right: The beewolf larva spends the winter underground in a silken cocoon. Antibiotic-producing bacteria protect the larva from harmful bacteria and fungi.

left: In this photomontage, the antibiotics are visualized by means of mass spectrometric imaging and projected on the cocoon in false colors. Red and yellow surfaces correspond to high concentrations of antibiotics, while blue and violet indicate lower levels.

Alliance on a Silken Thread

Many insects rely on bacteria for vital support. The microorganisms produce survival cocktails for their larvae, help them break down indigestible food components or supply essential vitamins. **Martin Kaltenpoth** and his team at the **Max Planck Institute for Chemical Ecology** in Jena are elucidating fascinating details about the symbiotic relationships between insects and microbes.

TEXT **ELKE MAIER**

Photo: MPI for Chemical Ecology

Insects are the most successful of all animal groups, and have penetrated almost every corner of the planet. They have colonized practically all habitats, from water surfaces to deserts, and are as much at home on Himalayan glaciers as in supposedly well-closed bags of muesli in the back of the storage cabinet.

At the Max Planck Institute for Chemical Ecology in Jena, the bugs occupy a whole room full of climate cabinets and are accommodated in dozens of Tupperware containers. "These are firebugs," says Martin Kaltenpoth, taking a transparent box from a heated cabinet and showing a group of black- and red-patterned insects that scuttle around inside. What makes them special is that, millions of years ago, their ancestors entered into an alliance with bacteria.

Martin Kaltenpoth, head of the Independent Max Planck Research Group "Insect Symbiosis" since November 2009, is excited about the diversity of insects and their partnerships with microbes. "Symbiosis is a key factor for the ecological and evolutionary success of insects," he explains, "because it allows them to adapt to changing environmental conditions and exploit new food resources."

Leaf-cutting ants provide an interesting example of such an alliance. They use small snippets of leaves as a substrate to create underground fungal gardens as a source of nutrition for their colonies. To protect these carefully nurtured food resources, the ants also cultivate bacteria that produce different antifungal agents. These substances combat the harmful *Escovopsis* fungus in particular, which occurs as a parasite in fungal gardens and would destroy them were it not for the bacteria's strong chemical defense.

Other insects use microorganisms to exploit special food sources. Many termites, for example, eat wood and use intestinal symbionts to digest the cellulose. The unbalanced diet of the blood-sucking tsetse fly, carrier of sleeping sickness, lacks the essential vitamin B, but microbes make up for this deficiency. And many other insects, such as aphids, cicadas and weevils, also rely on third-party supply of certain substances. >

Tobias Engl collects bees from the institute's hives using an adapted vacuum cleaner, and supplies them to the beewolves as food for their young.

Only the female beewolves provide food for their young. They seek out potential victims with their large compound eyes, and attack only after using their antennae to check the scent of their prey.

"For many years, bacteria have generally been seen as a cause of disease," says Martin Kaltenpoth, "but in recent decades, studies have revealed more and more examples of how microbes can ensure the survival of their hosts." The bacterial symbiosis of leaf-cutting ants, for instance, was first discovered in 1999, even though the ants carry their microscopic helpers around in such quantities that they are visible to the naked eye.

During his doctoral studies, Martin Kaltenpoth discovered a burning fascination for insect symbioses. While working at the University of Würzburg, he was researching pheromone communication in the European beewolf, *Philanthus triangulum,* a species of digger wasp. In late 2003, he stumbled upon an unusual kind of symbiosis that still occupies him today.

Kaltenpoth is especially interested in discovering how symbiosis evolves between insects and bacteria. What are the advantages to each? And why did those particular partners choose each other? He selected the red firebug, *Pyrrhocoris apterus,* as an additional object of study. Both the beewolf and the firebug enjoy symbiotic relationships with microbes of the interesting group of Actinobacteria.

At the Max Planck Institute in Jena, the beewolves are kept in a brightly lit greenhouse, right next to tobacco, cabbage and busy lizzies. Here, they live through the summer in glazed wooden boxes so the scientists can observe their activities.

BEE PROVISIONS FOR THE LARVAE

These solitary wasps are notorious for their remarkable reproduction strategy. In summer, the females hunt honey bees. When they spot a worker bee gathering nectar, they strike, paralyzing the bee with poison and carrying it off to a nesting hole in the ground. For each egg, a female digger wasp will bring in up to five paralyzed bees. After hatching, the larvae feast upon the nourishing meal and then spin a silken cocoon in which to spend the winter.

This is a dangerous time. "Mildew thrives in the damp environment of the underground chamber with the bee remains," says Tobias Engl, a postdoctor-

Photos: Sven Döring (top), MPI for Chemical Ecology

Hassan Salem is studying the influence of intestinal bacteria on firebug growth. A comparison of firebugs with and without these bacteria shows that those that have the symbionts grow bigger and have a better chance of survival.

al researcher in Martin Kaltenpoth's group. During his doctoral studies at the University of Regensburg, he established the existence of ten different types of fungus in the beewolf nurseries. Consequently, while the larva lies motionless in its cocoon, it is in constant danger of becoming moldy; but as Martin Kaltenpoth discovered in his doctoral thesis, the digger wasps have developed a unique strategy to protect their offspring.

Scientists had already observed that the females excrete a viscous white substance from special glands in their antennae while building the brood chambers, and that they apply this substance to the chamber ceiling. "This coating later helps the hatching beewolves find their way to the surface," explains the Max Planck researcher.

While analyzing electron microscope images, however, Kaltenpoth realized that the substance also had another, completely different function. The images showed elongated structures that looked like bacteria, arousing the curiosity of Kaltenpoth and his colleagues in Würzburg. Using gene analysis, they set about studying the mysterious structures – and struck gold. The 16S rDNA gene, a kind of ID card for microorganisms, revealed that the structures were indeed bacteria. In fact, they were a previously unknown species of *Streptomyces*.

Firebugs sometimes gather in large numbers in places where there is abundant food. They feed on plant juices, linden seeds being their preferred diet.

Photos: Sven Döring (top), MPI for Chemical Ecology

BACTERIA AS PRODUCERS OF ANTIBIOTICS?

Thus began perhaps the most exciting chapter of beewolf research: "Because it is known that many streptomycetes produce antibiotics," says Kaltenpoth. Could this be true for the beewolf's bacteria as well? To find out, he and his colleagues focused on the larvae, since observations had shown that larvae in the brood chamber seek out the white substance and take it up. The researchers surmised that perhaps the beewolf young somehow used the antibiotics to defend themselves against mold fungus in their underground homes, a little like the leaf-cutting ants that use antibiotics to protect their underground gardens. >

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Photos: FEMS Microbiology Reviews – Martin Kaltenpoth (large image), MPI for Chemical Ecology (top), Sven Döring (bottom)

And indeed, their investigations showed that the bacteria occur in large numbers on the outside of the larval cocoons, where the scientists then also identified a number of antibiotics produced by the microbes. Using a new mass spectrometric imaging technique, they were even able to make the antibiotics visible on the cocoon. "The larvae use the symbionts to impregnate their cocoons," says Kaltenpoth. "They spin the bacteria into the outer silk layers in such a way that they form an effective barrier, while the larvae themselves avoid any antibiotic-related side effects." In other words, the beewolves use the germicidal metabolites of the microbes to protect their offspring from mold fungi.

BROAD-SPECTRUM ACTIVITY AGAINST BACTERIA AND FUNGI

The insects actually make use of combination prophylaxis, as revealed by the nine different antibiotics the scientists isolated from their cocoons, including streptochlorin and eight different piericidins. The efficacy of these substances was then verified using bioassays. "The mix of antibiotics combats a very broad spectrum of fungi and bacteria," says Martin Kaltenpoth. "That would not be possible with individual substances."

The researchers showed just how effective the protection is by means of a simple experiment. One group of larvae in a brood chamber was blocked from accessing the bacteria-laden white substance, with spectacular results. Without the bacteria, the survival rate of the beewolf young fell drastically, from over 80 percent to under 7 percent.

Yet the question remained: What advantage do the microbes derive from this relationship? "For one thing, they are protected in the antennal glands

1 The female beewolf is a skilled hunter. The venom from her sting paralyzes the victim, enabling the beewolf to carry it to the brood chamber and store it there like food in a larder. Unlike their larvae, the adults are vegetarians and live on flower nectar.

2 At the end of a passage measuring up to one meter are a number of brood chambers, each containing a single egg. When the larvae have eaten their supplies of honey bees, they spin a cocoon that is attached to the wall of the chamber only by a thin stalk. This reduces the risk of fungal infestation.

3 The pupal cocoons must be opened in order to analyze the antibiotic coating.

4 A look into the beewolf nursery: A cocoon is taken from a brood chamber using tweezers. A sealable plastic tube of the kind normally used for DNA analysis serves as the brood chamber, and a refrigerator simulates the winter season.

5 The stage of development of a beewolf pupa can be seen through the cocoon when held up against the light.

6 A longitudinal section through the antenna of a female beewolf. The double-lobed antennal gland reservoir contains the symbiotic bacteria (blue and red). It is surrounded by gland cells that secrete unidentified substances into the reservoir. The top edge is the antennal exoskeleton, and the antennal nerve is visible at the bottom edge.

and have little competition," conjectures Martin Kaltenpoth. "However, since they multiply rapidly there, they must also get nutrients from their host." To test this hypothesis, Tobias Engl is analyzing the chemical composition of the glandular secretion, separating the various ingredients using gas and liquid chromatography and gel electrophoresis in order to identify them one by one. The results of this analysis, however, are not yet available.

The Max Planck team also wants to understand the beewolf symbiosis on a genetic level and discover how the digger wasp's immune system reacts to the bacteria and how the bacteria are influenced by their host. To this end, Taras Nechitaylo and Sabrina Köhler are studying which genes are active in both partners, and what their functions are.

In a cool climate chamber in the basement of the institute, Martin Kaltenpoth takes a transparent plastic box down from the shelf. It contains a good two dozen small plastic tubes, each of them holding a pupa. "At the end of the season, we gather the cocoons and store them in the refrigerator over the winter," says Kaltenpoth. "Then in spring, we warm them up so the beewolves emerge." When he opens the cover, a familiar smell emerges: "Geosmin," he affirms. The musty, earthy smell is caused by the streptomycetes in the silken cocoons around the larvae. Similar microbes occur in the soil and cause the typical smell of damp earth.

SYMBIOSIS AROSE FROM A CHANCE ENCOUNTER

Kaltenpoth surmises that the unusual alliance may also have originated underground. "It is probable that streptomycetes took up residence at some stage on burrowing insects," he says. "While the bacteria benefited from chemical compounds on the insect's skin, the bacterial metabolites proved advantageous to the insect. And since each partner benefitted from the other, they evolved in concert."

The combination of unusual symbiosis and highly complex behavior might suggest that beewolves are the perfect object of study for biologists. However, they are far from being perfect lab animals. The problems begin with their food supply: Four beehives are kept behind the institute to ensure that the females have enough food for their broods. A beekeeper has been employed for the time-consuming task of caring for the bees. And during the high season, the technical assistant has to don the beekeeping gear twice daily and go out to harvest bees with a specially adapted hand-held vacuum cleaner. >

Photos: Sven Döring (2, left), MPI for Chemical Ecology


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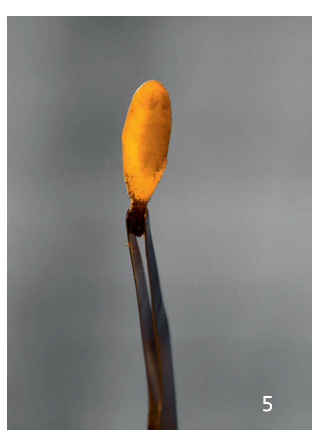
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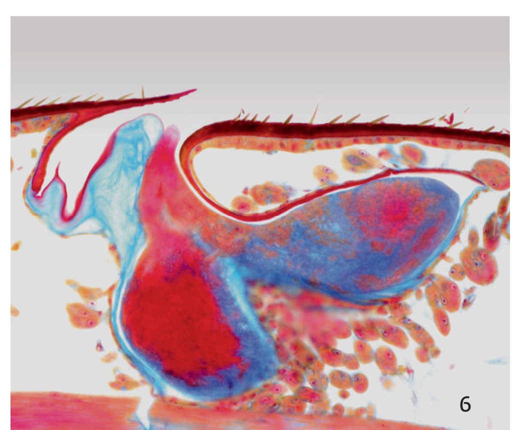
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1 In the laboratory, the firebugs live and reproduce in plastic boxes. For cross-species comparison, the scientists also breed cotton stainers, another bug that has a symbiotic relationship with bacteria.

2 The scientists store the bugs in alcohol to extract their DNA at a later stage.

3 A freshly hatched firebug nymph probes the surface of the egg to take up the intestinal bacteria.

4 Symbiotic bacteria under the fluorescence microscope: *Coriobacterium glomerans* from the midgut of a firebug.

The symbionts pose another difficulty: It seems that they only feel truly at home in the beewolf's antennal glands or in the care of their larvae. So far, attempts to cultivate them in the laboratory have been unsuccessful. Yet this is an important hurdle if scientists are to study their physiological characteristics more closely. "At one stage during my doctoral research, I was quite fed up with beewolves," admits Martin Kaltenpoth freely.

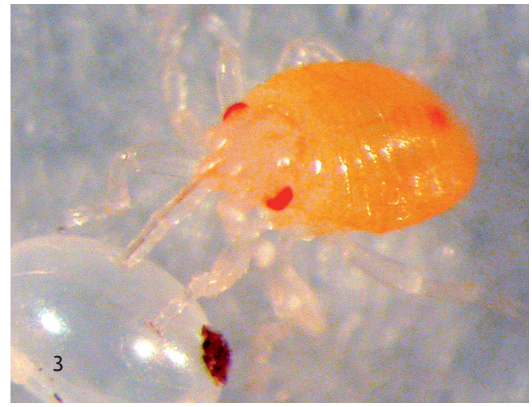
So his encounter with a small black and red insect on the Würzburg-based campus one day came at just the right time: "Now that's something to work with!" After all, the red firebug or *Pyrrhocoris apterus* is very undemanding and is content with just a little water and a few dry linden seeds. After some thorough research, the new object of study was finally established – and would become Kaltenpoth's second mainstay.

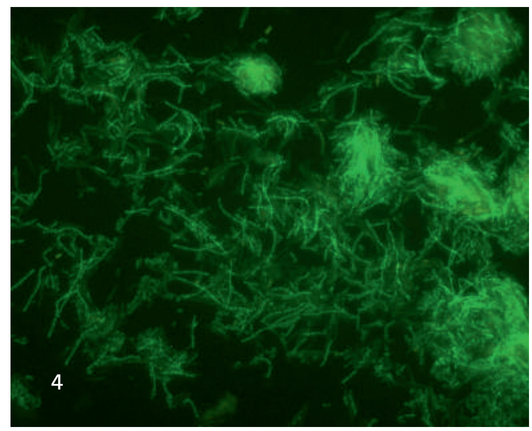
The scientific literature revealed that the brightly colored insects also played host to symbiotic Actinobacteria, although in a much less offbeat place than beewolves. In firebugs, the microbes colonize the digestive tract. Here, though, they occur in massive quantities, as Sailendharan Sudakaran, a doctoral student in the symbiosis group, discovered. He found that a single firebug can host up to one hundred million bacteria.

Hassan Salem, another doctoral student in Martin Kaltenpoth's team, then wanted to find out what the huge host of microbes is good for. He started by testing how the creatures get on without their symbionts. To raise a group of firebugs without gut bacteria, he sterilized the outside of the eggs, because the young normally pick up their symbionts from the surface of the eggs after they hatch.

As he discovered, the bugs are quite dependent on the tiny inhabitants of their gut. Without symbionts, they became stunted and their survival rate was very low – but why? "We presume that the bacteria help the firebugs digest the linden seeds," says Hassan Salem. These seeds are inedible for most insects, as they contain malvalic acid, a substance that inhibits the synthesis of fatty acids.

Photo: Sven Döring





Consequently, Salem is planning a new experiment, for which he has been busy crushing linden seeds and sunflower seeds in the laboratory. This is because firebugs without symbionts can't digest linden seeds, but could they manage on a diet of sunflower seeds, which contain no malvalic acid? The scientist plans to find out by extracting the acid from the crushed linden seeds and transferring it to the sunflower seeds. "Then, if the firebugs have problems with the sunflower seeds, we will know it's because of the malvalic acid," he explains.

INTESTINAL BACTERIA CULTIVATED IN THE LAB

Then the scientists can get to the crux of the matter, studying the relevant molecular mechanisms and finding the responsible genes. A major factor in their favor is the fact that the firebug symbionts, unlike those of the beewolf, can be cultivated in the lab.

As a result, Martin Kaltenpoth's team has great plans for their two objects of study, which have recently been joined by a bark beetle project. The pinhole borer, *Xyleborinus saxesenii,* may also avail of bacterial symbionts to protect fungal growths they use to feed their larvae. "To date, we know of only a few such examples of defensive symbiosis in the insect world," says Martin Kaltenpoth. He is convinced that many more fascinating partnerships between insects and microbes will come to light in the next few years.

Who knows? Perhaps one day the study of these partnerships will even deliver new drugs to fight against the growing number of resistant pathogens. After all, insects are well ahead of us in this area. Alexander Fleming only discovered the first antibiotic a little over 80 years ago – while beewolves have been using them for some 65 million years. ◂

GLOSSARY

Actinobacteria
Actinobacteria are a diverse group of Gram-positive bacteria that have a high proportion of guanine and cytosine bases in their DNA. The order includes the streptomycetes, which are used to produce antibiotics, but also important pathogens, such as those that cause tuberculosis, leprosy and diphtheria.

Digger wasps
Solitary insects that are closely related to bees. Some 10,000 species are known around the world, of which about 300 occur in Central Europe. Adult digger wasps derive their nourishment from flower nectar and pollen, while the larvae require food of animal origin. Consequently, the females provide for them fairly extensively, building brood chambers and providing the larvae inside with insects or spiders that they have paralyzed by stinging.