grow satisfactorily in natural peaty soils even without additional feeding on prey or soil fertilization. However, foliar fertilization by droplets of a mineral nutrient solution (Karlsson and Carlsson, 1984; Adamec et al., 1992; Adamec, 2002) had about the same positive growth effect as model feeding on prey, and proved that the absorbed mineral nutrients from prey, especially N and P, and not organic substances, were of principal importance for plant growth. Overall, depending on experimental conditions, feeding on prey or soil fertilization could increase the growth rate and mineral nutrient uptake of carnivorous plants as much as several times (for the review of experiments, see Adamec, 1997a and Ellison, 2006). However, on the basis of many greenhouse-growth experiments, it has been concluded that terrestrial carnivorous plant species differ greatly in their ability to use soil or foliar mineral nutrient supply for their growth and, accordingly, carnivorous plants have been classified into three main ecophysiological groups (Adamec, 1997a). Plants in the largest group of "nutrient requiring species" markedly increase their growth due to both soil and leaf nutrient supply and their root nutrient uptake may be stimulated by foliar uptake. These species grow relatively rapidly in rather wet habitats, sometimes with mildly increased soil nutrient content. Plants in the group of "root-leaf nutrient competitors" grow better and accumulate more nutrients due to both root and leaf nutrient uptake. However, competition occurs between root and leaf nutrient uptake. This group particularly includes some Australian *Drosera* species from drier areas. Plants in the third group of "nutrient modest species" have roots with a very low nutrient uptake capacity and rely on leaf nutrient uptake. This group includes some Australian pygmy sundews and also *Dionaea muscipula*.

7.2. NATURAL CONDITIONS

Field studies clearly show the ecological importance of carnivory for carnivorous plant growth and development under the conditions given. They include both competition and mortality, but also robbing of prey by opportunistic predators (kleptobionts, kleptoparasites) and washing out of nutrients from prey or washing away whole prey by heavy rains (Adamec, 1997a). In analogy with greenhouse experiments, natural capture of prey or feeding on extra prey in various terrestrial carnivorous plants under natural (or outdoor) conditions resulted in replicate growth increase which was comparable with that stated for greenhouse experiments (for the review, see Adamec, 1997a; Ellison, 2006). Moreover, the real ecological importance of carnivory in individual cases always depended on the amount of captured prey in which even closely placed individuals of the same species within the same micropopulation differed 10 times or more (Karlsson et al., 1987, 1994; Thum, 1989a, b). Thus, the amount of captured prey has proven to be the principal ecological factor for the natural growth and vigor of carnivorous plants. The differences in prey capture between the individuals might lead to size differentiation within the plant population (Thum, 1988). In this line, experiments on supplementary feeding of five European carnivorous plant species on prey proved that the plants were able to use much more prey for their enhanced growth or nutrient accumulation than they really could capture naturally (Thum, 1988; Chapin and Pastor, 1995; Hanslin and Karlsson, 1996). Therefore, the ecophysiological capacity to digest and utilize nutrients from prey is very high, but usually not fully used under natural conditions.

The classic cost–benefit model of plant carnivory by Givnish et al. (1984) predicts that carnivory will be beneficial only in nutrient-poor soils. To test this model, Ellison (2006) pooled the available data on 29 studies on growth experiments of carnivorous plants (influence of prey and soil fertilization), both under greenhouse and field conditions. A meta-analysis of these data clearly showed a significant positive growth effect of prey capture or addition ($p = 0.02$), but no significant effect of soil fertilization ($p = 0.15$) or nutrient × prey interaction ($p = 0.81$). This means that the effect of mineral fertilization of natural peaty soils may not lead to growth increase in carnivorous plants, though it was proven in some studies (e.g., Svensson, 1995) that an efficient use of prey is not confined to nutrient-poor soils (sensu Karlsson et al., 1991). As the level of soil fertilization was very different in single studies (see Adamec, 1997a) and could also be supraoptimal, it is hardly possible to generalize these experiments.

Prey capture is much more important for seedlings and small plants than for adult ones. Owing to their small size, prey capture by seedlings is considerably limited, but it leads, in successful individuals, to much faster growth and attaining maturity, and, therefore, to prolific flowering and seed set (Thum, 1988). Faster trap growth then allows more efficient capture of larger prey (i.e., positive feedback). Probably, capture of prey in adult plants supports flowering and seed set to the same extent as vegetative growth, but it markedly speeds up reaching the minimum plant size necessary for flowering. As a result of capturing prey, terrestrial carnivorous plants also markedly strengthen their competitive abilities (Wilson, 1985).

Under natural conditions, the ecological importance of carnivory concerning mineral nutrition mainly depends on what proportion of needed mineral nutrients (as seasonal nutrient gain or consumption) carnivorous plants take up directly from prey during their seasonal growth (Adamec, 1997a). From the measured rates of seasonal prey capture and usually a 76% efficiency of nutrient uptake from prey (after Dixon et al., 1980), the calculated values of the proportion listed in Table 1 are rather variable among different plant species, but also within a species, as dependent on differential seasonal prey capture. Overall, carnivorous plants at various sites can compensate by carnivory as much as 7–100% of their seasonal gain of N and the same amount of P, but only a small proportion of K (1–16%) and perhaps less Ca and Mg. These data indicate that the main ecological consequence of carnivory is to obtain the greatest proportion of seasonal N and P gain from prey as much as possible, because N and P are often the most (co-)limiting nutrients in peaty soils. Nevertheless, as shown by Hanslin and Karlsson (1996) for three *Pinguicula* species, the mean *direct* N uptake from extra-added prey amounted to only 39% of the total increased N amount, while the rest (i.e., 61% N) was taken up *indirectly* from the soil, as a result of the root uptake stimulation (see Section 6). Owing to low proportion of K, Ca, and Mg uptake from prey, the stimulated uptake of these elements from soil by roots should be the greater. Generally, it is possible to conclude that carnivory is ecologically very important for most species under natural conditions.

8. Ecophysiology of Aquatic Carnivorous Plants

8.1. INTRODUCTION

About 50 species belonging to the genera *Aldrovanda* (waterwheel plant, Droseraceae) and *Utricularia* (bladderwort, Lentibulariaceae) are submerged aquatic or amphibious carnivorous plants (Juniper et al., 1989; Taylor, 1989; Guisande et al., 2007). Unlike the dominant majority of aquatic noncarnivorous plants, all aquatic carnivorous species are strictly rootless and, therefore, can take up mineral nutrients for their growth from the ambient medium and from captured prey *only* via their shoots. Nevertheless, they fulfill all three principal functional criteria generally placed on carnivorous plants (see above). Traps of aquatic species exhibit rapid movements, which are among the most rapid within the plant kingdom, and represent fascinating objects for a biological study (Juniper et al., 1989). However, the main focus of ecophysiologists has always been the study of processes in terrestrial, rather than aquatic, carnivorous species (cf. Juniper et al., 1989; Adamec, 1997a). Moreover, mainly due to methodological problems, the ecophysiological study of the latter group has lagged much behind that of the former group. As both the ecological groups of carnivorous plants are rather dissimilar in their principal morphological and physiological features and also *per se* (submerged *vs.* terrestrial life *sensu* Colman and Pedersen, 2008), it is reasonable and justifiable to distinguish between these groups when making ecologically or physiologically oriented reviews.

8.2. ECOLOGICAL CHARACTERISTICS OF HABITATS OF AQUATIC CARNIVOROUS PLANTS

Aquatic carnivorous plants usually grow in shallow standing or slowly streaming humic (i.e., dystrophic) waters, often together with vascular aquatic noncarnivorous plants. It may be assumed that the former plant group also tolerates very high concentrations of humic acids and tannins (very dark waters), while the latter plant group usually does not. At these sites, the sum of the concentration of humic acids and tannins is commonly within the range 5–20 $mg.l^{-1}$ and may even extend to 60 $mg.l^{-1}$ (Adamec, 2007a, 2008b). The waters are usually poor in mineral N (NH_4^+, NO_3^-) and P (the concentration of both commonly 5–20 $\mu g.l^{-1}$), but also in K (<0.5 $mg.l^{-1}$). If the site is not impacted by human activity, the concentrations may be 5–10 times lower (see Adamec, 1997a; Guisande et al., 2007). The concentrations of SO_4^{2-}, Ca, Mg, and Fe, however, are usually >1 $mg.l^{-1}$ (Guisande et al., 2007) and do not limit plant growth. A partly decomposed, nutrient-poor litter of reeds and sedges usually accumulates in these waters. The slowly decomposable litter gradually releases mineral nutrients, humic acids, tannins, and CO_2. Hence, the waters are usually rather high in free $[CO_2]$(0.1–1 mM; Adamec, 1997a, b, 2007a, 2008b, 2009). A high $[CO_2]$ >0.15 mM was found to be the principal water chemistry factor supporting vigorous growth and propagation of stenotopic *A. vesiculosa* (Adamec, 1999). The same relationship between $[CO_2]$ and growth also holds true in aquatic noncarnivorous plants. In addition, reduced concentrations of dissolved oxygen, within the range 0.0–12 $mg.l^{-1}$, were found at many sites of aquatic carnivorous plants (Adamec, 1997b, 1999, 2007a; Guisande et al., 2000, 2004; Adamec and Kovářová, 2006; Guiral and Rougier, 2007). Moreover, the latter authors found a marked daily oscillation of $[O_2]$. The majority of aquatic carnivorous species usually grow in soft to moderately hard (total alkalinity 0.2–2 $meq.l^{-1}$), acid or neutral waters (pH 5–7.5), but some temperate-zone species may also grow in hard and alkaline waters (pH 8–9.3; see Adamec, 1997a). Two widespread aquatic species, *U. australis* and *U. minor*, were able to grow in a very wide range of pH, with the former species within 4.3–8.3 and the latter within 3.5–9.3 (cf. Adamec, 1997a, 2008b; Navrátilová and Navrátil, 2005). Thus, in eurytopic species at least, water pH alone is not important for their field growth.

While considering photosynthetic cost–benefit relationships, Givnish et al. (1984) postulated that for terrestrial carnivorous plants, carnivory is only beneficial in nutrient-poor, moist, and sunny habitats. However, many aquatic carnivorous species in their typical habitats do not comply, as irradiance is often very low (<5% of that in the open; Adamec, 2008b) though comparable with that for other aquatic noncarnivorous species.

8.3. GROWTH CHARACTERISTICS OF AQUATIC CARNIVOROUS PLANTS

Aquatic carnivorous plants markedly differ morphologically and ecophysiologically from terrestrial species: they are always rootless, floating freely below the water surface, or are weakly attached to lose sediments, submerged or partly amphibious (Taylor, 1989; Guisande et al., 2007). Most species have a linear and modular shoot structure consisting of regularly changing nodes with filamentous leaves and tubular, fragile internodes. In some species, the leaves are arranged in true whorls. Only several species (e.g., *U. volubilis*) are rosette-shaped plants. The majority of linear-shoot species have homogeneous (monomorphic), nondifferentiated green shoots bearing traps (e.g., *A. vesiculosa*, *U. vulgaris, U. australis, U. inflata*). Several species (e.g., *U. intermedia, U. floridana*) have dimorphic shoots differentiated into green photosynthetic ones (usually bearing only a few or no traps) and pale carnivorous (trapping) ones with many traps. These species are intermediate in body plan between the aquatic *Utricularia* species with monomorphic shoots and terrestrial species (e.g., *U. uliginosa, U. livida*) with aboveground, flat green leaves and belowground, pale carnivorous shoots bearing traps (Taylor, 1989).

Aquatic carnivorous plants exhibit some growth characteristics differentiating them distinctly from all terrestrial carnivorous species (Adamec, 1997a). First, adult plants maintain the length of the main shoot approximately constant throughout the season: they show very rapid apical shoot growth, but their basal shoot segments age and die at about the same rate ("conveyer-belt" shoot growth system). Thus, the new biomass is allocated into branching or flowering only. Under favorable conditions, the apical shoot growth rate of aquatic species with linear shoots was 1.0–1.2 whorls.day^{-1} in *Aldrovanda* (Adamec, 2000; Adamec and Kovářová, 2006) and even greater in field-grown *U. vulgaris* (1.4–2.8 nodes. day^{-1}, Friday, 1989) or *U. australis* (2.8–3.5 nodes.day^{-1}, Adamec and Kovářová, 2006). Surprisingly, the apical growth rate of *U. australis* was 2.9–4.2 nodes.day^{-1} in oligotrophic water also, though the relative growth rate (RGR) was zero (Adamec, 2009). Thus, very high apical growth rate in aquatic species may not be connected with high RGR. It is also an important strategy in competition with epiphytic algae, which usually densely cover their older shoots (Friday, 1989). In the slowly growing subtropical *U. purpurea*, however, the apical growth rate was only 0.25 nodes.day^{-1} (Richards, 2001). Unlike all terrestrial carnivorous plants, representing typical S-strategy, high RGRs were reported for a few aquatic species under favorable conditions, which rather suggests their R-strategy. The doubling time of biomass (i.e., $\log_e 2$ /RGR) in field-grown *Aldrovanda* was only 8.4–21.5 day (Adamec and Kovářová, 2006) or 12.9–23.0 day (Adamec, 1999), and 12.8 day in an outdoor culture (Adamec, 2000), 9.1–33.2 day in field-grown *U. australis* (Adamec and Kovářová, 2006), or 12.4–23.1 day in greenhouse-grown *U. vulgaris*, *U. geminiscapa, and U. purpurea* (Pagano and Titus, 2004, 2007). Pagano and Titus (2007) proved about a two- to threefold RGR increase in the three *Utricularia* species as a result of [CO_2] increase. Comparable values 6.4–34.7 days were recorded by Nielsen and Sand-Jensen (1991) for aboveground biomass in

12 rooted submerged, noncarnivorous species. In contrast, the corresponding values for terrestrial species of the genera *Drosera*, *Genlisea*, and *Sarracenia* are much greater (21–104 days, mean about 35–40 days), suggesting their considerably slower growth (Adamec, 2002, 2008c; Farnsworth and Ellison, 2008).

The very rapid growth of rootless aquatic carnivorous plants in nutrient-poor habitats requires ecophysiological adaptations that enable the plants to access the highly limited supplies of mineral nutrients from water. These adaptations include carnivory, efficient nutrient reutilization from senescing shoots, and a very efficient nutrient uptake from water (Kamiński, 1987; Kosiba, 1992; Friday and Quarmby, 1994; Adamec, 2000, 2008a, b; Englund and Harms, 2003).

Another important growth characteristic that facilitates propagation is the production of shoot branches which subsequently develop into separate new individuals (Adamec, 1999). The branches of *Aldrovanda* always develop into new plants, but in some aquatic *Utricularia* species, once initiated, branches may not develop beyond the early stages (Adamec, 2009). It is generally accepted that the number of branches per shoot is the principal growth parameter to be used as a criterion for plant vigor and propagation rate and therefore also reflects the suitability of a habitat for plant growth (Kamiński, 1987; Adamec, 1999, 2000, 2009; Adamec and Kovářová, 2006). In some aquatic carnivorous species studied, branching rate (number of internodes between two branches) was regular under optimum conditions and species specific (*Aldrovanda*: mean 6.2, range 3–11, Adamec, 1999; *U. stygia*: 12.2±0.4 in photosynthetic shoots, 6.7±0.2 in carnivorous shoots; *U. intermedia*: 16.8±0.4 in photosynthetic shoots, 5.9±0.1 in carnivorous shoots, Adamec, 2007a; *U. australis*: 22.1±1.2 or 10.5±0.4, Adamec, 2009). The latter data indicate that although branching rate is genetically fixed, it is under ecological regulation. If branching rate is divided by the apical shoot growth rate, this parameter – the branching frequency – characterizes the real time involved in initiating successive branches on the shoot. Thus, branching frequency is a good criterion for RGR (Adamec and Kovářová, 2006). The authors found a similar branching frequency (4.7–5.5 days.branch^{-1}) for field-grown *Aldrovanda* and *U. australis.* In both the species, however, competitive processes occurred between the production of new whorls and branches. Evidently, knowledge of branching traits is crucial for understanding the growth characteristics in aquatic *Utricularia* species.

8.4. TRAP ECOPHYSIOLOGY OF AQUATIC *UTRICULARIA* – MYSTERIES ASSOCIATED WITH PREY DIGESTION. HOW IMPORTANT ARE COMMENSALS?

Utricularia suction traps are hermetically closed bladders functioning on the basis of underpressure (e.g., Sydenham and Findlay, 1975; Juniper et al., 1989; Guisande et al., 2007). The trap size in aquatic species is within the range of 1–6 mm, rarely up to 12 mm (Taylor, 1989); these are larger than the traps of terrestrial species in the genus.

Though they are the smallest of all carnivorous plants, they are considered the most sophisticated traps functionally (Juniper et al., 1989). Contrary to the traps of other species, solutes and suspended particles sucked-in from the ambient water are retained hermetically in the lumen until the trap is senescent. Four types of glands (hairs) occur inside or outside the traps; abundant and large internal quadrifid and bifid glands are principal for trap physiology (see Juniper et al., 1989; Guisande et al., 2007).

Commensal microorganisms (mainly bacteria, algae (*Euglena*), ciliates, rotifers; e.g., Richards, 2001) occur and propagate in the traps of many aquatic *Utricularia* species. The question of their role in trap functioning and possible benefit for plants is often discussed. Presumably, some of these commensals participate to various extent in prey digestion by producing their own enzymes (Richards, 2001; Sirová et al., 2003), which has been demonstrated for phosphatases in commensal bacteria and unicellular algae (Płachno et al., 2006; Sirová et al., 2009). A slightly reduced growth of the wetland species *U. uliginosa* after the feeding on *Euglena* culture as prey (Jobson et al., 2000) suggests that the real relationship may even be slightly parasitic. Nevertheless, for some aquatic *Utricularia* species with low trapping efficiency (e.g., *U. purpurea*) in barren waters, commensal communities in traps seem to be more beneficial for the plants than the trapping of prey alone (Richards, 2001; Sirová et al., 2009). As shown very recently, commensals presumably participate in providing the traps without prey with N and P (Sirová et al., 2009). In traps without prey, which had sucked in some detritus or phytoplankton from the ambient water during incidental firings, a miniature microbial food web may run. Its main components are bacteria, Dinophyta, ciliates, and rotifers. Similar interactions were found in the digestion fluid in *Sarracenia* pitchers (Gray et al., 2006). Moreover, in filtered fluids from empty traps of two field-grown *Utricularia* species, high concentrations of organic carbon (60–310 $\mathrm{mg.l^{-1}}$), both glucose and fructose (8–24 $\mathrm{mg.l^{-1}}$), organic N (7–25 $\mathrm{mg.l^{-1}}$), and soluble P (0.2–0.6 $\mathrm{mg.l^{-1}}$) were detected (Sirová et al., 2009). The concentrations usually increased with trap age and correlated with commensal biomass. Traps presumably support the run of this microbial food web energetically by supplying organic matter, which is in relative excess for the plant, and as a trade-off for this, they obtain growth-limiting N and P from decomposed detritus or phytoplankton. Thus, aquatic *Utricularia* species, which grow in very oligotrophic habitats with low prey availability, are rather “bacterivorous” or “detritivorous” than carnivorous. The N_2 fixation mediated mainly by cyanobacteria occurred on the outer trap surface in *U. inflexa* (Wagner and Mshigeni, 1986). Owing to the specific chemical conditions, it could also run inside the traps and provide the traps with N.

Surprisingly, zero O_2 concentration was consistently detected in the fluid of excised and intact traps (without prey) of six aquatic *Utricularia* species bathed in an oxygenated medium, regardless of trap age and irradiance (Adamec, 2007b). Thus, there is normally anoxia inside the traps, which can incidentally be interrupted due to trap firing (trapping of prey or another irritation) for short periods of time.

The potential aerobic respiration of the inner glands and trap walls is so high that all O_2 is exhausted to zero within 10–40 min. The traps can pump out water and reset the underpressure within 30 min, which requires high amounts of energy derived from aerobic respiration and is prevented by respiration inhibitors (Sydenham and Findlay, 1975). Yet it is not clear how the traps (glands) provide sufficient ATP energy for their demanding functions under anoxia, though a mitochondrial mutation of cytochrome *c* oxidase found in *Utricularia* should provide greater energetic power for the traps (Laakkonen et al., 2006). In traps with captured prey, anoxia causes prey to die of suffocation, while all trap commensals are adapted to facultative anoxia (Adamec, 2007b).

A pH value of 5.0±0.1 occurred in trap fluid in four aquatic *Utricularia* species independently of digestion of prey (Sirová et al., 2003). This suggests that trap fluid pH is well regulated. Though several types of hydrolytic enzymes were described from *Utricularia* traps using biochemical and cytochemical methods (protease, esterase, acid phosphatase; see Juniper et al., 1989), only the activity of phosphatases at pH 4.7 was determined as significant by an *in situ* analysis of empty trap fluid in four aquatic *Utricularia* species (Sirová et al., 2003). Trap activities of α- and β-glucosidases, β-hexosaminidases, and aminopeptidases at the pH of 4.7 were usually lower by one or two orders of magnitude, and were usually higher in the culture water at the same pH. Thus, a large proportion of the trap enzymatic activity, with the exception of phosphatases, entered the traps from the ambient water after firing. Generally, the activity of all enzymes was independent of prey digestion and was not inducible by prey. The absence of aminopeptidases (proteases) in traps could be compensated by the autolysis of prey tissues. Consistently high trap activities of phosphatases in all species imply that P uptake from prey or detritus might be more important than that of N for the plant.

8.5. PHOTOSYNTHESIS OF AQUATIC CARNIVOROUS PLANTS

While the (maximum) net photosynthetic rate (P_{max}) per unit DW or area of leaves of slowly growing terrestrial carnivorous species is, on an average, 2–5 times lower than that in different functional groups of terrestrial noncarnivorous species (Ellison, 2006; see Section 5), P_{max} in aquatic species (seven species, 40–120 mmol O_2 kg^{-1} fresh weight.h^{-1}) is comparable with the highest values found in aquatic noncarnivorous species (30–110 mmol.kg^{-1} fresh weight.h^{-1}, Adamec, 1997b, 2006). Thus, very high P_{max} is typical for aquatic carnivorous species with rapid growth, but is also a prerequisite for this rapid growth as the rapid, permanent decay of senescent shoot segments causes a great loss of structural and nonstructural carbohydrates (Adamec, 2000). In amphibious carnivorous species with dimorphic shoots, P_{max} of photosynthetic shoots can increase considerably if the shoots become emergent (Colman and Pedersen, 2008).

Traps of aquatic species as physiologically very active organs have high respiration rates (RD) and represent great photosynthetic costs, as hypothesized by Givnish et al. (1984). In six aquatic *Utricularia* species, trap RD per unit fresh weight (5.1–8.6 $mmol.kg^{-1}.h^{-1}$) was 1.7–3.0 times greater than that in leaves on carnivorous or photosynthetic shoots and nearly maximum P_{max} in photosynthetic leaves exceeded that in the traps (5.2–14.7 $mmol.kg^{-1}.h^{-1}$) 7–10 times (Adamec, 2006). Thus, very high RD:P_{max} ratio in traps of these species (50–140%), unlike that in leaves (3.6–8.2%), means that there are high maintenance and photosynthetic costs of traps: in *U. stygia* and *U. intermedia* with dimorphic shoots, the trap RD could amount to 34–44% of the total plant respiration, while 63% was observed in *U. australis* with monomorphic shoots (Adamec, 2006, 2007a, 2008b). However, in *U. macrorhiza*, mean trap RD was only about 10% higher than that in leaves and trap P_{max} in lake water was as much as 41–67% of that in the leaves (Knight, 1992); similarly 67% in *Aldrovanda* (Adamec, 1997b).

Aquatic carnivorous plants usually grow in waters with high $[CO_2]>0.1$ mM. This fact is very important as all aquatic species tested so far use only CO_2 for photosynthesis (see Adamec, 1997a, b; Adamec and Kovářová, 2006; Pagano and Titus, 2007). The recent finding of slight HCO_3^- use in *U. australis* induced by growing at high pH of about 9.2 (Adamec, 2009) deserves further study. Generally, in several aquatic carnivorous species growing in the field or culture, CO_2 compensation points (CO_2 CPs) fell within the range 1.5–13.2 μM (Adamec, 1997a, b, 2009; Adamec and Kovářová, 2006; Pagano and Titus, 2007); similar values of 1.5–10 μM are reported in aquatic noncarnivorous plants (Maberly and Spence, 1983). In 17 culture-grown species or accessions of aquatic carnivorous plants of both the genera, CO_2 CPs (mean 5.3 μM, range 1.9–13.6 μM) were similar to those found in these species growing *in vitro* (mean 5.2 μM, range 2.5–8.8 μM; Adamec, 2007, unpubl.). A highly significant relationship was found between the CO_2 CP values and $[CO_2]$ in the culture water. Similarly, CO_2 CPs in *U. australis* growing at 17 sites of different trophic levels ranged within 0.7–6.1 μM (mean 2.6 μM), but neither correlated significantly with the internal or water chemistry factors nor the capture of prey (Adamec, 2009).

The influence of prey capture on P_{max} and RD was investigated in *Aldrovanda* and *U. australis* growing in an outdoor culture (Adamec, 2008a). Though both the species fed on zooplankton grew significantly faster than unfed ones, feeding increased P_{max} by 59% in *Aldrovanda,* but decreased it by 25% in *U. australis.* CO_2 CP was unchanged due to feeding in *Aldrovanda,* but increased from 5.2 to 9.2 μM in *U. australis.* The RD values stayed unchanged in both the species. Thus, the hypothesis by Givnish et al. (1984) on stimulation of photosynthesis by catching prey has not been supported, although more data are needed. Carnivory should partly compensate for photosynthetic CO_2 uptake, but the uptake of organic carbon from prey in aquatic carnivorous plants has never been quantified. Yet, organic carbon uptake from prey in aquatic species may be ecologically important under CO_2 shortage (see Adamec, 1997a): field-grown *Aldrovanda* was also able to grow at pH>9.0 when catching numerous prey (Adamec, 1999), and greenhouse-grown *U. vulgaris* fed on prey grew better and branched more only at higher pH values of 7.6–9.1 (Kosiba, 1992).

8.6. MINERAL NUTRITION IN AQUATIC CARNIVOROUS PLANTS

Although rootless aquatic carnivorous plants grow in mineral-poor habitats, they have macroelement composition similar to rooted aquatic noncarnivorous species (cf. Dykyjová, 1979; Adamec, 1997a, 2008a, b). Their tissue nutrient content (% DW) in young shoots is usually 1.0–4.0 for N; 0.12–0.50 P; 1.5–5.0 K; 0.15–3.0 Ca; and 0.2–0.7 for Mg. *Utricularia australis* growing in very oligotrophic waters with low prey availability kept a relatively high shoot nutrient content (Adamec, 2008b). Regardless of a marked polarity of tissue N, P, and Ca content along shoots and the great differences between leaves and traps (Adamec, 2000, 2008b), the mean shoot content of these five macroelements in aquatic carnivorous plants is about 1.5–3 times greater than that in terrestrial carnivorous plant leaves (cf. Ellison, 2006; see Section 5), and could reflect much faster growth in aquatic species. A marked polarity of tissue N, P, Ca content, indicating a very efficient N and P reutilization from senescing shoot segments, was found in rapidly growing aquatic species (Adamec, 1997a, 2000, 2008b). The Ca polarity was opposite, whereas K and Mg contents were constant. Culture-grown *Aldrovanda* reutilized 88% N and 67% P (Adamec, 2000), and field-grown *U. australis,* on an average, reutilized only 48% N and 72% P (Adamec, 2008b). However, the slowly growing *U. purpurea* reutilized only 37% N and 71% P (see Adamec, 1997a). Moreover, a very effective reutilization of N and P can be assumed in autumnal shoots forming turions (Adamec, 2000). Thus, aquatic species permanently lose only a relatively small part of their N and P in their senescent shoots (like terrestrial species; Adamec, 1997a), but all K, Ca, and Mg. Zero K reutilization in senescent shoots of aquatic species greatly contrasts with very efficient K reutilization in terrestrial ones (41–99%, see Section 5; Adamec, 2002).

Considerable differences in tissue nutrient content occur between shoots and traps in aquatic carnivorous plants of both the genera. The content of N, Ca, and Mg is usually greater in photosynthetic shoots than in the traps, while the opposite is true for P and K demonstrating a considerable "mineral" cost of carnivory (Adamec, 2008b; 2007, unpubl.). In *U. australis* with monomorphic shoots and mean proportion of trap DW 38%, traps in adult shoots contained about 30% total N, 53% P, and 51% K within the shoots (Adamec, 2008b).

Aquatic carnivorous species grow in oligomesotrophic waters in which [NH_4^+] usually strongly dominates over [NO_3^-]. *Aldrovanda* and some aquatic *Utricularia* species preferentially took up NH_4^+ than NO_3^- from diluted NH_4NO_3 solutions (Adamec, 1997a, 2000; Fertig, 2001). Phosphate uptake by *Aldrovanda* apical shoot parts was two times faster than that by basal parts, but the finding that *Aldrovanda* takes up K+ only via the basal parts (Adamec, 2000) still requires a deeper explanation. Besides, aquatic species can also take up a substantial amount of mineral nutrients from prey. Yet, only Friday and Quarmby (1994) have quantified an efficiency of N uptake from prey in aquatic species. In *U. vulgaris* fed on mosquito larvae, they estimated the efficiency of N uptake to be at least 83% of the total prey N. About 52% total plant N was obtained from the prey. Probably, the efficiency of N uptake from prey in *Utricularia* traps may be even higher than that in terrestrial species (cf. Adamec, 2002, see Section 6). P was also taken up rapidly from the prey, but P reutilization from old shoot segments was much better than that of N.

There are several data showing marked effects of prey utilization on the growth of aquatic species, both in a culture and in the field (for the review see Adamec, 1997a, 2000, 2008a; Englund and Harms, 2003). Feeding led to longer shoots, greater DW, faster apical shoot growth, greater RGR, and, especially, increased branching as a main means of propagation. Thus, carnivory in aquatic species is at least as important as in terrestrial ones. However, it is still unclear how this growth effect of carnivory is induced because tissue N and P contents in apical or young shoot segments in prey-fed plants were lower when compared with unfed plants (Adamec, 2000, 2008a).

Another important ecological parameter associated with mineral nutrition is the proportion of seasonal (daily) N and P gain obtained from prey. In *U. macrorhiza,* the proportion of seasonal N gain from carnivory was estimated to be about 75% (Knight, 1988). In robust *U. foliosa* growing at a nutrient-poor site with extremely low prey availability in Florida, the mean proportion was only about 0.9% N and 3.5% P (Bern, 1997). At sites with greater prey availability, these values could be one order of magnitude higher. Using a model containing literature-based data (Adamec, 1997a, 2000, 2008b; Adamec and Kovářová, 2006), it is possible to calculate which proportion of the daily N and P gain can be obtained due to capturing one small *Cyclops* (DW 25 μg) daily in *Aldrovanda* and *U. australis*. In rapidly growing plants (plant biomass doubled in 15 days), this covers about 15% daily N and 4% P gain in smaller *Aldrovanda*, while only about 0.62% N and 0.56% P gain in larger *U. australis*. However, if plant growth is zero and the plants maintain constant biomass, which is the case under unfavorable growth conditions, then it covers up to 100% daily N and about 16% P gain in *Aldrovanda* and about 1.8% N and 2.6% P gain in *U. australis.* Like in terrestrial species, the estimated values show that the ecological importance of N and P uptake from prey depends primarily on the quantity of captured prey. Capture of prey in aquatic species is thus one of the decisive factors for their rapid growth and especially for their propagation.

8.7. REGULATION OF INVESTMENT IN CARNIVORY IN *UTRICULARIA*

In aquatic *Utricularia* species, the proportion of traps to the total plant biomass as the structural cost (investment) in carnivory is 10–65%, but this proportion is regulated flexibly by the plants to minimize the costs of carnivory according to habitat factors: particularly, water chemistry, prey capture, and irradiance (Knight and Frost, 1991; Friday, 1992; Bern, 1997; Guisande et al., 2000, 2004; Richards, 2001; Englund and Harms, 2003; Manjarrés-Hernández et al., 2006; Porembski et al., 2006; Kibriya and Jones, 2007; Adamec, 2007a, 2008b). Moreover, this regulation may be different in various species. Yet, in most cases, increased mineral nutrient availability either in the ambient water or prey led to decreased investment in carnivory in terms of trap number per leaf or proportion of trap biomass. The number of traps per leaf in *U. foliosa* decreased in waters with increasing [NO_3^-], but simultaneously decreasing prey capture (Guisande et al., 2004). Thus, it is not possible to separate both the factors. However, the number of traps per leaf in the same species correlated statistically significantly (inversely proportional) with shoot P and especially N content (Bern, 1997). Out of all nutrient factors investigated in field-grown *U. australis*, only tissue N content in young shoot segments significantly (negatively) correlated with trap proportion (Adamec, 2008b). These results consistently support the "nutrient" hypothesis that all external nutrient factors that decrease tissue N content in young shoots (poor prey capture, low [NH_4^+], high [CO_2], etc.), increase trap production in young shoots and *vice versa*, as a negative feedback regulation. This finding is consistent with the suggestion of Guisande et al. (2004) that ambient N sources are a limiting factor regulating investment in carnivory. However, Kibriya and Jones (2007), studying *U. vulgaris*, proposed a central regulatory role for P. This negative feedback also helps stabilize the tissue contents of other mineral nutrients. As prey capture also supports plant growth, growth rate itself is obviously a component of this endogenous regulatory system. Moreover, some data show that the "nutrient" regulation of trap proportion in aquatic *Utricularia* is subject to photosynthetic regulation (Bern, 1997; Englund and Harms, 2003; Adamec, 2008b). At low photosynthetic rate (low irradiance, [CO_2]), trap proportion is relatively low or even zero.

9. Is Plant Carnivory Beneficial: When and Why? Nutritional Cost–benefit Relationships of Carnivory

In their natural habitats, carnivorous plants grow *together* with noncarnivorous species (e.g., bog and fen species of graminoids, cyperoids, and Ericaceae), and it is evident that both the plant groups are subjected to exactly the same ecological conditions; the same conclusion may also hold true for aquatic carnivorous plants. Carnivory is thus only one of many possible adaptation strategies to combined unfavorable conditions occurring in nutrient-poor and wet soils. Yet when and why can this ecological adaptation be beneficial? Based on studying carnivorous bromeliads, Givnish et al. (1984) predicted in their cost–benefit model that carnivory would only be beneficial for plants growing in sunny, moist, and nutrient-poor habitats, in which the benefits of carnivory would equal or rather exceed the costs associated with carnivory. The model focused on enhanced photosynthesis as the primary physiological consequence and benefit of carnivory and made three predictions (see also Givnish, 1989; Ellison and Gotelli, 2001; Ellison, 2006, Guisande et al., 2007). First, carnivorous plants should have an energetic advantage in competing with noncarnivorous plants. Further, the primary energetic benefit of carnivory should be either an increased photosynthetic rate per unit leaf mass or total leaf mass supported. Finally, the absolute benefit of carnivory should saturate and the marginal benefit should decline with increasing investment in carnivory when factors other than nutrients (e.g., light, water) are limiting. The main structural and "nutrient" costs of carnivory are the production of traps. However, traps are also associated with photosynthetic and energetic (metabolic) costs. First, P_{max} of traps is lower than that of leaves, but the data are still scarce (Knight, 1992; Adamec, 1997b, 2006; Ellison and Gotelli, 2002; Pavlovič et al., 2007). Second, RD of traps (as maintenance cost), due to their intensive physiological processes, is much greater than that of noncarnivorous parts of the leaves (Knight, 1992; Adamec, 2006).

Although photosynthesis is a key process and prerequisite of plant growth, the focus of Givnish et al. (1984) on photosynthesis increase as a primary consequence and benefit of carnivory has probably been rather overestimated. As terrestrial carnivorous species being S-strategists are evolutionarily adapted to low RGR, the capacity to accelerate their growth is limited (Adamec, 2002, 2008c; Farnsworth and Ellison, 2008) and, thus, they do not require high P_{max} per unit biomass. This conclusion clearly follows from the study by Shipley (2006) on close correlation of P_{max} values with RGRs in plants. Moreover, on the basis of the fact that P_{max} may not increase due to carnivory and owing to relatively low foliar N and P content in carnivorous plants (see Section 5), it is possible to assume that slow growth of carnivorous plants is rather limited by a shortage of mineral nutrients necessary for growth processes in growing centers. Thus, the primary physiological benefit of carnivory could be to provide N and P for essential growth processes, such as cell division, DNA replication, and proteosynthesis in young, miniature tissues in shoot apices (Adamec, 2008a), though the measurement of tissue N and P content in "bulk" shoot apices did not confirm this assumption (Adamec, 2000, 2008a). In this line, the primary physiological benefit of carnivory in terrestrial species could also be the stimulation of root nutrient uptake (see Section 6).

In summary, the ecological cost–benefit relationships of carnivory could therefore be expressed (instead of photosynthesis, Givnish et al., 1984) in terms of *relative gain* of limiting mineral nutrients coming from carnivory (or *efficiency* of mineral nutrient investment in traps). All carnivorous plants have to invest some amount of mineral nutrients in the production of traps (M_1 for a given nutrient) within the growing season, which is a mineral nutrient cost of carnivory. A portion of this amount (M_2) is lost with the senescent biomass; the difference $M_1 - M_2$ is reutilized (see Section 6). The traps containing M_1 mineral nutrients are able to gain a certain amount of mineral nutrients from prey (M_3) over their trap lifespan (a trap does not necessarily survive for an entire growing season). By assuming mineral nutrient uptake from prey to be the main *direct* ecophysiological benefit of carnivory, the M_3:M_2 ratio then characterizes the *direct* nutritional benefit (and also efficiency) of carnivory. It expresses how many atoms of, e.g., N is taken up from prey per one atom of N lost with the senescent trap biomass, but not per the total trap N amount. However, to ignore the total trap nutrient amount (M_1) in the nutrient-based cost–benefit relationships of carnivory is a simplification, as carnivorous plants must first invest this amount to trap production, although the reutilizable amount ($M_1 - M_2$) is not lost for the plant. Therefore, some differences in the interpretation of this model may occur among evergreen, temperate, and annual carnivorous species. Nevertheless, in terrestrial carnivorous species, a greater proportion of mineral nutrients gained as a result of carnivory is taken up by roots from the soil, as a result of the stimulation of root nutrient uptake (see Section 6). Thus, the direct nutritional gain from prey (M_3) should be added to the amount of nutrients gained *indirectly* by the plant due to this root stimulation (M_4) as *indirect* ecophysiological benefit of carnivory. Then, the M_4:M_2 ratio characterizes the *indirect* nutritional benefit of carnivory, the sum $M_3 + M_4$, the *total* ecophysiological benefit of carnivory in terms of nutrient gain, and, consequently, the expression $(M_3 + M_4)/M_2$ characterizes the *total* nutritional benefit of carnivory, as the sum of the *direct* and *indirect* nutritional benefits of carnivory (see Fig. 1). A simpler interpretation of these parameters might be for plants with traps distinctly separated from photosynthetic leaves (e.g., *Genlisea*, *Utricularia*) than for those with combined traps and leaves (e.g., *Drosera, Pinguicula*). Furthermore, it is possible to assume that, in mineral-richer soils, a greater proportion of all mineral nutrients to the total plant amount will be taken up by roots from the soil (or from the ambient water in aquatic species), and thus, the nutritional contribution of carnivory will be declining sigmoidally

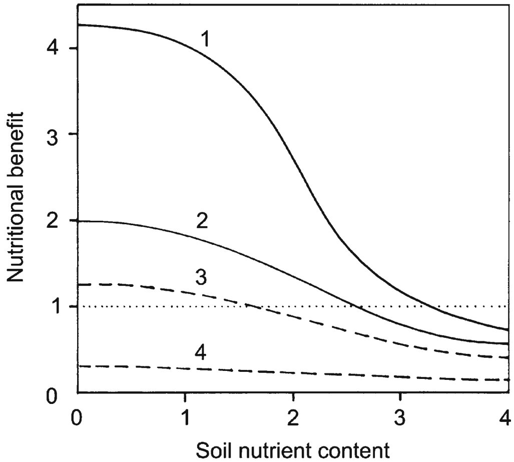

Figure 1. Schematic model for the nutritional benefits of carnivory as dependent on available soil mineral nutrient content (arbitrary units). Nutritional benefits in this scheme include either direct or total nutritional benefits (for their explanation, see the text). 1, total nutritional benefit for N, P, and K, high prey capture; 2, total nutritional benefit for N, P, and K, low prey capture; 3, direct nutritional benefit for N and P and total nutritional benefit for Mg, high prey capture; 4, direct nutritional benefit for K, Ca, and Mg, high prey capture.

below 1. The value of the total nutritional benefit equal to 1 denotes the level under which carnivory for a given nutrient is not ecologically beneficial.

According to this definition, the direct, indirect, and total nutritional benefits of carnivory are zero in carnivorous plants without any prey, however rapid their growth and nutrient uptake are. This must be respected at experimental estimations of these parameters: nutrient amounts in unfed controls should be subtracted from those in variants with prey (*sensu* Hanslin and Karlsson, 1996; Adamec, 2002). So far, these parameters have not been presented and complete data for their calculation are lacking. Generally, there are two approaches to estimate or assess the nutritional benefits of carnivory. In all cases, for the estimation of these parameters, the data on the proportion of trap nutrient amount (or at least DW) to that of the total plant and on the efficiency of mineral nutrient reutilization from traps (leaves) are necessary. One approach to estimate both the direct and indirect benefits is based on detailed data from a growth experiment on feeding on prey or supply of nutrient solution onto the traps (Hanslin and Karlsson, 1996; Adamec, 2002). To estimate the indirect nutritional benefit, the presented values of "efficiency of the use of nutrients supplied onto the leaves" (Adamec, 2002) might also be used. Second, in natural populations of carnivorous plants, the estimated values of the proportion of seasonal nutrient gain from carnivory (see Section 7.2, Table 1), together with the relationship between the seasonal nutrient gain and total plant nutrient amount may be used for assessing the direct nutritional benefit.

In mineral-poor soils, at high natural rates of prey capture, on the basis of known efficiencies of nutrient uptake from prey and nutrient reutilization (see Section 6), and assuming the proportion of trap DW to the total one to be about 40% in *Drosera* spp. or 80% in *Pinguicula* spp., the total nutritional benefit of carnivory at high prey capture rates should be several units (2–10) for N, P, and K, around 1 for Mg, but only about 0.5 for Ca, while the direct values for all nutrients should be much lower (Fig. 1). The assessments of direct values for a growth experiment on three *Drosera* species fed on nutrient solution (Adamec, 2002) were about 0.4 for N, 0.2 K, 0.1 Mg, and 0.07 for Ca, while the total values were about 4.0 for N, 11 P, 5.5 K, 1.1 Mg, and 0.5 for Ca. Similarly, the experimental data on

Table 1. Mean or range of seasonal mineral nutrient gain coming from carnivory (in percent) in terrestrial carnivorous plant species under natural conditions obtained from different authors.

Species	N	P	K	Reference
Pinguicula vulgaris	26–40	36	7–16	Karlsson (1988); Karlsson et al. (1994)
Pinguicula alpina	8–14	12–19	1.3–1.9	Karlsson (1988); Karlsson et al. (1994)
Pinguicula villosa	7–15	6–10	3–12	Karlsson (1988); Karlsson et al. (1994)
Drosera rotundifolia	63	95	1.1	Thum (1988)
Drosera intermedia	92	100	1.6	Thum (1988)
Drosera erythrorhiza	11–17	–	–	Dixon et al. (1980)
Drosera erythrorhiza	100	100	2–3	Watson et al. (1982)

feeding three European *Pinguicula* species on fruit flies (Hanslin and Karlsson, 1996) may lead to an assessment of the direct values for N within 0.8–2.7, while the total ones within 1.7–7.0. *Drosera rotundifolia* and *D. intermedia* capture prey very efficiently, and covering 90% of their seasonal N and P gain and 1.5% K gain from carnivory (Table 1; Thum, 1988) may lead to the direct nutritional benefit of about 3.6 for N, 8.1 P, but only 0.08 for K. In aquatic *U. macrorhiza*, the total value of about 4.3 for N can be assessed from the study by Knight (1988).

To what extent does this model reflect reality? Obviously, this nutritional model may be useful in comparing and quantifying both the nutrient losses in senescent traps as the nutritional cost, and the direct and indirect nutrient gains as the benefits. The model confirms the experimentally based results on that the direct mineral nutrient uptake from carnivory is usually several times lower than the indirect one due to root uptake stimulation and that this disproportion is relatively greater for K, Mg, and Ca than for N and P (see Section 6; Adamec, 1997a, 2002; Hanslin and Karlsson, 1996). It follows clearly from the model that individual mineral nutrients taken up from carnivory differ greatly in their relative benefit for the plants: the nutrients taken up very efficiently from prey carcasses (N, P, K) and, simultaneously, being reutilized very efficiently from senescing leaves (N, P, K) are candidates for mineral nutrients that brought an ecological advantage for plants with this adaptation and evolutionarily supported carnivory. In contrast, Mg and Ca reutilization is usually very poor and also, due to their low tissue content in prey and zero Ca uptake from prey, direct uptake of these two nutrients from prey could not "drive" carnivorous plant evolution. Instead, to ensure the seasonal Ca and Mg gain by the plants and thus avoid a possible Ca- and Mg-based growth limitation, efficient physiological mechanisms were developed to stimulate Ca and Mg uptake by roots. In conclusion, to be nutritionally beneficial, carnivorous plants do not only need to capture prey efficiently (per trap biomass) but also need to maximize nutrient uptake from prey and minimize nutrient losses in senescing traps. The fact that terrestrial carnivorous plants show a very efficient N, P, and K reutilization from shoots supports this concept. The "nutritional" concept of carnivory does not deny the outcomes of the photosynthetic concept (Givnish et al., 1984). Rather, it prefers the importance of mineral nutrients for carnivory.

Overall, looking at worldwide species diversity of terrestrial carnivorous plants, it can be concluded that the majority of species (about 70–80%) do obey the ecological predictions given by Givnish et al. (1984) and grow more or less in sunny, moist, and nutrient-poor habitats (e.g., Juniper et al., 1989; Taylor, 1989; Rice, 2006; Studnička, 2006). However, the other terrestrial species can be considered facultatively (or even strictly) sciophilous (i.e., shade adapted) and grow within the herbal understorey under the canopy of taller accompanying vegetation (e.g., *Utricularia*, *Genlisea*, *Pinguicula*), or in shaded rocky walls (*Pinguicula*), or in tropical rainforests under a canopy of trees (*Nepenthes, Drosera, Triphyophyllum*). In contrast, *Drosophyllum lusitanicum* is an atypical xerophytic species (Adlassnig et al., 2006).

Within the ecological group of about 50 species of aquatic carnivorous plants (see Section 8.2), the absence of sunny habitats is presumably much more common than in terrestrial ones, as many aquatic *Utricularia* species can either be considered sciophilous or grow in rather shaded habitats or in dark waters (often <5% of incident irradiance; Adamec, 2008b). Yet, they are still carnivorous, but under shade conditions, they could greatly reduce their growth rate. However, when irradiance falls below a critical limit or CO_2 is under shortage, their investment in trap production is minimal or zero (Bern, 1997; Englund and Harms, 2003; Adamec, 2008b). Generally, the above-mentioned predictions on habitat requirements given by Givnish et al. (1984) should not be applied for aquatic carnivorous plants. Instead, it is possible to suggest that carnivory in aquatic environments will only be beneficial for plants in nutrient-poor and CO_2-rich habitats at above-threshold irradiance over 5–10% of that in the open.

10. Phylogeny of Carnivorous Plants: How Many Times in the History?

As follows convincingly from modern molecular-taxonomic studies, recent carnivorous plants evolved in five to six lineages from its preliminary ancestors (probably protocarnivorous plants; Spomer, 1999), independently on each other within evolution (i.e., polyphyletic origin; e.g., Müller et al., 2004; Heubl et al., 2006; Porembski and Barthlott, 2006). However, due to the lack of fossil material, the original ancestral types are still mostly unknown. For a long time, the paleaospecies *Palaeoaldrovanda splendens* from Late Cretaceous (75–85 mya; Degreef, 1997) was considered the oldest known carnivorous plant, representing the ancestral type of the recent *A. vesiculosa*. Recently, a fossil angiosperm plant *Archaeamphora longicervia* has been described from the Early Cretaceous Yixian Formation (125 mya), NE China, as a possible ancestor of the modern *Sarraceniaceae* (Li, 2005). Morphologically, it resembles *Sarracenia purpurea* and *Heliamphora* very much. Thus, it probably represents the oldest carnivorous plant and the only fossil record of pitcher plants. By assuming the age of Angiosperms to be about 280 mya (see Li, 2005), these findings show that the origin of carnivorous plants was relatively very early in the evolution of Angiosperms. On the other hand, carnivory was lost secondarily in recent genera of the family Ancistrocladaceae and almost lost in Dioncophyllaceae (except *Triphyophyllum peltatum*; Heubl et al., 2006).

Within carnivorous plants, the greatest attention has recently been paid to studying the molecular-taxonomic and evolutionary relationships in Lentibulariaceae comprising about 325 species of three genera (e.g., Jobson et al., 2003; Müller et al., 2004, 2006; Laakkonen et al., 2006). In two genera of the rootless *Utricularia-Genlisea* clade, but not in *Pinguicula,* extremely high DNA mutation rates were found (Müller et al., 2004, 2006). This high mutation rate in *Utricularia* and *Genlisea* is associated with the smallest genome size in these two genera within Angiosperms (Greilhuber et al., 2006) and is explained as the metabolic consequence of direct uptake of organic substances (e.g., amino acids). Nevertheless, the rootless aquatic

A. vesiculosa (Droseraceae) has exactly the same type of mineral and organic nutrition as aquatic *Utricularia* species (see Sections 8.5 and 8.6), but its DNA mutation rate is extremely low, and as such could be considered a "living fossil" (Maldonado San Martín et al., 2003; Hoshi et al., 2006). It seems, therefore, that the reasons for such a behavior will be elsewhere. In *Utricularia* (but not in *Genlisea*) species, a mutation in the mitochondrial cytochrome *c* oxidase has recently been found (Laakkonen et al., 2006). This mutation would permit the plants to increase energy output (for rapid trap movement), but with a 20% reduction in energy efficiency of the respiratory chain. On the basis of their model, the authors suggest that this mutation leading to greater RD could represent a metabolic benefit, was evolutionarily advantageous, and could contribute to faster evolution of this genus.

11. Concluding Remarks: Inspiration for Further Research

As shown in the review, carnivorous plants have evolved several times during plant evolution independently of each other, mainly as an ecological adaptation to combination of mineral nutrient poverty in wet soils, usually in sunny habitats. Thus, there was a permanent and strong selective pressure in plant evolution to adapt to these stressful conditions. The main ecophysiological strategy of terrestrial carnivorous species as S-strategists is slow growth and very effective mineral nutrient economy. This strategy, together with gaining mineral nutrients from carnivory, does not require a high photosynthetic rate – as compared with noncarnivorous species – and enables them to survive even in very barren wet habitats. Following from the typical tissue mineral nutrient content in animal prey carcasses, it is N and P (partly also K) taken up from prey that are of the greatest importance for plant growth. The benefit of carnivory always depends on the quantity of captured prey so that successful "trappers" can compensate from prey up to 60–100% of their seasonal N and P gain, but only 1–16% of K. Besides the direct mineral enrichment of carnivorous plants in nutrients coming from prey, foliar nutrient uptake from prey very markedly stimulates root nutrient uptake representing the main physiological effect of carnivory.

Aquatic carnivorous species are ecophysiologically quite dissimilar to their terrestrial counterparts. The principal growth traits in rootless aquatic species with linear shoots – very rapid apical shoot growth and high RGR even in barren habitats – are associated with very steep physiological polarity along the shoots and require a combination of several ecophysiological processes. They include the capture of animal prey, very high P_{max}, very efficient mineral nutrient uptake from water, and efficient mineral nutrient reutilization (except K) from senescent shoots.

Generally, it is possible to conclude that carnivory is *almost indispensable* for naturally growing carnivorous plants.

To obtain further insight into the ecophysiology of growth and nutrition of carnivorous plants, the following directions of research could be considered and the questions raised could be answered.

1. Basic properties of mineral ion uptake need to be studied in isolated roots, e.g., mineral nutrient uptake affinity and capacity for different mineral ions, and compared with those in roots of noncarnivorous plants.
2. The stimulation of root nutrient uptake by foliar uptake represents the main physiological effect of carnivory, but its essence is still unknown. It is not known whether it is mediated by increased allocation of photosynthates or mineral nutrients from leaves to roots or whether it represents primary or secondary effects of utilization of prey.
3. The effect of growth enhancement due to carnivory: which physiological effects are primary and which are secondary? What is the role of tissue N and P content in this growth enhancement? If carnivory does not increase P_N per unit shoot biomass, it is possible to assume that the positive growth effect is caused by stimulation of cell divisions in juvenile tissues in shoot apices.
4. Steep growth polarity in aquatic carnivorous plants and their very rapid apical shoot growth associated with the steep physiological polarity imply the involvement of physiological processes that are insufficiently understood (e.g., phytohormone distribution).
5. In aquatic carnivorous plants, what is the affinity of shoots for mineral nutrient uptake from water? Is shoot nutrient uptake from the ambient water stimulated by prey capture (in analogy with terrestrial species)? What is the efficiency of mineral nutrient uptake from prey?
6. On the basis of the ecophysiological peculiarities of aquatic carnivorous plants, the cost–benefit model of carnivory needs to be elaborated for this particular plant group.
7. What is the role of organic nutrition in carnivory?
8. Food webs have been described in traps of many carnivorous plant species. What is the role of commensals living in the traps for nutrient uptake by plants?

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REVERSING THE ROLES OF PREDATOR AND PREY

A Review of Carnivory in the Botanical World

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1. Carnivory in the Plant World

Plants, like all organisms on the planet, live to perpetuate their own existence and to reproduce. The transmission of genetic material to future generations is the driving force behind life. In habitats where light, and water, and the essential nutrients are easily available, plants must merely allocate resources to contend with the challenges associated with reproduction, and defending themselves against the environment and herbivores. However, in more stressful habitats where nutrients are in short supply, plants have developed novel solutions to increase their chances of survival.

1.1. HABITATS SELECTING FOR CARNIVORY BY PLANTS

Despite its benefits, carnivory has its metabolic costs. Significant resources must be expended in creating what are at times elaborate structures that are inefficient at conducting the normal operations in which a plant engages, specifically that of photosynthesis. Furthermore, metabolic resources from the plant must be expended in the creation of pigments, nectar lures, odors, and perhaps compounds such as digestive enzymes. Such expenses cause carnivory to be ineffective in most settings; it is even a losing proposition in conditions that are not limited by available soil and water-borne nutrients. Cost-benefit models of carnivory indicate that carnivorous plants are more likely to occur in sunny locations where water is abundant and nutrients are in short supply (Givnish et al., 1984). In most carnivorous plant species, this model is reflected in the wild (Rice, 2006a). On the whole, two environmental situations encourage predation by plants:

1. Soils with very high organic content, but with unavailable nutrients. Examples of such settings include boglands around the world. Although the organic content of the soils in these habitats may be very high, approaching 100%,

the soils are extremely acidic, with a pH of 3–4 (Clarke and Moran, 2001; Crum, 1988; Schnell, 2002). The high acidity is often maintained by extensive growths of *Sphagnum* moss (Crum, 1988; Schnell, 2002). In such conditions, bacterial mineralization activity is suppressed, and dead organic material is not reduced to raw nutrients.

2. Soils that are either poorly developed or have extremely low organic content. Many carnivorous plants in Australia, Africa, and South America live in seasonally wet sites, in a substrate consisting of nearly pure sand or laterite (Lowrie, 1989; Taylor, 1989). Many species of *Pinguicula*, *Utricularia*, and *Genlisea* around the world live on sheer rock outcrops, inselbergs (isolated rock hills emerging from flatter topography), or ferricretes (a highly weathered type of compacted soil) (Fischer et al., 2000; Legendre, 2000; Taylor, 1989). Species in the genera *Nepenthes* and *Triphyophyllum* live in poor soils in tropical forests, where most of the available nutrients are already sequestered in the existing biomass (Clarke, 1997; McPherson, 2008).

To this simplified analysis, one could add a variety of other extreme conditions. Numerous aquatic carnivores can be found suspended in waters in habitats associated with both situations described above (Breckpot, 1997; Rice, 2006a; Taylor, 1989). In spite of predictions by Givnish et al. (1984), entirely epiphytic habits have been adopted by carnivorous plants in the genera *Utricularia*, *Pinguicula*, and *Nepenthes* (Clarke, 1997; Legendre, 2000; Taylor, 1989). Finally, soils derived from limestone or serpentine are associated with carnivorous plants in Southeast Asia, Mexico, and the Pacific Northwest of the USA (Clarke and Moran, 2001; Legendre, 2000; Rice, 2006a; Schnell, 2002). Serpentine soils are low in macronutrients while high in metals such as magnesium, iron, chromium, nickel, and cobalt (Juniper et al., 1989; Kruckerberg, 2006), conditions which are harmful to many plants. The mechanism for metal tolerance in carnivorous plant is not well understood.

1.2. DEFINITIONS OF CARNIVORY

The attributes necessary for a plant to be considered carnivorous are not universally agreed upon. The first published, rigorously defined criteria consisted of two parts, and required only that (1) a plant have at least one adaptation for attraction, capture, or digestion of prey, and (2) that the plant be able to absorb the nutrients from the prey (Givnish et al., 1984). Juniper et al. (1989) noted that a plant involved in the carnivorous syndrome might display attributes related to attraction, retention, trapping, killing, digestion, and absorption of prey. Juniper et al. (1989) also noted that only two components are necessary: the possession of both traps and digestive organs. A definition frequently adopted is that the plant must attract, trap, digest prey, and nutrients from the digested prey must be subsequently absorbed; to these four criteria could be added that the plant must clearly benefit from the nutrients obtained by the act of carnivory (Schnell, 2002).

Despite the apparent soundness of such definitions, the carnivory of plants is often taken purely on faith. Indeed, these primary characters (i.e., attraction, trapping, digesting, absorption, proven benefit) have been conclusively demonstrated to be in effect for only a small fraction of those species that are currently proclaimed as carnivorous.

For example, consider the genera *Drosera* and *Pinguicula*, which include a large number of basal-rosetted plants with sticky surfaces. The carnivory of such species is never questioned (Lloyd, 1942; Lowrie, 1987, 1989, 1998; Rice, 2006a; Schnell, 2002), even though the only studies comparing the attracting effects of *Drosera* and *Pinguicula* to comparably sized inert traps (Antor and García, 1994; Harms, 1999; Watson et al., 1982; Zamora, 1990, 1995) failed to show any significant active luring by such plants (Ellison and Gotelli, 2009). It is unclear whether prey of these species are actually attracted to the leaf rosettes or whether they simply blunder onto the sticky leaves. If these plants do not attract prey, should they no longer be considered carnivorous?

The production of digestive enzymes by putatively carnivorous plants is another matter of great discussion, particularly with respect to sticky-leaved plants (Anderson and Midgley, 2003; Hartmeyer, 1997; Rice, 1999) and some of the pitfall trap genera, i.e., *Brocchinia*, *Catopsis*, *Darlingtonia*, and *Heliamphora* (Givnish et al., 1984; Jaffé et al., 1992; Rice, 2006a). There is no doubt that many carnivorous plants produce their own digestive enzymes (see Section 3.1), but do they all do so? Some plants that have a variety of carnivorous attributes (sometimes in great abundance, such as the extraordinary *Darlingtonia californica*) do not produce their own digestive enzymes; instead, such plants rely on bacteria and invertebrates to achieve the breakdown of captured prey (Juniper et al., 1989; Rice, 2006a). *Sarracenia purpurea* pitchers, which persist for approximately 2 years, produce digestive enzymes for only a short period (Gallie and Chang, 1997).

To emphasize the results of newer findings, especially regarding the roles of commensals, a revised, three-part definition for carnivory in plants is formulated below:

1. Clear adaptations to capture prey are present. Such adaptations may include specialized structures (i.e., the trap types as discussed in Section 2) and also enhancements to improve the luring and capture of prey (i.e., extrafloral nectaries, attractive UV or pigmentation patterns, odors, hairs that guide prey, etc.).
2. A mechanism is present by which prey are degraded into a form that can be assimilated by the plant. The digestive mechanism may be enzymes produced by the plant, decomposition by bacterial activity, or other organisms in a mutualist relationship with the plant (i.e., arthropods as in the cases of *Darlingtonia* and *Roridula*).
3. A pathway is available that allows nutrients to be absorbed into the plant, thus contributing to the plant's competitive and reproductive fitness. For most carnivorous plants, nutrients are transmitted via diffusion through trap fluids.

In some cases (i.e., *Roridula*), nutrients are first passed through the digestive tracts of mutualistic organisms and then deposited, as fecal matter, onto the leaves, where they enter the plant through cuticular gaps.

2. A Common Strategy

Although perceived as an exception to the rule and an almost fantastic one at that, carnivory is not altogether rare in the plant kingdom. Currently, carnivory is undisputed in 13 genera (Rice, 2006a), and with carnivory defined using the three criteria as in Section 1.2, there are 17 carnivorous genera with 700 species (Rice, 2008b).

Clearly, carnivory has evolved separately in at least five distinct botanical groups (see Table 1). Furthermore, since some of the groups (such as the Caryophyllales) contain carnivores of strikingly different strategies and forms, it is quite likely that carnivory has evolved independently many more times than this (Ellison and Gotelli, 2009, and sources therein).

Table 1. Families, genera, and species counts in five distinct evolutionary groups of carnivorous plants. In the bromeliads, only the species thought to be carnivorous are counted.

Group 1: Caryophyllales		
Dioncophyllaceae	*Triphyophyllum*	1
Droseraceae	*Aldrovanda*	1
	Dionaea	1
	Drosera	187
Drosophyllaceae	*Drosophyllum*	1
Nepenthaceae	*Nepenthes*	124
Group 2: Ericales		
Roridulaceae	*Roridula*	2
Sarraceniaceae	*Darlingtonia*	1
	Heliamphora	18
	Sarracenia	11
Group 3: Lamiales		
Byblidaceae	*Byblis*	7
Lentibulariaceae	*Genlisea*	21
	Pinguicula	96
	Utricularia	225
Group 4: Oxalidales		
Cephalotaceae	*Cephalotus*	1
Group 5: Poales		
Bromeliaceae	*Brocchinia*	2
	Catopsis	1

In a striking example of convergent evolution, these disparate botanical lineages have evolved traps involving the same five foraging strategies: pitfall traps, sticky traps, lobster pots, suction traps, and snap traps.

2.1. PITFALL TRAPS

The pitfall trap involves no moving parts, and can be effective even in its most primitive form. All that is required is a pit into which insects are ushered by gravity. In the carnivorous bromeliads, the entire plant forms the trapping urn; in other carnivorous plants, leaves (or portions thereof) are modified into the pitfall trap (see Fig. 1).

Every genus of pitfall plants has evolved refinements, which improve the capturing efficiency of the trap. Floral mimicry (complete with extrafloral nectaries), slippery surfaces, downward-pointing hairs, landing pads conveniently placed for flying prey, and overhanging ledges, which prevent escape, are but a few of these evolutionary innovations (Clarke, 1997, 2001; Lloyd, 1942; Rice, 2006a; Schaefer and Ruxton, 2008; Schnell, 2002). Digestive enzymes are often, but not always, present in the pitcher fluids. Symbiotic inquilines are universal.

Pitfall trap genera include *Brocchinia*, *Catopsis*, *Cephalotus*, *Heliamphora*, *Nepenthes*, *Sarracenia*, and *Darlingtonia* (but see Section 2.3).

Elements of pitfall traps frequently appear in noncarnivorous plants, for example in the many urn-like bromeliads, the perfoliate leaves of *Dipsacus*, the myrmecophilic structures in *Dischidia*, and in the pollination biology of many pouch-like floral structures in Orchidaceae, Araceae, and Aristolochiaceae (Bell, 1991; Rice, 2006a). An example of pitfall traps in the animal world are provided by the larvae of ant lions (Neuroptera), which consume prey that fall into conical pits in dusty or sandy soils.

2.2. STICKY TRAPS

In its simplest form, the sticky trap is merely a sticky surface to which insects adhere (see Fig. 2). The adhesive mechanism is aqueous mucus (or resin, in the case of *Roridula*) produced by stalked glands. Struggling by the captured prey usually results in its being covered with so much mucus that it suffocates, otherwise death is by exhaustion. Genera with sticky traps include *Byblis*, *Drosera*, *Drosophyllum* (but see Section 2.6), *Pinguicula*, *Roridula,* and *Triphyophyllum* (Lloyd, 1942; Lowrie, 1998; Rice, 2006a).

Plant motion may be exhibited in some species – adjacent tentacles often bend toward the prey to convey additional mucus, and the entire leaf may even curl over the capture site. The leaves of some *Pinguicula* species dimple under the prey, while the margins of the leaves may curl inward. Whether or not prey successfully escape is usually determined on a much shorter timescale than that of

Figure 1. Pitfall traps. *Top left* – a trap of *Cephalotus follicularis*, partially buried in moss in Australia; *top right* – the endangered *Sarracenia alabamensis* with a syrphid fly*; bottom left* – a green lynx spider (*Peucetia viridans*) hauling a dead wasp out of a *Sarracenia flava* pitcher in North Carolina (USA); *bottom right* – the pitfall/lobster pot carnivore *Darlingtonia californica* in California (USA).

the leaf movement, so the plant movement probably only helps in speeding digestion of those creatures that are successfully captured. Speedy digestion also ensures that the captured nutrients are absorbed by the plant instead of being washed away by rain or stolen by kleptoparasites.

Figure 2. Sticky traps. *Top* – flies captured by *Pinguicula macroceras* in Montana (USA); *bottom left* – prey captured by *Byblis gigantea* in Australia; *bottom right* – *Drosera linearis* leaf coils around a captured fly.

Digestive enzymes are produced either by the stalked glands or additional sessile glands; otherwise, mutualist organisms (i.e., predacious hemipterans) perform the role of digestion.

The mucous glands of *Drosophyllum lusitanicum* are different from other sticky trap plants in that mucus is freely transferred completely onto the prey. As the prey tumbles down the erect, thread-like leaves, it is coated with suffocating mucus (Rice, 2007).

A notable variation is the catapult-action of some sticky trap plants, as exemplified by *Drosera glanduligera*. This species has long, jointed, marginal tentacles capable of rapid, thigmotropic action. On stimulation, a tentacle flexes so rapidly that prey are practically flung into the middle of the glandular leaf (Rice, 2006a, citations therein).

The continuum between noncarnivorous and carnivorous plants is clearest in the sticky trap plants. A vast number of plants have sticky, glandular surfaces. Even the indisputably carnivorous *Triphyophyllum* is only a part-time carnivore, exhibiting its predatory nature only rarely (Bringmann et al., 2002). Examples of sticky predators in the animal world include many gelatinous marine invertebrates such as sea anemones and jellies.

2.3. LOBSTER POTS

The lobster pot trap, also called an eel trap, involves a mechanism in which the prey is lured (or blunders) into a chamber, and once having entered is unable to find the escape route (Lloyd, 1942).

Refinements of the lobster pot trap can seem almost insidious (see Figs. 1 and 3). For example, prey entering the opening of a *Darlingtonia californica* trap find themselves in a large chamber lit on all sides by transparent windows. The exit to freedom is difficult to reach, and to achieve it, the day-flying insect would have to fly through an exit portal toward the relatively dark ground. Instead, the organism is enticed by the brightly lit windows, which only confuse and exhaust the prey. A seemingly inviting escape tunnel, also brightly lit by clear windows, leads not to freedom but instead into a bath of fluids populated with carnivorous inquilines (Lloyd, 1942; Rice, 2006a; Schnell, 2002).

Sarracenia psittacina, the only lobster pot trap in a genus of pitfall traps, has a greatly expanded pitcher wing that terminates at the opening of the lobster pot, and which probably functions much as a "drift fence," such as used in wildlife monitoring to increase the effectiveness of funnel or pitfall traps (pers. observation).

Carnivorous plants that use the lobster pot strategy are all the members of the genus *Genlisea*, *Sarracenia psittacina*, and arguably *Darlingtonia californica* (Rice, 2006a, 2007). No noncarnivorous plants demonstrate genuine lobster pot characteristics, although the plants listed at the end of Section 2.1 and *Colura zoophaga* in Table 2 arguably have similar characteristics in their methods of temporarily confusing or retaining organisms. The only animal known to hunt by this strategy is man.

Figure 3. Snap, suction, and lobster pot traps. *Top* – prey captured by *Dionaea muscipula* in North Carolina (USA) revealed after digestion is complete; *bottom left* – bladders of *Utricularia intermedia* feeding in California (USA); *bottom right* – *Genlisea violacea* leaf rosettes with bifurcated, tunnel-like traps.

Table 2. A selection of paracarnivorous plants with literature citations 1=Frank and O'Meara (1984), 2=Schnell (2002), 3=Figueira et al. (1994), 4=Romero et al. (1998), 5=Juniper et al. (1989), 6=Rice (2008a), 7=Radhamanim et al. (1995), 8=Fritsch et al. (2007), 9=Darnowski et al. (2006), 10=Barthlott et al. (2000).

Pitfall Traps	
	Bromeliads (various genera)[1]
	Dipsacus fullonum[2]
	Paepalanthus bromelioides[3]
Sticky Traps	
	Aracamunia liesneri[4]
	Capsella bursa-pastoris (seed)[5]
	Ibicella lutea[6]
	Passiflora foetida[7]
	Philcoxia spp.[8]
	Proboscidea spp.[6]
	Stylidium spp.[9]
Lobster Pots	
	Colura zoophaga[10]

2.4. SUCTION TRAPS

Suction traps are small bladder traps, usually less than 10 mm across, produced by the genus *Utricularia* (Taylor, 1989). These bladders are aquatic (occurring either in open water or in waterlogged soils), and operate by pumping out water so that they are at a negative pressure. Prey cause a trap door to be opened inwardly, and are drawn into the trap with the rapid inflow of water that equilibrates the bladder pressure (see Fig. 3).

Many details of the trapping process are not understood – it is unknown if *Utricularia* traps actually attract prey, and the functions of the many adornments to the bladders are matters of pure conjecture (Taylor, 1989).

There are no analogies to suction traps in noncarnivorous plant genera, but the cryptic ambush predators called frogfish (Antennariidae) lure and suck in prey using a similar strategy (Pietsch, 1984).

2.5. SNAP TRAPS

Snap traps, sometimes called spring traps or bear traps, are similar to foothold traps used to capture mammals. On stimulation of sensitive trigger hairs, the two lobes of the trap snap shut, capturing the prey (see Fig. 3). Further stimulation by the captured prey induces the trap to close more tightly and then initiate digestion processes. For details on trap refinements evolved by snap trap carnivorous plants, see Lloyd (1942) and Schnell (2002).

The only snap trap carnivores are the aquatic *Aldrovanda vesiculosa* and the terrestrial *Dionaea muscipula*, two species in monotypic, closely related genera. There are no snap trap analogs in the noncarnivorous plant genera, although the palea and lemma of *Molinia caerulea* (Poaceae) flowers can reportedly capture insects via a snapping action, but to no apparent benefit (Lloyd, 1942). Animal snap trap predators abound, e.g., alligator snapping turtles, which ambush-hunt with gaping jaws.

2.6. HYBRID TRAPS AND UNEXPLOITED STRATEGIES

Darlingtonia californica is discussed above both as a pitfall trap and a lobster pot because it has elements of both strategies in its morphology. Other carnivorous plants also mix methodologies to various degrees. *Nepenthes aristolochioides* has characteristics that suggest both pitfall traps and lobster pots (Nerz, 1998; Rice, 2007), while the pitcher walls of *N. inermis* are coated with a sticky, slimy fluid so as a whole the trap incorporates elements of both pitfall and sticky traps (Clarke, 2001). Viscoelastic properties have similarly been observed in *N. rafflesiana* pitcher fluids (Gaume and Forterre, 2007).

The five strategies described in Sections 2.1–2.5 may seem to have exhausted all the possible methods that plants, as sessile organisms, might use to trap and kill their prey. However, it is entirely possible that as yet unknown carnivorous plants use other methods. Carnivorous fungi (*Arthrobotrys* sp.) hunt prey by simple sticky traps, but also by constricting rings that function like loop snares (Yang et al., 2007). No carnivorous plants use piercing/retentive spines to kill prey, although noncarnivorous species in the Loasaceae (see Section 4.2) are adorned with grappling-hook hairs (Bowles, 2008; Rice, 2006c; and references therein).

3. Enzyme Production, Paracarnivory, and Commensal Organisms

Once prey is captured and killed, it must be digested. Digestive enzymes are often produced by the plant, but mutualist organisms may contribute to (or achieve entirely) the digestive function.

3.1. PLANT-PRODUCED ENZYMES

In spite of the considerable metabolic costs associated with their production, digestive enzymes have been detected in members of most of the carnivorous plant genera (Frazier, 2000; Juniper et al., 1989). In fact, enzyme production seems to be lacking only in the sticky trap species in the genus *Roridula* and possibly some members of *Byblis* (Anderson and Midgley, 2003; Wallace and McGhee, 1999), the monotypic lobster pot genus *Darlingtonia* (Schnell, 2002), and the pitfall traps in *Brocchinia*, *Catopsis*, and *Heliamphora*, with the possible exception of *Heliamphora tatei* (Jaffé et al., 1992; Lloyd, 1942; Rice, 2006a; Schnell, 2002).

Many enzymes have been identified in carnivorous plants. Proteases have been found in *Dionaea*, *Drosera*, *Sarracenia*, and *Utricularia*; phosphatases occur in *Aldrovanda*, *Dionaea*, *Drosera*, *Sarracenia*, *Pinguicula*, and *Utricularia*; esterases are produced by *Drosera*, *Pinguicula*, *Sarracenia*, and *Utricularia*, amylases are in *Pinguicula* and *Sarracenia*; and nucleases have been found in *Dionaea* and *Pinguicula* (Heslop-Harrison and Knox, 1971; Juniper et al., 1989; Parkes, 1980; Robins and Juniper, 1980; Scala et al., 1969).

The chemistry of *Nepenthes* pitfall trap fluids has been a matter of considerable study (Frazier, 2000). Two as-yet unclassified proteinases (nepenthesin I and II) are known from *Nepenthes* pitchers (Clarke, 2001, and references within). Recently, studies have been detecting further enzymes, such as chitinases, lipase, phosphatases, esterases, and others (Eilenberg et al., 2006; Hatano and Hamada, 2008, and references therein).

3.2. THE ISSUE OF PARACARNIVORY

As any schoolchild would attest, *Dionaea muscipula* is unsurpassed in its exhibition of the carnivorous strategy. While impressive, pitfall traps are somewhat dramatic in their carnivory, and passive sticky trap plants are still less thrilling. As this continuum is extended further toward the realm of conventional plants, at some point the plants grade from carnivory to noncarnivory. Plants that nearly, but do not quite, satisfy the criteria of carnivory are referred to as paracarnivorous (Schnell, 2002) or protocarnivorous (Darnowski et al., 2006). In some cases, the difference between paracarnivory and true carnivory is a matter of opinion and the interpretation of the science available. It is quite possible that some species currently considered to be paracarnivorous will in the future be universally embraced by the scientific community as carnivorous.

3.3. SYMBIOSES: MUTUALIST, COMMENSAL, AND PARASITIC ORGANISMS

Relationships between organisms of different species are complex, and the literature that treats them is extensive. However, four important concepts from that discipline are relevant here: symbioses, mutualisms, commensalisms, and parasitisms. Although some of these terms are often used interchangeably, in fact they have different meanings (Boucher et al., 1982; Bronstein, 1994).

In a symbiotic relationship, two organisms live close together – the relationship may be facultative or obligate for one or both species, and may be for short or long terms (Moran, 2006). If one organism benefits at the expense of another, the symbiotic relationship is parasitic (Roughgarden and Diamond, 1986). If one organism benefits while the other organism is neither helped nor harmed (i.e., inquilines feeding on detritus in the urn of a noncarnivorous bromeliad), the symbiosis is commensal. Finally, if both parties benefit (i.e., inquilines living in the fluids of a pitfall trap plant that cannot produce its own enzymes), the symbiosis is a mutualism. The differences among these relationships are important as they help shape our concepts of carnivory in plants.

Parasitic relationships are well known in carnivorous plants. Ants may steal prey from the leaves of *Pinguicula* (Zamora, 1995), and green lynx spiders (*Peucetia viridans*) may haul captured prey out of the tubes of *Sarracenia* pitchers (pers. observation). The red crab spider *Misumenops nepenthicola* is an ambush predator that captures arthropods attracted to the traps (Clarke, 1997), probably without benefit to the plant. Other parasitic relationships can damage reproductive fitness, such as spiders that hide in *Sarracenia* or *Darlingtonia* flowers to attack potential pollinators (pers. observation).

Commensal relationships are even more widespread. Pitfall traps provide homes for many commensal organisms, especially those suited for an aquatic lifestyle. Mites are common, such as the slime mite *Sarraceniopus darlingtoniae*, which is endemic to the pitchers of *Darlingtonia* (Fashing, 2004), and species of *Naiadacarus*, *Nepenthacarus*, and *Zwickia* in *Nepenthes* pitchers (Fashing, 2002; Fashing and Chua, 2002).

In cases where the host plant produces few or no digestive enzymes, the symbiotic relationships shift from commensal to mutualist, but even enzyme producing pitfall plants probably benefit from such inquilines. Bacteria are the most universal of such organisms, but larvae of insects that feed on captured prey occur in every genus of pitfall trap plants (Bradshaw and Holzapfel, 2001; Clarke, 1997, 2001; Gibson, 1999, 2001; Rice, 2006a; Schnell, 2002). The digestion performed by chironomid midge larvae (*Metriocnemus edwardsi*) and other infauna is critical for facilitating carnivory in *Darlingtonia californica* (Rice, 2006a; Schnell, 2002). Digestion activity by infauna can occur over multiple trophic levels; the larvae of *Toxorhynchites* mosquitoes, *Wilhelmina* calliphorid flies, and *Lestodiplosis* flies are predators that feed on *Nepenthes* pitcher infauna – including each other (Clarke, 1997)!

A well-known mutualism involves an ant (*Camponotus schmitzi*) found only in close association with *Nepenthes bicalcarata* (Clarke, 1997). This ant forms its colonies in the hollow tendrils that support the pitchers; they gain access to the chambers by chewing a small access hole. They can swim, and dive into the pitchers to haul out large captured prey. This may be of value to the plant by preventing nutrient overload that could damage the plant by putrefaction; the ants also drive herbivorous weevils from the plant, protecting its growth tips from damage (Merbach et al., 2007).

Many symbioses are difficult to classify as either commensal, parasitic, or mutualist. The crab *Geosesarma malayanum* and several species of frogs are associated with *Nepenthes* (Clarke, 1997). One frog species lays its eggs in the fluids of *Nepenthes* pitchers – the frogs metamorphose into the adult state while still enclosed in their eggs (Clarke, 1997). Frogs are also frequently found, both alive and dead, in *Sarracenia* pitchers (personal observation; Schnell, 2002).

Sticky trap plants have many symbiotic relationships with true bugs (Hemiptera). These predatory insects (e.g., *Cyrtopeltis* sp., *Setocoris* sp.) are frequently found on *Drosera* and *Byblis* in Australia (Hartmeyer, 1996; Lowrie, 1998). The hemipterans *Pameridea marlothii* and *P. roridulae* are critical mutualist partners that enable carnivory for *Roridula dentata* and *R. gorgonias* (Anderson, 2001; Anderson and Midgley, 2003). During times of poor foraging, *Pameridea* bugs suck sap from *Roridula* tissues; meanwhile, the *Pameridea* are in turn preyed on by a specialist spider *Synaema marlothi* (Anderson and Midgley, 2007).

Care must be taken to avoid overinterpreting the presence of true bugs on glandular or hairy plants; hemipterans are wide-ranging predators that may be found foraging on a variety of noncarnivorous or paracarnivorous plants such as *Brugmansia*, *Helianthus*, *Ibicella*, and *Stylidium* (Rice, 2008a; pers. observation).

Grading from parasitism to herbivory, a number of organisms consume the tissues of carnivorous plants, some in novel ways. The larvae of *Exyra* moths feed on the tissues on the interior of *Sarracenia* pitchers; as they do so the pitcher top collapses, thus protecting the larvae from possible predators (Schnell, 2002). Larvae of the moth *Eublemma radda* feed on the pitchers of *Nepenthes*, causing pitcher collapse within a few weeks (Charles Clarke, pers. communication, 2008; Kitching, 2000). Larvae of the moth *Trichoptilus parvulus* feed on the leaves of the sundew *Drosera capillaris*, first by consuming the glandular tentacles, and then the leaves; even insects trapped by the plant are devoured (Eisner and Shepherd, 1965).

4. The Nature of the Prize

Clearly, carnivorous plants are foraging for certain minerals that are deficient from their soils. However, as several hundred species of carnivorous plants are known, and since many types of soil-based nutrient deficiencies exist, it would be unwise to declare with confidence exactly what nutrients carnivorous plants are lacking. Similarly, it would be foolish to assume that all carnivorous plants are foraging for the same nutrients.

It is typically assumed that carnivorous plants are foraging for macronutrients such as nitrogen, phosphorus, and potassium, but it is possible that other elements such as calcium and sulfur are also targeted for absorption (Adamec, 1997, 2002; Juniper et al., 1989). Magnesium is also effectively absorbed from animal prey (Adamec, 2002).

To obtain the needed nutrients, plant predators have evolved to prey on many types of organisms. Examining the prey spectrum reflects the diversity of approaches taken by carnivorous plants.

4.1. INVERTEBRATE PREY OF CARNIVOROUS PLANTS

Aquatic snap trap and suction trap carnivorous plants (i.e., *Aldrovanda* and *Utricularia*, respectively) are predators that capture and digest any organisms that are large enough to trigger the trapping mechanism, while small enough to fit into the trap (Lloyd, 1942; Taylor, 1989). Even relatively large prey, such as mosquito larvae or slender annelids several cm long, can be consumed by *Utricularia* traps over time. In such cases, the captured end of the organism is digested, and as the tissues erode more of the still-struggling creature is slowly drawn in by trap suction (personal observation; Lloyd, 1942). This is perhaps one of the more truly horrific ways to die!

The submerged traps in the lobster pot genus *Genlisea* capture a similar wide spectrum of prey including nematodes, crustaceans and other arthropods, as well as algae (Darnowski, 2010; Lloyd, 1942; Plachno et al., 2005; Studnicka, 1996, 2003a, 2003b). Laboratory studies indicate that *Genlisea* traps have some sort of attractive agent that apparently induces a tropism in protozoans, suggesting that some *Genlisea* species may actively hunt protozoan species (Barthlott et al., 1998). However, the observations of other prey in *Genlisea* utricles would make it inappropriate to say that the genus specializes in protozoans (Darnowski, 2010). Even so, this behavior expands the prey spectrum of carnivorous plants to another nonanimal kingdom of organisms.

The terrestrial snap trap *Dionaea muscipula* is a nonselective carnivore that feeds on any arthropod that it can trap, especially ants, spiders, grasshoppers, and winged insects (Schnell, 2002, and references therein). The traps are brightly colored and provide a small nectar reward to foraging insects; however, many organisms (such as mollusks, arachnids, isopods, and grasshoppers) captured by *Dionaea* probably enter the trap only because of general foraging activity (pers. observation).

Like other carnivorous plants, pitfall trap and terrestrial lobster pot carnivores capture a huge range of prey. A single study of the prey found in *Nepenthes* pitchers resulted in a treasure trove of 150 species (Erber, 1979), and *Sarracenia purpurea* pitchers have been observed capturing prey representing up to 150 families (13–14 orders), mostly of insects, but also including slugs, spiders, and other organisms (Cresswell, 1991; Schnell, 2002; Wray and Brimley, 1943).

Sticky trap carnivores also have relatively unspecialized dining habits. In broad terms, the most common prey for these plants are usually winged insects (Achterberg 1973; Dixon et al., 1980; Green et al., 1979; personal observation). Prey range in size from tiny midges attracted to the fungal-scented leaves of *Pinguicula*, to large predatory dragonflies captured by carnivores that have large leaves held high above the ground. Small species with only basal leaves, as is the case for many *Drosera* and *Pinguicula*, also feed heavily on ground-foraging, crawling invertebrates.

Inexplicably, the leaves of *Pinguicula*, which are armed with only weakly adhesive glands on very short stalks, frequently capture surprisingly large and even muscular prey such as crane flies (Tipulidae), bottle flies (Calliphoridae), and crickets (Gryllidae) (personal observation).

In some cases, ostensibly carnivorous plants are observed gathering nutrients from surprising sources. *Nepenthes lowii* produces lumpy masses of white excrescences on the undersides of its pitcher lids. Apparently, this is a sacrificial product that encourages birds to fly onto the pitchers, feed on the excrescences, and as a matter of course, defecate into the lavatory-like pitcher mouth, which is conveniently situated under the bird's anus (Clarke, 1997, Clarke and Moran, 2001). Meanwhile, *Nepenthes ampullaria*, which forms pitchers in great clusters on the forest floor, may harvest much of its nitrogen from plant detritus raining down from the forest canopy (Clarke and Moran, 2001). This same strategy may be used by some species of *Heliamphora* (Stewart McPherson, pers. communication, 2008). In a clear case of herbivory by a plant, *Pinguicula* rosettes can capture and digest large amounts of living *Pinus* pollen on their sticky, flat leaves; *Pinguicula vulgaris* may obtain as much as 50% of its proteins from captured pollen grains (Barthlott et al., 2007; Juniper et al., 1989). Algae are found inside the traps of various aquatic species, but it is unclear if the algae are prey, competitors, or some other kind of symbiont (Adlassnig et al., 2006; Peroutka et al., 2008; Plachno et al., 2005; Plachno and Wolowski, 2008; Richards, 2001).

4.2. VERTEBRATE PREY OF CARNIVOROUS PLANTS

While certainly not typical prey, higher organisms are occasionally captured by carnivorous plants. Such trapping events are for the most part rare in the extreme, and are best treated as anomalous curiosities.

Vertebrate organisms can occasionally be captured by *Dionaea*; plants growing outside their native range, but in wildland settings in California, have been observed feeding on Pacific tree frogs (*Pseudacris regilla*) (Peter D'Amato, pers. communication, 2008; Robert Ziemer, 2008, personal communication). Presumably, *Dionaea* plants in their native range feed on comparable native frogs (e.g., *Pseudacris*, *Hyla* spp.).

Utricularia can capture and kill, but perhaps not consume, minute aquatic vertebrates such as fish fry and tadpoles (Darnowski, 2007; Lloyd, 1942).

Vertebrates are more frequently trapped by pitfall traps. Schnell (2002) notes the occasional occurrence of frog skeletons in *Sarracenia* traps. The pitchers of *Nepenthes rafflesiana* and other species have been noted to contain the digested remains of captured frogs and lizards (Clarke and Moran, 2001); this activity may be the only example of vertebrate capture and digestion by a carnivorous plant that occurs frequently enough to be considered normal. *Nepenthes rafflesiana* and *Nepenthes rajah* have been observed containing the remains of small rodents, possibly *Mus* sp. or *Chiropodomys* sp. (Brodie and Lee, 2000). Although such events are spectacular, mammals of any size are not part of the normal diet of any carnivorous plants.

Tales persist of sticky-leaved carnivores occasionally capturing large prey such as rabbits, squirrels, and birds, although such stories are typically discounted (Lowrie, 1981). Unlike the almost woody traps of massive pitfall traps, even the most enormous sticky trap carnivores are comparatively delicate. The physics of the trapping mechanism, which depends on the weak adhesion of polysaccharide mucus, precludes the effective capture of a large animal unless it was already essentially near death. Even *Drosera gigantea*, which often occurs in massive, dense growths in the wild, could be penetrated safely by even a small bird (personal observation).

Roridula is reported to have captured birds (Anderson, 2001). As this is the only genus of sticky trap with a resin-based adhesive, the trapping event is more plausible. However, the digestive mechanism for *Roridula* requires the intervention of capsid commensals, and since it is unlikely that such insects could feed on dead birds, the trapping of birds by *Roridula* can probably be ruled out as actual carnivory.

Great care should be taken when evaluating the rare occurrences of large animal capture by carnivorous plants, or even what appears to be predation of conventional prey by noncarnivorous plants. Accidents happen, and even though animals may be found dead on a plant, it does not mean that the event is part of a carnivorous syndrome. For example, desert plants in the Loasaceae frequently capture and kill flying insects with grappling-hook and piercing hairs (Bowles, 2008; Metzler, 2006; Rice, 2006c), and at least three bat species have been observed impaled on the spines of *Eucnide urens* (Hardy, 1949; Stager, 1943). Nesting birds are often found impaled on the leaf spines of the Andean bromeliad *Puya raimondii* (Givnish et al., 1984), and the stiffly recurved hooks on the fruit of (noncarnivorous) burdock plants (*Arctium* spp.) – clearly evolved for dispersal on the fur of passing mammals – have captured both hummingbirds and small bats, which subsequently died on the plant (Bowles, 2008, and references therein).

4.3. PREY SPECIALIZATION

In the animal world, specialization among predators is common. In contrast, predators in the plant world usually cannot afford to be too selective. This is presumably a cost associated with being a sessile predator; analogously, sit-and-wait ambush predators in the animal world (such as trapdoor spiders) tend to be nonspecialists. Even so, there are some indications of prey selectivity (Ellison and Gotelli, 2009).

Nepenthes plants produce two types of pitcher – highly ornate pitchers that occur on or near the ground, and less adorned pitchers higher on the plant, sometimes even in the forest canopy (Clarke, 1997, 2001). Ants are the predominant prey of *N. rafflesiana* upper pitchers, but they also capture a variety of flying insects including flies, beetles, moths, wasps, bees, and true bugs, many of which are pollinating agents of other plants. In contrast, lower pitchers tend to capture only ants and termites (Moran, in Clarke, 1997). *Nepenthes macfarlanei* lower pitchers have a higher frequency of capturing flightless, ground-prowling nocturnal arthropods such as ants, cockroaches, spiders, centipedes, and mites – most of which are also predators (Schmid-Hollinger, 1997). Studies in which upper and lower pitchers were switched indicate that the differences in prey capture are due to height above ground, and also intrinsic differences between the two pitcher types (Moran, 1993, 1996).

The species *Nepenthes albomarginata* has pitchers adorned with a bright ring of tomentose tissue just below the pitcher opening. This tissue is highly attractive to Nasutitermitinae termites, which seek out the pitchers for mass feedings. A small percentage inevitably falls into the pitchers (Moran, in Clarke, 1997; Clarke and Moran, 2001). Although the plant is gorged, losses to the termite colony are small, and this situation may actually be best described as a mutualism (see Section 3.3).

It might seem intuitive that erect North American pitfall plants would tend, because of the bright colors of pitchers and the nectar enticements provided by the traps, to attract those insects involved in plant pollination such as butterflies, wasps, flies, and bees. However, *Sarracenia minor* may actually be a specialized hunter of ants (Ellison and Gotelli, 2009; Schnell, 2002). Similarly, some observers have suggested that *Cephalotus* traps are filled with a disproportionate number of ant prey (Lloyd, 1942), although other observers have found many other types of prey in their traps, including isopods, flies, and other arthropods (Gibson, 1999).

The snap trap of *Dionaea muscipula* selects prey based on size. On initial closure, tiny prey can easily escape through the openings between the teeth of the loosely clasping lobes. Only larger prey – which carry more of a nutrient reward – are retained and, by their frantic motions, continue to stimulate the trap to complete the digestion process (Darwin, 1875; Ellison and Gotelli, 2009; Schnell, 2002).

5. On the Matter of Sex

With the exception of some self-pollinating species, the known pollination mechanisms of all carnivorous plants involve animal agents; such pollinators include the usual array of bees, flies, moths, butterflies, beetles, hummingbirds, and other agents (Clarke, 1997, 2001; Lowrie, 2001; Salmon, 2001; Taylor, 1989). However, the need for a successful and safe interaction of plant and pollinator poses a quandary. How does the predatory plant separate prey from pollinator?

5.1. SEPARATION BY TEMPORAL BARRIERS

In this simple strategy, the plant produces its flowers during a time it is not actively carnivorous. Clearly, this is not a method that could be effective for those tropical species that are continually carnivorous.

An exemplar for this strategy is *Sarracenia alata*. Most of its pitchers are produced in the spring and early summer. By the arrival of winter, the pitchers are so damaged by wind, rain, fires, insects, and passing wildlife that they can no longer trap prey efficiently (Rice, 2006a; Schnell, 2002). In the spring, while the pitcher leaves are still in a damaged condition, the plant produces its tall inflorescences. Even as the plant is being pollinated, new pitchers are forming, but these do not open until after the pollination season is more or less complete (Rice, 2006a; Schnell, 2002).

Another example of this strategy is exhibited by tropical heterophyllous species of *Pinguicula*. These plants survive the annual dry season by regressing to reduced rosettes with noncarnivorous leaves. These plants often respond to the arrival of the wet season by initiating flowering, while the carnivorous leaves are at most in a state of development (Legendre, 2000). Tuberous *Drosera* of Australia spend the arid summer in dormant tubers, and emerge to an actively growing state during the wet season. In some species, most notably *Drosera rosulata*, *D. erythrorhiza* subsp. *squamosa*, some populations of *D. whittakeri* (i.e., *Drosera whittakeri* var. *praefolia*), and *D. zonaria*, the first structures to emerge above ground are the flowers, which are then followed by the carnivorous leaves (Lowrie, 1987, 1998).

Triphyophyllum peltatum produces its carnivorous tendrils prior to the shoot elongation that precedes flowering. It has been traditionally hypothesized that the production of such carnivorous leaves is timed so as to give the plant an additional metabolic boost prior to flowering, but Bringmann et al. (2002) demonstrated that the carnivorous phase was not obligate before flowering, at least in cultivation. A reinterpretation of the timing may be that, at least in part, the plant is providing a temporal barrier between its pollinators and its traps.

5.2. SEPARATION BY SPATIAL BARRIERS

In many cases, carnivorous plants produce flowers at the same time that they are in a predatory state. Many carnivorous plants have responded by evolving long peduncles that situate the flowers far from the carnivorous leaves.

This method is particularly effective when the space separating the flowers from the leaves also includes a change in habitat character. For example, the flowers of *Dionaea* are a few to several decimeters above the ground, while the carnivorous leaves during the flowering season are ground-hugging and tucked among the low vegetation into which pollinators are less likely to venture (Rice, 2006a; Schnell, 2002). After pollination is complete, *Dionaea* traps are produced on much longer, erect petioles. The changing leaf length is often assumed to place the traps in a better hunting location as competing grasses and forbs mature, but since *Dionaea* leaves capture many ground crawling prey, it may be more a matter of no longer needing to protect pollinators from danger.

Other carnivorous genera with very long peduncles include *Brocchinia*, *Catopsis*, *Cephalotus*, many *Drosera*, *Heliamphora*, and *Nepenthes* (Clarke, 1997, 2001; Lloyd, 1942; Rice, 2006a).

5.3. SEPARATION BY TAXONOMIC BARRIERS

Probably, the most effective strategy to protect pollinators from consumption is to ensure that the kinds of organisms that are pollinators will be different from the kinds of organisms that would be prey.

The clearest examples of this approach can be found in those species with traps that are submerged in soil or water, while the flowers are positioned above ground. The aquatic hunters *Aldrovanda*, *Genlisea*, and *Utricularia* are at no risk of capturing their pollinators.

However, the implementation of this strategy may be more subtle. Many species of *Pinguicula* have brightly colored flowers with long nectar spurs. The winged pollinators of such plants are unlikely to land in the grasses and be captured by the traps. Instead, the plants prey on smaller flying insects and crawling invertebrates that are attracted to the fungal-smelling leaves (Rice, 2006a; Schnell, 2002). The flowers of some *Sarracenia* species are scented fruity, musky, or with a strong feline urine odor – these odors are lacking from the pitchers (Rice, 2006a, Schnell, 2002). Tuberous *Drosera* and some South African *Drosera* tend to be highly fragrant, apparently to lure pollinators directly from flower to flower, minimizing the risks posed by the many dangerous sticky leaves (Fleischmann et al., 2007; Lowrie, 1987).

Sarracenia psittacina may use both spatial and taxonomic barriers in protecting its pollinators. Many of its leaves are close to the ground, thus attracting ground-crawling arthropods. Indeed, some plants are even submerged for much of the year (Schnell, 2002). However, in some situations the leaves are nearly erect and might be attractive to pollinators. Correspondingly, the flowers are held very high above the plant, on tall wiry scapes.

5.4. SEXUAL DYSFUNCTION

Some carnivorous plant species do not appear to use any mechanism to insulate pollinators from the perils of the carnivorous leaves.

Two pitfall plants, *Sarracenia rosea* and *S. oreophila*, seem to have very little in the way of safety barriers between flower and trap. The flowers of both species are much the same color as the traps, are largely odorless, and are held near the openings of fully functional trapping leaves. Furthermore, the leaves easily trap organisms of the same size as those that pollinate the flowers. *Sarracenia oreophila* seems in particular to be focused on dysfunction, as its green flowers mature while nestled among a swarm of gaping green pitcher mouths (pers. observation;

Schnell, 2002). Are there other cues, such as UV reflectance patterns or structural characteristics that have not yet been identified?

Several genera of flypaper plants seem to have similar problems. *Byblis*, *Drosophyllum*, some *Drosera*, and *Roridula* all have flowers, which are only moderately removed from the carnivorous leaves. How do pollinators of the flowers of *Byblis liniflora* navigate from one flower to the next, without encountering one of the many dangerous leaves in the process? The flowers of some of these species are buzz-pollinated by large insects; is it possible that this ensures that the plants are pollinated by muscular insects that may escape sticky capture (Andreas Fleischmann, pers. communication, 2008)? On the other hand, it is possible that some sticky trap plants might be using their flowers for dual purposes. Some South American *Drosera* produce highly fragrant flowers practically nestled among the leaves (Fleischmann et al., 2007). The scents may guide pollinators safely from flower to flower, but perhaps the flowers also lure less careful insects to the carnivorous leaves?

Darlingtonia californica is a final addition to this list of unresolved pollinator--prey conflicts. The identity of its pollinator has long been a matter of speculation (Nyoka and Ferguson, 1999), although flowers have been observed being entered by the ground-nesting bee *Andrena nigrihirta* (Rice, 2006b). *Darlingtonia* pitchers last well into their second year, so spring pollinators must negotiate a treacherous landscape filled with carnivorous traps from the previous year. These pitchers can be nearly as tall as the flowers, exude nectar, and are brightly colored. How these traps are not mistaken for flowers by the pollinator is not yet known.

It may simply be that in some cases, there are no special protections for the pollinators. Instead, it might be that the plants merely rely on the fact that their trapping efficiencies are low. The relatively small number of tuberous *Drosera* species mentioned in Section 5.1 that flower before producing leaves suggests that the pressure to protect pollinators from capture is not particularly strong.

6. Concluding Notes

The biology and ecology of carnivorous plants is complicated in the extreme. The earliest interpretation of carnivorous plants was that they were kindly plants put on the planet by a Creator in order to provide shelter and water for small creatures such as insects and frogs during times of hardship (Rice, 2006a). Subsequently, they tended to be caricaturized as predacious marvels of a dangerous nature. A newer perspective is that they are integrated into the food webs in which they grow, yes as predators, but also as providers of food and home to many other organisms. Science has nearly come full circle! Our perspectives and understanding continue to grow.

Unfortunately, the many anthropogenic stresses on carnivorous plants are severe. Global climate change, habitat destruction, range fragmentation, changes to fire regime and hydrology, pollution, and poaching by enthusiasts are all contributing to a rapid decline in carnivorous plants on a global scale (Rice, 2006a). With carnivorous plants disappearing from the planet, we find ourselves in an increasingly dark, lonely, and broken world.

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