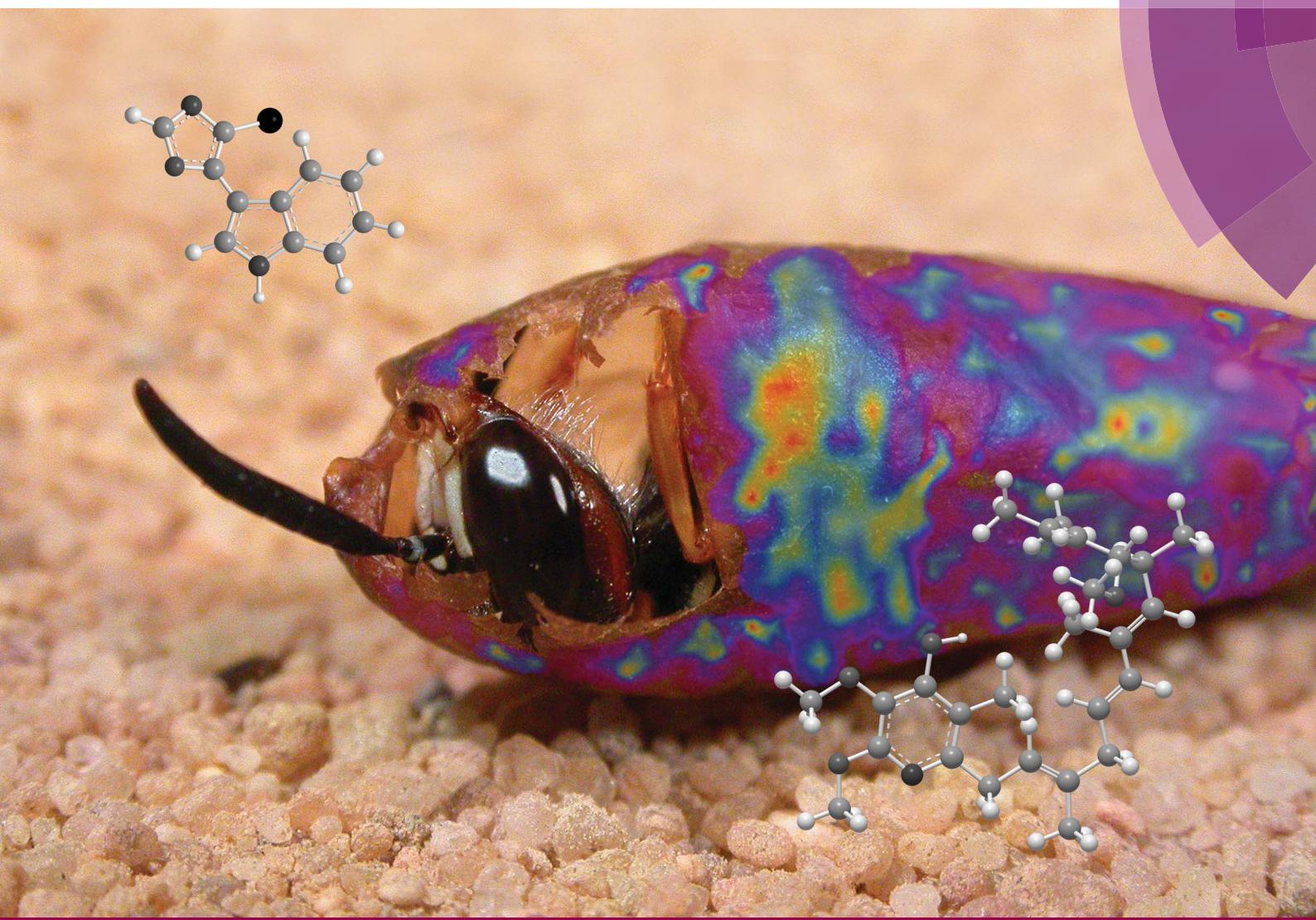


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REVIEW ARTICLE

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Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms

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Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms[†]

 Laura V. Flórez,[‡] Peter H. W. Biedermann,[‡] Tobias Engl[‡] and Martin Kaltenpoth^{‡§*}

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Many organisms team up with microbes for defense against predators, parasites, parasitoids, or pathogens. Here we review the described protective symbioses between animals (including marine invertebrates, nematodes, insects, and vertebrates) and bacteria, fungi, and dinoflagellates. We focus on associations where the microbial natural products mediating the protective activity have been elucidated or at least strong evidence for the role of symbiotic microbes in defense is available. In addition to providing an overview of the known defensive animal–microbe symbioses, we aim to derive general patterns on the chemistry, ecology, and evolution of such associations.

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1 Introduction

All organisms are threatened by antagonistic encounters with predators, pathogens, parasites, and/or parasitoids, which exert strong selective pressures on evolving efficient defense strategies. Such protective adaptations include behavioral, mechanical, and structural defenses against predators,¹ as well as a sophisticated immune system providing protection from microbial intruders and parasitoids.² In addition, many animals across a broad range of taxa use an arsenal of chemicals to defend themselves against various antagonists.^{3,4} Many of these defensive compounds are produced by the animals themselves, but it is becoming increasingly evident that microbial symbionts can make important contributions to their host's defense.^{5,6}

While symbiosis research has traditionally focused on the nutritional aspects of mutualistic associations between animals and microorganisms, more recent research has revealed the importance of defensive alliances with microorganisms for their hosts' ecology and evolution.^{5,6} In general, there are four different ways in which microbial symbionts can contribute to their host's protection from antagonists (Fig. 1): (i) microbial partners can improve the overall vigor of their host and thereby enable it to allocate an increased amount of resources into defense. This is likely true for many, if not all, nutritional symbioses, even though it is not often discussed in this context, given the usually more obvious (and more dramatic) direct effects of nutritional symbiosis on host survival and fecundity. (ii) Microbial symbionts can provide protection to their host by competitively excluding pathogenic microbes.⁷ (iii) The interaction with symbiotic microorganisms can stimulate or prime

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the host's immune system and thereby enhance resistance against pathogens, parasites, or parasitoids.⁸ (iv) Microbes can produce bioactive compounds or their precursors and thereby contribute to their host's defensive chemistry.^{9,10} In the context of natural products chemistry, defensive symbioses of the last category are the most interesting, as they often involve novel compounds of potential interest for application in human medicine, agriculture, or food technology.

In the present review, we aim to provide an overview of the known defensive symbioses between Metazoa and microorganisms, with an emphasis on associations where host protection is mediated by symbiont-produced secondary metabolites. We are building on previous reviews of microbial protective symbioses in particular groups of animals, including marine organisms,^{11–14} insects,^{15–19} and nematodes,^{20–22} as well as on reviews covering the metabolites produced by symbiotic bacteria.^{9,10} Generally, we focus particularly on symbioses for which the defensive chemistry

has been elucidated, and a protective benefit for the host has been demonstrated or is at least very likely. Most of these involve associations with bacteria, but a few defensive alliances with fungi and dinoflagellates have also been described. As might be expected, bioactive compounds derived from polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) are particularly widespread in defensive symbioses, occurring in marine systems like sponges, corals, ascidians and bryozoans, as well as in terrestrial associations involving nematodes and insects. However, a diverse range of other compound classes with interesting activities occur across symbiotic associations and habitats, including organic acids, phenolics, ribosomal peptides and terpenes (Table S1†). Following our review of the literature on defensive microbial symbioses in animals, we conclude with a synthesis section aimed at deriving general patterns on the chemistry, ecology and evolution of defensive animal-microbe symbioses.



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Tobias Engl studied Chemistry and Biology at the University of Regensburg, Germany, obtaining his diploma in biology in 2008. His dissertation project in the Evolutionary Ecology group of Dr Erhard Strohm in Regensburg focused on anti-fungal defenses of the European beewolf. Since 2011, he is a postdoctoral researcher at the Max Planck Institute for Chemical Ecology in Jena, Germany, extending his focus to the chemistry of symbiont-mediated defense in beewolves as well as the ecological and evolutionary implications of insect-microbe interactions in general.



Martin Kaltenpoth obtained his MSc and PhD degrees from the University of Würzburg, followed by postdoctoral research at the University of Regensburg and the University of Utah in Salt Lake City. In 2009, he established the Insect Symbiosis Research Group at the Max Planck Institute for Chemical Ecology in Jena. From April 2015, he will be a full professor for Evolutionary Ecology at the University of Mainz. His research focuses on the evolution, chemical and molecular ecology of insect-microbe symbioses, with a special interest in defensive alliances.



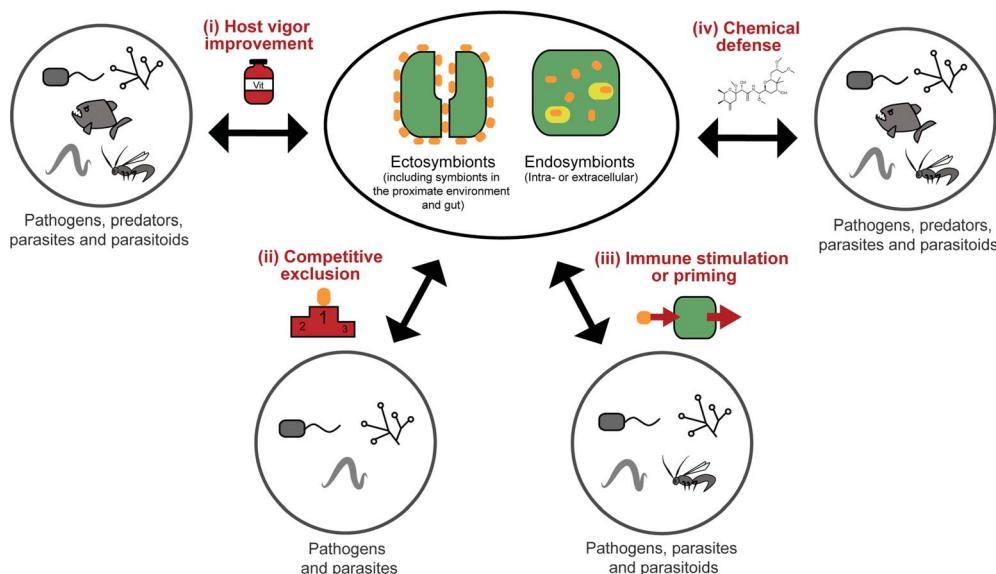


Fig. 1 Types of defense mechanisms in animals mediated by ectosymbionts (including those in the gut and in the proximate environment of the host) or endosymbionts (intra- or extracellular) against different possible antagonists (described or likely effective against).

2 Defensive animal–microbe symbioses

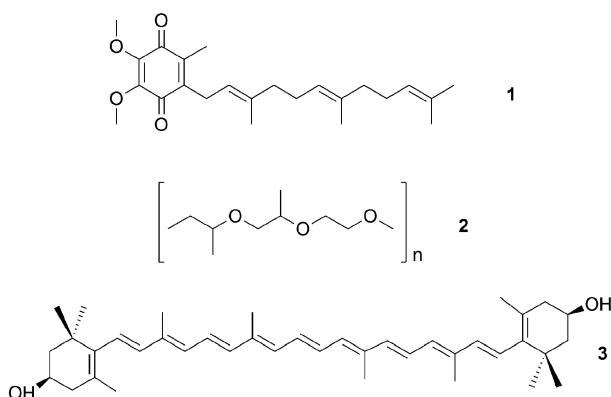
2.1 Marine invertebrates

2.1.1 Sponges. Due to their soft bodies and immobile lifestyle, many sponges heavily rely on chemical defenses. This is reflected in a rich repertoire of secondary metabolites that can be self-produced, sequestered from the food, or provided by symbiotic partners.²³ In fact, many sponges harbor a diverse community of microorganisms that can be transient, digested as a nutrient source, or stably associated with the sponge.^{24,25} Past and recent developments in molecular techniques have enormously improved our understanding of sponge symbioses, by providing the opportunity to localize individual bacterial cells in host tissue through fluorescence *in situ* hybridization (FISH), characterizing microbial communities taxonomically by high-throughput amplicon sequencing as well as functionally by metagenomics, sorting of uncultivable bacteria through fluorescence-assisted cell sorting (FACS) and elucidating their metabolic capabilities by single cell genomics. However, the task of characterizing relationships between sponges and key members of their microbial community as well as elucidating the nature and function of bioactive metabolites in an ecological and evolutionary context remains challenging.²⁶ In particular, experimental manipulation of sponge–microbiota associations to reveal symbiont contributions to host fitness is often impossible and remains limited to very few amenable systems. As several recent reviews summarize the literature on natural products from microbes associated with or isolated from sponges,²⁷ on methodological developments and approaches to study the possible bacterial origin of sponge-derived defensive compounds,^{28–30} on sponge symbioses in

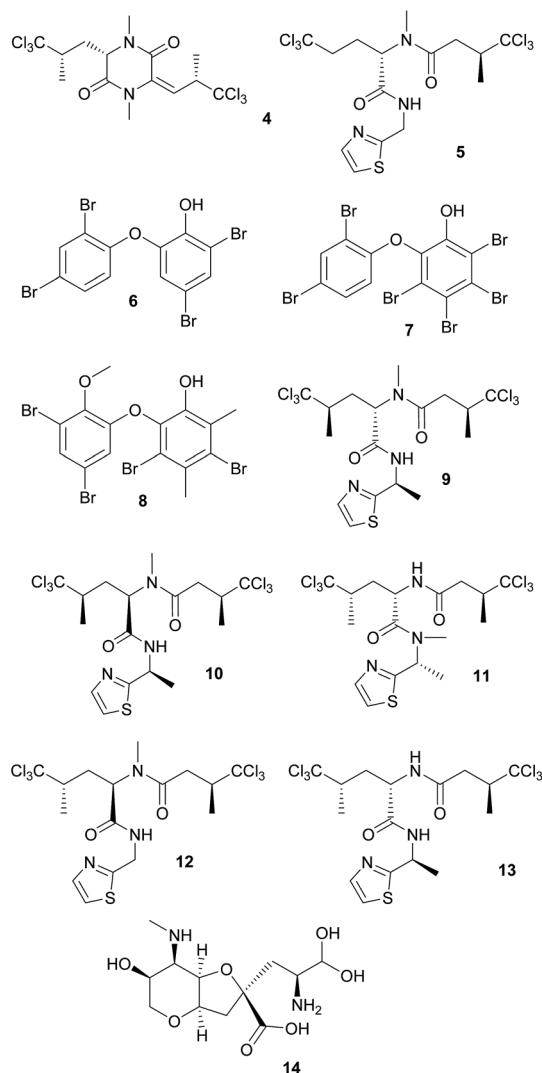
general,^{25,31,32} and on metabolites isolated from marine organisms including sponges³³ as well as their potential applications,^{34–36} we focus here on examples where the mutualistic nature of defense, the involved chemistry, and the ecological context have been studied.

Culture-dependent approaches to isolate defensive symbionts. Some of the first insights in sponge defensive symbioses were gained by culture-dependent approaches. Konya *et al.*³⁷ followed the reports of surface-associated compounds influencing the settlement of invertebrate larvae causing fouling, and the idea that bacteria might produce these compounds. Concordantly, they succeeded in isolating an *Alteromonas* strain from the sponge *Halichondria okadai* that inhibited the settlement of *Balanus amphitrite* cyprids. The active compound was identified by bioassay-guided fractionation as ubiquinone-8 **1**. Several structurally related compounds like other ubiquinones but also vitamin K inhibited larval settlement as well.³⁷ Using a similar approach, Dash *et al.*³⁸ isolated *Winogradskyella poriferum* from *Lissodendoryx isodictyalis*, which directly inhibits the settlement of *B. amphitrite* and *Hydroides elegans* larvae and additionally reduces the growth and biofilm formation of several bacteria that are known to induce larval settlement on sponges. The active compound was identified as a poly-ether **2** of variable chain length.³⁹ However, the specificity and prevalence of both associations and their effect on host fitness remain unknown. A different function was reported by Miki *et al.*⁴⁰ for two *Flexibacter* sp. isolated from the sponge *Reniera japonica*. The bacteria produce the carotenoid 3R,3'R-zeaxanthine **3**, which is a potent quencher of singlet molecular oxygen and a scavenger of free organic radicals, suggesting a protective role against reactive oxygen species (ROS).





Defensive cyanobacterial symbionts in dictyoceratid sponges. The first culture-independent approaches for the identification of symbiont-produced defensive chemicals in sponges relied on the physical separation of host and symbiont cells. Unson and Faulkner used flow cytometric cell sorting and subsequently located the sesquiterpenes herbadysidolide and spirodysin only



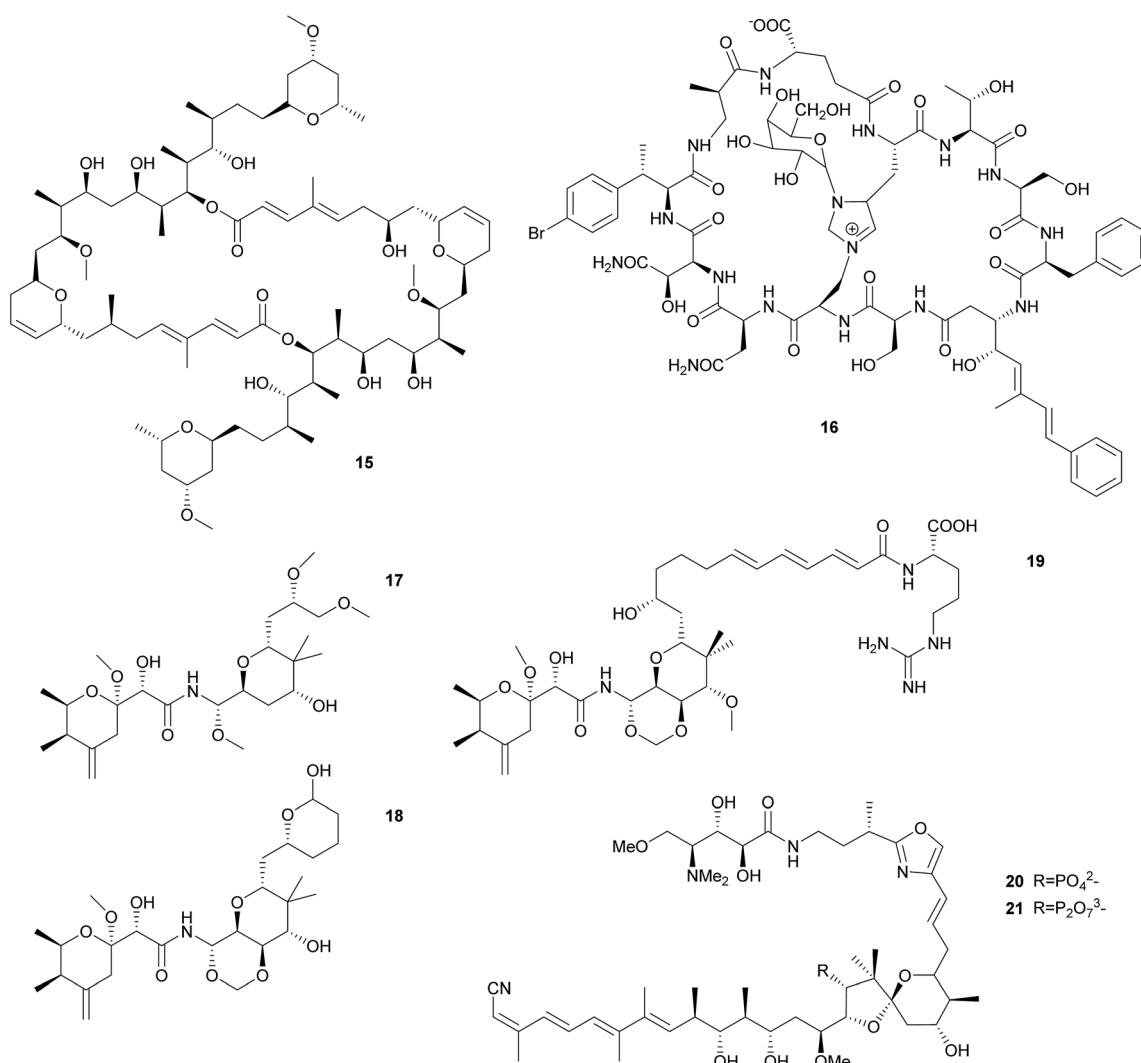
in tissue of the sponge *Dysidea herbacea* itself, whereas the polychlorinated diketopiperazines dihydrodysamide C 4 and demethyl-dihydrodysamide C, as well as 13-demethylsodysidin 5 were only present in the fraction containing the symbiotic cyanobacterium *Oscillatoria spongeliae*.⁴¹ Flowers *et al.* repeated the experiment using density gradient centrifugation, verifying the earlier findings and additionally locating didechlorodihydrodysamide C within *O. spongeliae*.⁴² They further found a cyanobacterial cell fraction devoid of the chlorinated compounds, indicating either different physiological states or strains of the symbionts. The symbiont-derived compounds were tested for their bioactive potential and shown to strongly deter fish-feeding, suggesting that they are involved in defense against predators in the natural environment.⁴¹ Interestingly, *D. herbacea* can also carry a different strain of *O. spongeliae* that – instead of the chlorinated compounds – produces polybrominated biphenyl ethers 6–8 that not only deter fish-feeding,⁴³ but also show antimicrobial activity.⁴⁴ Importantly, these results provided the first description of different sponge chemotypes due to variation in the metabolic profiles of their symbionts, a pattern that was subsequently found repeatedly across several sponge taxa as well as other marine invertebrates.⁴⁴

Using fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR) amplification, *O. spongeliae* was also identified as the producer of polychlorinated peptides like dysidenin 9, iso- 10 and neodysidenin 11, 13-demethylsodysidin 12 and nor-dysidenin 13 in *D. herbacea*,⁴⁵ some of which have been shown to be toxic for fish.⁴⁶ The primers and probes used for the detection of the dysidenins were based on the biosynthetic gene cluster derived from the cyanobacterium *Lyngbya majuscula* that produces the homologous compounds barbamide and nordysidenin. The PCR results revealed that not all *O. spongeliae* strains contain the dysidenin gene cluster, resulting in different chemotypes of the sponge host depending on the symbiont strain.⁴⁵ In a more extensive screen, Ridley *et al.* found species-specific secondary metabolite profiles in four dictyoceratid sponge species, comprising either chlorinated peptides, brominated diphenyl ethers or nonhalogenated compounds, mainly sterols.⁴⁷ Phylogenetic analyses supported a general pattern of co-speciation of the sponges with their respective *O. spongeliae* symbionts, but also revealed a likely host switch. Additional studies confirmed that the presence of unique symbiont strains in different sponge species of the family Dysideidae⁴⁸ conferred the characteristic chemical profiles to their hosts and supported the occurrence of host switches and independent infection events.⁴⁹ Furthermore, *D. herbacea* individuals can harbor an additional symbiont of the genus *Synechocystis*, which produces the potent neurotoxin dysiherbaine 14.⁵⁰ In analogy to the *Oscillatoria* symbionts, *Synechocystis* strains vary in their ability to synthesize dysiherbaine, thereby resulting in different host chemotypes. However, the ecological significance of symbiont-mediated dysiherbaine production for the host remains elusive.

Production of bioactive polyketides by sponge symbionts. Polyketide synthases (PKS)⁵¹ and non-ribosomal peptide synthetases

(NRPS)⁵² are enzyme complexes that synthesize secondary metabolites based on a stepwise elongation of the product, catalyzed by often repetitive and conserved modules that are encoded in a single operon. The conserved nature of the individual modules provides the opportunity for PCR-based screens with degenerate primers and allows for *in silico* predictions of possible metabolite structures based on the architecture of the gene cluster.^{28–30,53} PKS and NRPS gene clusters and/or their products have been reported for several different sponge taxa. From the sponge *Pseudoceratina clavata*, Kim *et al.* isolated multiple *Salinispora* spp. that contained a rifamycin-like PKS gene cluster and showed strong *in vitro* antibiotic activity.⁵⁴ Concordantly, rifamycin B and SV could be isolated *in vitro*, and specific primers detected the biosynthetic genes in most isolated strains. The caribbean sponge *Plakortis simplex* contains the polyketide plakortin and several derivatives, in addition to the glycosphingolipids plakosides and simplexides, as well as the crasserides and bacteriohopanoids, all of which are mainly or exclusively known from *Sphingomonas* bacteria.⁵⁵ Together, these

compounds exhibit a wide spectrum of biological activities that might be involved in chemical defense of the sponge against microbes (plakortins: antimicrobial/antimalarial^{56–58}), fish or other predators (crasserides,⁵⁹ plakortethers⁶⁰), or in regulating its microbial community by modulating the host's immune system (plakosides,⁶¹ simplexides⁶²). An attempt to isolate the plakortin biosynthesis genes failed, but yielded an unusual polyketide-fatty acid synthase hybrid that supposedly synthesizes an acyl chain with various functional groups, probably containing a sulfate group.⁶³ Fisch *et al.* also exploited the conserved sequence of the ketosynthase (KS) domain to screen metagenomic fosmid libraries from the sponges *Psammocinia* aff. *bulbosa* and *Mycale hentscheli* for candidate bacterial gene clusters involved in the production of psymberin (=ircinastatin A) and mycalamide A, respectively.⁶⁴ These compounds were long known to exert anti-viral^{65,66} and selective cytotoxic activity against certain tumor cell lines.⁶⁷ KS sequences were successfully amplified from both sponge metagenomes, and the entire psymberin locus



from *P. aff. bulbosa* was sequenced, but the producing bacteria have not been identified.

Sponge-associated symbionts in the candidate genus *Entotheonella* proved to be an especially rich source of polyketides. Using differential centrifugation, Bewley *et al.* were able to separate the bacteria associated with the lithistid sponge *Theonella swinhoei* into three fractions, containing unicellular cyanobacteria, unicellular heterotrophic bacteria, and filamentous heterotrophic bacteria, respectively.⁶⁸ The antifungal and cytotoxic⁶⁹ macrolide swinholide A 15 was isolated from the unicellular heterotrophic fraction, while a cyclic peptide was isolated from the filamentous heterotrophic bacteria. The latter shows high structural similarity to the antifungal theonogrammid⁷⁰ and was later named theopalauamide 16 and also characterized as antifungal.⁷¹ Schmidt *et al.* characterized the filamentous symbiont from different *T. swinhoei* chemotypes on the 16S rRNA level and found very closely related species in the chemotypes containing theopalauamide, theonogramide and theonellamide A, respectively.⁷² The name '*Candidatus Entotheonella palauensis*' was proposed for the strain from the theopalauamide producing chemotype.

The subsequent exploration of the *Entotheonella* symbionts in *T. swinhoei* revealed an extraordinarily large biosynthetic repertoire, including the potential for the production of theopederin A, onnamide A, polytheonamides, as well as keramamides, cyclotheonamides, nazumamide, and proteusins.⁷³ Interestingly, the identification of a bacterium of the genus *Pseudomonas* as the producer of the polyketide pederin 17 in a beetle and the elucidation of its biosynthesis (see 2.2.2) was a useful starting point to identify the genes responsible for polyketide biosynthesis in *T. swinhoei*, due to the structural similarity of pederin and the cytotoxic theopederin A 18⁷⁴ as well as the cytotoxic and antiviral onnamide A 19.⁷⁵ PCR-based screening and subsequent sequencing of metagenomic cosmid libraries of different *T. swinhoei* chemotypes revealed the onnamide gene cluster,⁵³ which was confirmed to be of bacterial origin and closely resembles the pederin cluster. This cluster was only detected within the sponge Y chemotype, which contains solely pederin-like metabolites.⁷⁶ Later, Freeman *et al.* reported the ribosome-produced polytheonamides as additional bacterial products from *T. swinhoei*,⁷⁷ which form unimolecular ion channels⁷⁸ and are active against Gram-positive bacteria. Wilson *et al.* finally attributed the metabolic genes of both onnamide and the polytheonamides to *Entotheonella* by analyzing single cells *via* differential centrifugation and fluorescence-assisted cell sorting, followed by multiple displacement amplification and whole genome sequencing of individual bacterial cells.⁷³ Interestingly, the genome sequences of *Entotheonella* revealed two very similar strains that both carried a plasmid containing the onnamide and polytheonamide genes, but differed remarkably with regard to chromosomally encoded secondary metabolite gene clusters. In addition to the plasmid-localized clusters, the biosynthetically rich TSY1 strain carried 28 secondary metabolite biosynthetic gene clusters, including those for the synthesis of keramamides, cyclotheonamides, nazumamide, and proteusins, as well as a non-functional

konbamide cluster. By contrast, the TSY2 strain carried 'only' seven additional biosynthetic gene cluster, with nearly no overlap in the secondary metabolite repertoire with TSY1. This diversity in biosynthetic potential was found to extend to the *Entotheonella* symbionts across several sponge taxa, indicating that *Entotheonella* strains in the newly described bacterial phylum 'Tectomicrobia' will likely serve as a rich source for future discoveries of novel natural products.

Similar to *Theonella*, the sponge genus *Discodermia* contains a diversity of bioactive secondary metabolites produced by symbiotic microbes. In fact, *Entotheonella* symbionts have been reported from different *Discodermia* species,⁷⁹⁻⁸¹ which present a large diversity of PKS clusters.^{79,81} Additionally, the cytotoxic cyclic peptides calyxamide A and B, structurally similar to the above mentioned keramamides, were isolated from *Discodermia calyx*.⁸¹ However, it has only been possible in a single case to unambiguously connect secondary metabolite production to a specific bacterium in a *Discodermia* host: Wakimoto *et al.* sequenced the gene cluster responsible for the production of calyculins from the metagenome of *D. calyx*, localized the PKS cluster using FISH within filamentous bacteria and isolated these by laser microdissection.⁸² PCR on the isolated bacteria confirmed the PKS localization and identified the symbionts *via* 16S rRNA analysis as an *Entotheonella* species. Interestingly, the authors were also able to characterize a means for storage of a defensive compound in a form that is harmless for the host. The usually cytotoxic calyculin A 20 is phosphorylated by the *Entotheonella* symbionts and stored as the less toxic diphosphate 21. Upon wounding of the sponge, the phosphocalyculin is rapidly converted by a host-derived enzyme to the more than a thousand times more toxic calyculin, thus representing an activated chemical defense mechanism.⁸²

Fungal defensive symbioses in sponges. In contrast to the wealth of knowledge on protective bacterial symbionts in sponges, convincing evidence for defensive fungal symbionts is lacking.^{25,83} This is insofar surprising as the number of fungal species isolated from sponges⁸⁴ and their potential for secondary metabolite production is tremendous.^{33,85} A few studies have addressed the symbiotic aspect of sponge-fungi relationships, and shown maternal transmission of a yeast in the sponge *Chondrilla*,⁸⁶ horizontal gene transfer between fungi and sponge mitochondria,⁸⁷ as well as fungal recognition proteins in sponges.⁸⁸ Another indication of the potentially symbiotic nature of fungi in sponges is the presence of specific fungal 18S rRNA sequences in sponge databases.²⁴ Furthermore, sponge-associated fungi were found to contain a large diversity of PKS and NRPS genes,⁸⁹ but their possible roles in the defense of the host remain enigmatic.

2.1.2 Cnidarians. Many corals are intimately associated with algal symbionts as well as a diverse community of bacteria. In particular, dinoflagellate symbionts of the genus *Symbiodinium* are well-known for their important contributions to the coral hosts' metabolism by providing photosynthetically derived nutrients,⁹⁰ as well as by recycling and assimilating ammonia produced by the host.⁹¹ Furthermore, endolithic algae of the genus *Ostreobium* can contribute carbon sources to their host,⁹⁰ and diazotrophic bacteria have



been found to fix atmospheric nitrogen in the Caribbean coral *Montastrea cavernosa*.^{92,93} In addition to these nutritional benefits, the microbial associates of corals can play important roles in the defense of their hosts against pathogens and predators. Concordantly, several studies provided theoretical and empirical evidence for the involvement of coral-associated bacterial communities in the defense against microbial pathogens.^{94–98} However, to our knowledge, the chemical basis of bacteria-mediated defensive activities remains unknown. Therefore, we will focus here on the dinoflagellate symbionts of corals and their involvement in the production of two groups of defensive compounds, bioactive diterpenes and secosterols.

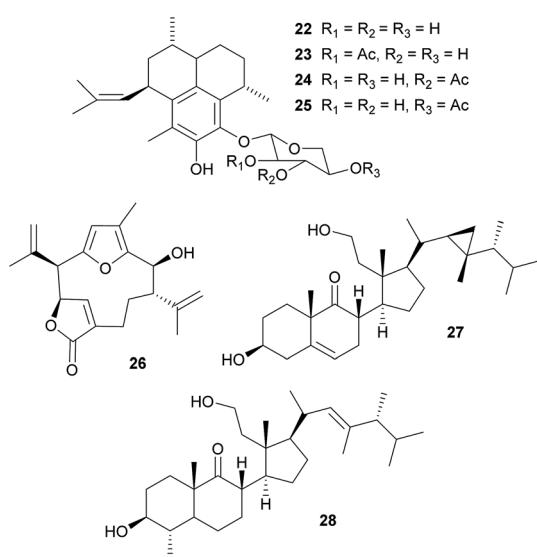
Defensive diterpenes. Like many other sessile marine animals, corals are a rich source of bioactive secondary metabolites that play an important role in the defense against predators.^{99–101} Among these, the pseudopterosins 22–25 are a group of tricyclic diterpene glycosides with potent antiinflammatory and analgesic activity that were originally isolated from the soft coral *Pseudopterogorgia elisabethae*.^{102–104} Enrichment of *P. elisabethae*'s symbiotic dinoflagellates of the genus *Symbiodinium* by differential centrifugation revealed the predominant localization of the pseudopterosins in the symbiont fraction, suggesting that they are produced by the dinoflagellates.¹⁰⁵ Concordantly, incubation of this fraction with either $\text{NaH}^{14}\text{CO}_3$ or tritiated geranylgeranyl diphosphate ($^3\text{H-GGDP}$) resulted in labeled pseudopterosins.¹⁰⁵ A similar strategy of symbiont cell enrichment and subsequent radioactive labeling with $^3\text{H-GGDP}$ revealed the *Symbiodinium*-mediated production of kallolide A 26 in *Pseudopterogorgia bipinnata*.¹⁰⁶ Interestingly, only one out of four different *Symbiodinium* strains exhibited kallolide production *in vivo*, indicating differences in chemical properties and defensive capabilities across different symbionts.¹⁰⁶ Even though the adaptive significance of the symbiont-produced

pseudopterosins and kallolides for the coral hosts has not yet been demonstrated *in vivo*, extracts of both coral species (*P. elisabethae* and *P. bipinnata*) were unpalatable to the generalist fish predator *Thalassoma bifasciatum*,¹⁰¹ highlighting the potential importance of the *Symbiodinium*-produced bioactive compounds for the antipredator defense of the coral host.

Secosterols. Secosterols isolated from corals, sponges, and ascidians can exhibit a diverse range of biological activities, including antiproliferative, antifouling, antiinflammatory, antimicrobial, ichthyotoxic and antiviral.¹⁰⁷ In the octocoral *Pseudopterogorgia americana*, bioassay-guided fractionation revealed the deterrent activity of 9,11-secogorgosterol 27 and 9,11-secodinosterol 28 against predatory fish in laboratory and field assays.¹⁰⁸ Even though the source of the secosterols in *P. americana* has not been unambiguously identified, zooxanthellae isolated from other marine organisms (including a coral) were reported to produce gorgosterol and dinosterol.¹⁰⁹ Furthermore, gorgosterol is transformed to 9,11-secogorgosterol by enzyme preparations of *P. americana* colonies.¹¹⁰ Thus, it seems likely that dinoflagellate symbiont-produced precursors are modified by host enzymes to synthesize the defensive secosterols.

Protective symbionts in *Hydra*. The epithelial surfaces of freshwater polyps in the genus *Hydra* harbor stable and species-specific bacterial assemblages^{111,112} that are shaped by the host *via* antimicrobial peptides.¹¹³ By generating germ-free animals and reinfecting them with individual bacterial taxa or combinations thereof, a recent study revealed that the symbiotic community of *Hydra vulgaris* plays an important role in protecting the host against fungal infestation.¹¹⁴ Although the mechanistic basis of the protective effect remains to be elucidated, both *in vitro* and *in vivo* studies point to a combined activity of the host and its microbiota in pathogen defense.¹¹⁴

2.1.3 Bryozoans. Bryozoans are a group of sessile marine animals with a dispersive larval stage, comprising close to 6000 described species to date. Although only a small fraction of this biodiversity has been investigated chemically, a large number of compounds with bioactive properties have already been described that may play a role in defense against predators, competitors, parasites, or pathogenic bacteria and fungi.¹¹⁵ Based on (i) the structural similarity to microbially produced substances, (ii) the occurrence of similar compounds in taxonomically distinct bryozoan lineages, and/or (iii) the differences in secondary metabolite profiles across populations of the same species, several defensive compounds isolated from bryozoans have been hypothesized to be of microbial origin, including the phidolopins and other nitrophenols of *Phidolopora pacifica*, *Diaperocia californica*, *Heteropora alaskensis*, *Tricellaria ternata*, and *Hippodiplosia insculpta*,¹¹⁶ the brominated convolutamides, convolutamines, convolutamydines, convolutindole, volutamides, amathamides, and amathaspiramides in *Amathia* spp.,^{115,117–119} the perfragilins of *Biflustra perfragilis*,^{120,121} as well as various secondary metabolites isolated from *Flustra foliacea*.¹²² However, we will focus here on cases where more direct evidence for a microbial involvement in secondary metabolite production has been provided.



Bryostatins. The cosmopolitan bryozoan *Bugula neritina* is chemically defended against predators by a cocktail of cyclic polyketides, the bryostatins.^{123,124} While these compounds are present in low concentrations in adult *B. neritina* colonies, the abundance of bryostatin 10 **29** and bryostatin 20 **30** is strongly increased in young larvae.¹²⁵ By binding to the diacylglycerol binding site of protein kinase C's regulatory domain,¹²⁶ the bryostatins exert toxicity and deterrence to fish, corals, and sea anemones and thereby protect *B. neritina* larvae from predation.^{125,127-130} Importantly, attacked and rejected larvae show high rates of settlement, demonstrating a direct fitness benefit from chemical protection.^{129,130} After settlement and metamorphosis, bryostatin levels rapidly decrease, indicating a switch from chemical to structural defense as the colony matures.¹²⁵

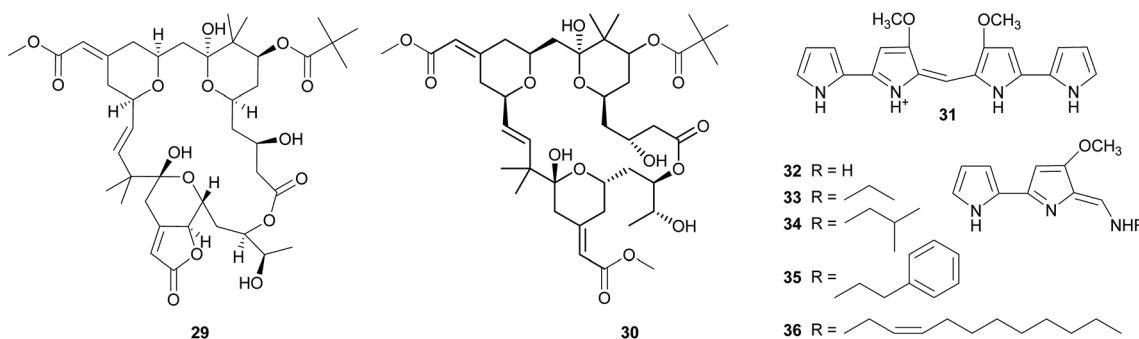
Soon after the structure elucidation of bryostatin 1, this compound was suspected to be of bacterial origin rather than produced by *B. neritina* itself.¹³¹ Concordantly, earlier studies had already reported on rod-shaped bacteria that are consistently associated with adult and larval *B. neritina*.^{132,133} Based on the 16S rRNA sequence, these bacteria were later described as a new taxon within the γ -Proteobacteria and named '*Candidatus Endobugula sertula*'.¹³⁴ A series of subsequent studies provided convincing evidence that the bryostatins are indeed produced by '*Ca. E. sertula*', thereby constituting one of the best documented cases of defensive symbiosis between animals and microorganisms in the marine environment. Davidson *et al.*¹³⁵ used *in situ* hybridization to co-localize the symbiotic bacteria and a polyketide synthase (PKS) gene fragment putatively involved in bryostatin synthesis. Simultaneous fluorescent detection of '*Ca. E. sertula*' and the bryostatins later revealed the dynamics of bryostatin production during the life cycle of *B. neritina*.¹¹⁵ As expected under the hypothesis of symbiont-mediated bryostatin synthesis, reduction of symbiont titers in adult *B. neritina* by antibiotic treatment resulted in a strong decrease in bryostatin concentrations.¹³⁵ The offspring of antibiotic-treated colonies likewise showed strong reductions in symbiont abundance and bryostatin concentrations, and symbiont-free larvae failed to deter predatory fish.¹²⁸ Interestingly, however, settlement and growth of juvenile *B. neritina* was not affected by symbiont elimination, indicating that the

defensive capacities of the symbionts are the only or at least the most important benefit for the host.¹²⁸

Efforts to elucidate the genomic basis of bryostatin production resulted in the discovery of a single large PKS gene cluster (*bry*) in a *B. neritina* genomic library enriched for bacterial DNA.^{136,137} This gene cluster is expressed in '*Ca. E. sertula*' cells in the pallial sinus of *B. neritina* larvae, and expression is not detectable after symbiont elimination through antibiotic treatment, providing further evidence that it is indeed encoded by the '*Ca. E. sertula*' genome.¹³⁵ Bioinformatic predictions supported the biosynthesis of the bryostatin core structure by the *bry* gene cluster,^{124,138} and heterologous expression of *bryP* and *bryA* confirmed the functionality of these genes.^{139,140} The symbionts of two sibling species of *B. neritina* exhibited high similarity in structure and sequence (98%) of the *bry* gene cluster, indicating a common ancestry.¹³⁷

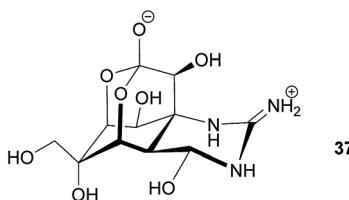
The occurrence of bryostatin-producing symbionts was confirmed for two sibling species of *B. neritina* as well as for *Bugula simplex*.^{141,142} Surprisingly, a third sibling species of *B. neritina* was devoid of bryostatin-producing symbionts,¹⁴³ but still exhibited deterrence to a fish predator, providing evidence for additional defensive compounds produced by the bryozoan itself or an as yet unknown symbiont.¹²⁸ In *Bugula pacifica* and *B. turbinata*, symbionts closely related to '*Ca. E. sertula*' and '*Ca. E. glebosa*' (the symbiont of *B. simplex*) were discovered, but no bryostatin activity could be detected.¹⁴⁴ Interestingly, extracts from *B. pacifica* showed broad-spectrum antibacterial activity, suggesting that defensive compounds other than bryostatins are present and may be produced by the symbionts.¹⁴⁵ Three additional *Bugula* species – *B. dentata*, *B. stolonifera*, and *B. turrita* – appeared to be devoid of the symbionts.¹⁴⁴ The patchy occurrence of *Endobugula* symbionts across host species indicates a dynamic symbiotic association with frequent host switches or symbiont acquisitions/losses. Given the deficiency in recombination of the symbionts,¹²⁴ changes in defensive chemistry by symbiont switches or replacements might be advantageous in the arms race against co-adapting predators. Alternatively, the symbiotic partnership may respond by changing the absolute or relative composition of the bryostatin cocktail, which can influence its activity against predators.¹²⁸

Tambjamines. The tambjamines **31–36** are a group of 4-methoxypyrolic natural products that occur across several



taxonomically distinct groups of marine organisms, including bryozoans,^{146,147} nudibranchs,¹⁴⁸ and ascidians.^{149,150} Based on this disparate distribution and the occurrence of identical or closely related compounds in bacteria,^{151,152} the tambjamines were suspected to be of microbial origin. The discovery of the tambjamine-producing marine bacterium *Pseudoalteromonas tunicata*¹⁵² and its association with a range of marine animals¹⁵³ – including bryozoans, mussels, ascidians, fish, corals, and sponges^{153–155} – support this hypothesis. Recently, the molecular basis of tambjamine production in *P. tunicata* was elucidated by heterologous expression of the *tam* gene cluster in *E. coli*.¹⁵⁶ The tambjamines show toxicity and/or deterrence against predatory fish as well as antimicrobial activity, indicating that they might confer protection from both pathogens and predators.^{152,157–161} Some predatory nudibranchs, however, are resistant to the adverse effects of tambjamines; in fact, they sequester the bioactive compounds from their bryozoan or ascidian diet and use them for their own defense.^{148,160}

2.1.4 Nemerteans. Tetrodotoxin 37 (TTX) is a highly potent neurotoxin that is found across a wide range of marine organisms, as well as a few terrestrial animals.¹⁶² Its chemical structure consists of a guanidine derivative connected to a highly oxygenated carbon skeleton with 30 known analogues.¹⁶² The prevalent hypothesis is that it serves as an antipredatory agent,¹⁶² although it is also known to be employed by some predators to paralyze their prey.¹⁶³ Owing to the broad array of unrelated metazoans that contain TTX, it has been suggested that the compound is not of endogenous origin but produced by microbial symbionts.^{164,165} In fact, there is strong evidence demonstrating that a number of different bacteria are capable of synthesizing the molecule,^{166,167} but insufficient support for a true symbiotic association of these bacteria with the respective host species. Recently, the nemertean ribbon worm *Cephalothrix simula* was found to contain high concentrations of TTX and several of its analogues.¹⁶⁸ Shortly after, Magarlamov *et al.* isolated TTX-producing *Bacillus* species from *C. simula* individuals and used immunohistochemical methods to tie TTX production to the bacteria and localize the compound in the maturing spores.¹⁶⁶ If substantiated and combined with more detailed analyses on the nature and consistency of the *Bacillus*–*C. simula* association, these results could provide the first demonstration of symbiont-produced defensive TTX. Interestingly, *C. simula* is known to be a food source of the pufferfish, the organism from which TTX was first described, so the fish might sequester TTX from its nemertean diet.



2.1.5 Mollusks. Several mollusks also contain TTX, the prime example being the blue-ringed octopuses of the genus *Hapalochlaena*, which also carry several bacterial species that

produce TTX in culture.^{162,169} However, the nature of the bacteria–octopus association as well as the source of TTX in further mollusks has not been unambiguously identified, so the microbial origin remains speculative.^{162,170}

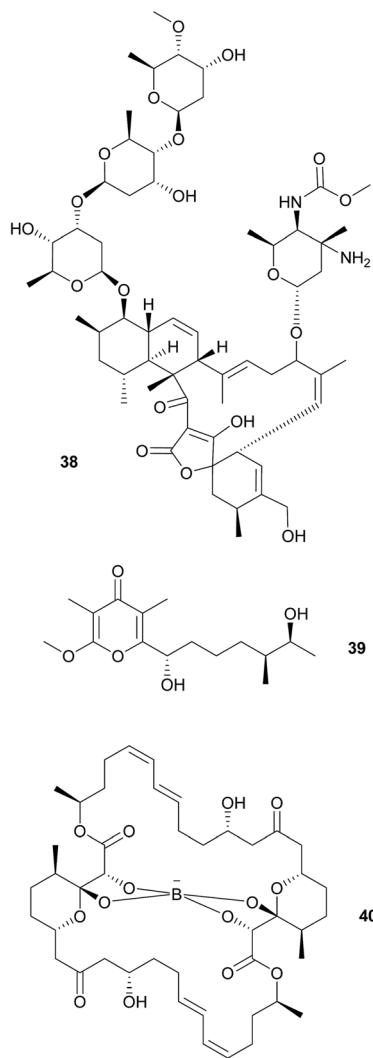
Gastropods. An interesting case of symbiont-mediated structural protection has been described in the scaly snail *Crysomallon squamiferum*, a gastropod occurring at hydrothermal vents.¹⁷¹ The snail's foot is covered in hardened scales of multiple layers that likely confer protection against predation.^{171,172} The outer layer is composed of pyrite (FeS₂) and greigite (Fe₃S₄), whose biosynthesis has not been described in metazoans. Interestingly, a community of δ - and ε -Proteobacteria, which are known for their ability to recycle sulfur and mineralize iron sulfides, were found to live in association with the snail.¹⁷¹ Thus, it was suggested that the bacterial partners are responsible for depositing the outer scale layer and thereby confer protection to the snail host.¹⁷¹ However, another study based on the structural and chemical composition of the scales suggests that the snail itself controls the biomineralization *via* sulfur compounds derived from the hydrothermal vents.¹⁷³ To our knowledge, no study to date has taken an experimental approach that aims to manipulate the bacterial community associated with the snail, so the case remains unresolved.

As cone snails are well-known for their arsenal of protective peptide toxins, further microbe-derived defensive compounds were not expected. Surprisingly, however, Peraud *et al.* found a diverse actinomycete community associated with different cone snails of the genus *Conus* that displayed bioactive properties.¹⁷⁴ *Streptomyces* sp. CP32 isolated from *C. pulicarius* produces several benzyl thiazole and thiazoline compounds (aerugine, pulicatins A–G and watasemycins A & B) that exhibit antimicrobial, anti-inflammatory and antihypotensive activity.¹⁷⁵ Another *Streptomyces* isolate from *C. tribblei* that also produces pulicatin A was hypothesized to protect the snail surface against microbial colonization.¹⁷⁵ Eight nobilamides and two related compounds were identified in further isolates from *C. tribblei* and *Chicoreus nobilis*, some of which inhibit the TRPV1 cation channel that is a major mediator of pain and inflammation in vertebrates.¹⁷⁶ A *Gordonia* sp. isolate from a different *Conus* species produces a number of circumycin derivatives that show neuroactivity or broad antimicrobial activity.¹⁷⁷ Also, another *Streptomyces* sp. isolated from the recently discovered turrid gastropod *Lienardia totopotens* produces the antibacterial and cytotoxic lobophorins 38.¹⁷⁸ However, for the majority of these compounds, evidence for a beneficial effect on the host's fitness is lacking, so the possible mutualistic nature of the associations remains to be established. Unlike the previous cases, nocapryrones 39 are already long known from mollusk secreted mucus. Some are either toxic for various predators or induce escape reactions in conspecifics.¹⁷⁹ Interestingly, the *ncp* PKS gene cluster for three derivatives of this class of compounds, which are secreted in the mucus of *C. tribblei* and *C. rolani*, were identified in the bacterium *Nocardiopsis alba*.¹⁸⁰

Wood boring bivalve mollusks in the family Teredinidae (“shipworms”) harbor various symbionts in their gills¹⁸¹ and



gastric caeca,¹⁸² that are known to contribute to the host's carbon metabolism by providing cellulose degrading enzymes.¹⁸³ Furthermore, *Teredinibacter turnerae*, found in the gills of the shipworms, seems to be involved in structuring the community of shipworm-associated bacteria. The sequenced genome contains three PKS and six NRPS gene clusters,¹⁸⁴ one of which encodes for the biosynthesis of tartrolons **40** that occur across all shipworm tissues.¹⁸⁵ While the two isolated tartrolons (one as the free form and the other chelating a boron atom) show no activity against eukaryotic cells or the shipworm's native microbial community, they inhibit the growth of *B. subtilis* and marine pathogenic bacteria.¹⁸⁵

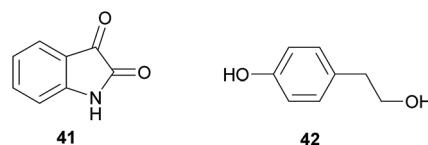


Cephalopods. The association of the Hawaiian bobtail squid, *Euprymna scolopes*, with the luminescent bacterium *Vibrio fischeri* is undoubtedly one of the best-studied symbiotic model systems, particularly with regard to the molecular basis of host-symbiont interactions mediating the specific establishment and maintenance of the association.^{186,187} The squid carries *V. fischeri* bacteria in a specialized light organ that helps to disguise the squid from predators and prey through 'counter-illumination'.¹⁸⁸ While not a chemical defense *per se*, the symbionts' light emission is a by-product of a biochemical

reaction in which luciferase catalyzes the reaction between an aliphatic aldehyde substrate (reduced flavin mononucleotide) and molecular oxygen.¹⁸⁹ The association with bioluminescent *V. fischeri* is not confined to *E. scolopes*, but also occurs in several other squid as well as fish species.¹⁸⁹

Presumably, symbiotic bacteria also reside within the accessory nidamental gland (ANG) of several squid genera, including *Loligo*, *Sepia* and *Euprymna*. The ANG houses a highly specific bacterial community of α - and γ -Proteobacteria as well as Bacteroidetes, with *Roseobacter* dominating in *Loligo* and *Sepia* species, and *Phaeobacter* in *Euprymna scolopes*.^{190–192} Sexual maturity in these squids is accompanied by the enrichment of symbiont-synthesized carotenoids, although the exact function of those carotenoids remains unknown. It is also uncertain whether a specific carotenoid-producing physiological stage in the bacteria is required for maturity of the females, or whether maturing females induce the bacteria to produce the carotenoids.^{193–195} During oviposition, the bacteria are transferred from the ANG to the eggs and likely serve as an inoculum resulting in dense bacterial populations within the egg capsules.¹⁹² However, no symbiotic bacteria were found on hatched embryos, indicating that the squids acquire their symbiotic microbiota *de novo* from the environment in every generation.^{196,197} Extracts from the ANG contained high amounts of unsaturated fatty acids and exhibited antimicrobial activity, as did egg extracts and bacterial isolates.^{198–200} In addition to active inhibition, the secreted bacteria might provide colonization resistance of the egg capsules by depleting nutritional resources.²⁰¹

2.1.6 Crustaceans. The best studied protective symbioses in crustaceans are among the earliest known examples of defensive alliances in animals. Gil-Turnes and colleagues were able to show that symbiont-produced chemicals protect embryos of both the shrimp *Palaemon macrodactylus* and the lobster *Homarus americanus* against phycomycetous fungi, including the pathogen *Lagenidium callinectes*.^{202,203} In *P. macrodactylus*, the symbiont was identified as an *Alteromonas* species that produces 2,3-indolinedione (istatine) **41**. This compound restored the protective effect in embryos that had previously been experimentally depleted of their symbionts.²⁰² In *H. americanus*, epibiotic Gram-negative bacteria protect the embryos from pathogenic fungi by producing 4-hydroxyphenethyl alcohol (tyrosol) **42**,²⁰³ which has also been described as a protectant of fungal plant symbionts against phytopathogens.^{204,205}



A symbiosis with both nutritional and defensive benefits occurs in marine isopods of the genus *Santia*.²⁰⁶ These crustaceans harbor a photosynthetically active episymbiotic community comprising Cyanobacteria of the genus *Synechocystis*. In order to provide their symbionts with suitable conditions for

photosynthesis, the isopods occupy exposed areas with sufficient sunlight. Two investigated populations or species (the actual status has not been determined) showed remarkable differences regarding their symbionts and the defense against predators. One population, whose large epibiotic *Synechocystis* symbionts confer a characteristic red coloration to their hosts, is usually ignored or rejected by predatory fish, while the other population carrying an inconspicuous brown *Synechocystis* strain is readily consumed.²⁰⁶ The symbiont seems to be vertically transmitted from mothers to newly emerged juveniles and – in addition to the difference in color – shows morphological strain variation across the two host populations.²⁰⁶ When experimentally removed from their surface, the isopods were equally consumed by fish. Methanol extracts of isopods with their red symbionts partially restored protection, indicating that symbiont-produced bioactive metabolites are involved in their host's defense against predators.²⁰⁶ However, the chemical basis of the protective effect remains to be elucidated.

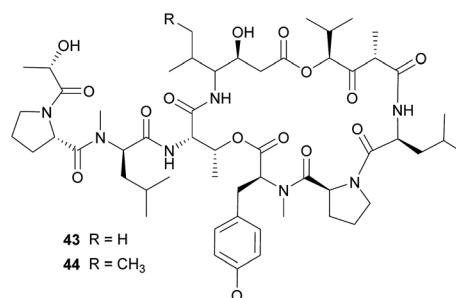
2.1.7 Tunicates. Tunicates are sessile or pelagic filter feeders that occur worldwide in marine environments. Among tunicates, the colonial ascidians are an especially rich source of secondary metabolites, many of which are believed to originate from microbial associates. Comprehensive reviews on ascidian natural products including many compounds of likely microbial origin – based on the structural similarity to metabolites of free-living bacteria – have been published previously.^{207–209} As for the other marine invertebrates, we will focus here on cases with experimental evidence for symbiont-produced secondary metabolites that are putatively involved in the defense of the host against antagonists. One group of compounds, the tambjamines, is present across diverse marine animals including tunicates, bryozoans, and mollusks. These compounds have already been discussed collectively in the section on bryozoa.

Didemnid ascidians. Colonial ascidians of the family Didemnidae have been studied extensively as producers of a rich repertoire of bioactive secondary metabolites, many of which are produced by microbial symbionts.^{12,207–210} We will focus here on five groups of compounds, for which a symbiotic origin has been demonstrated or is at least very likely: the cyanobactins (including patellamides, trunkamide, lissoclinamides, patellins, and many others), didemnins, patellazoles, bistramides, and palmerolides.

Many didemnid ascidians live in an obligate symbiosis with vertically transmitted Cyanobacteria of the genera *Prochloron* or *Synechocystis*.^{211–213} *Prochloron* symbionts have been found on the surface and/or in the common cloacal cavity of colonial didemnids such as *Lissoclinum patella*, *L. bistratum*, *L. voeltzkowi*, *L. punctatum*, *Trididemnum cyclops*, *T. clinides*, *Didemnum molle*, and *Diplosoma virens*,^{212,213} while *Synechocystis* is associated with ascidians of the genus *Trididemnum*.²¹¹ Through photosynthesis, the cyanobacterial symbionts make a major contribution to the hosts' energy demands, and they play an important role in the recycling of nitrogenous compounds.²¹⁴ In addition to these nutritional contributions, the symbionts have been implicated in the

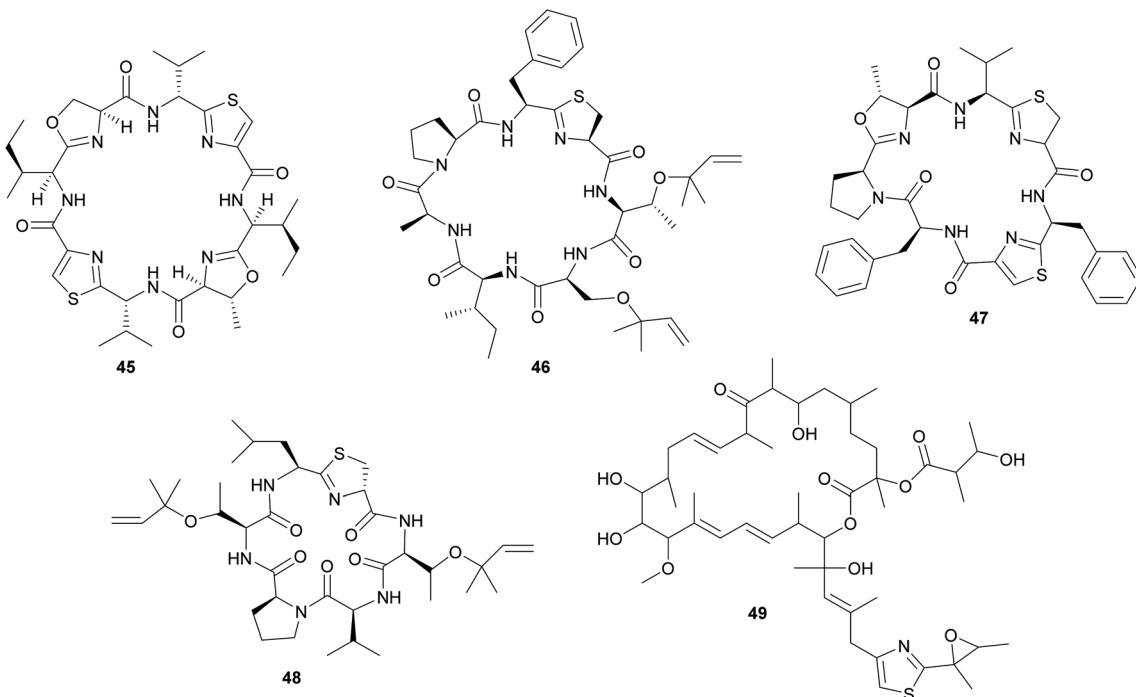
production of bioactive secondary metabolites that play a role in the defense of the host.^{207,209}

The didemnins, potent antiviral and antitumor cyclic peptides, were first isolated from the Caribbean ascidian *Trididemnum solidum*,^{215,216} which hosts the cyanobacterial symbiont *Synechocystis trididemni*.²¹¹ Behavioral assays demonstrated that *T. solidum* larvae are distasteful to predatory fish species, and two isolated didemnins (didemnin B 43 and nor-didemnin B 44) significantly deterred predators when applied at naturally occurring concentrations.^{158,217,218} Since didemnin B was also found in a phylogenetically distant ascidian and shows structural similarity to metabolites from free-living cyanobacteria, it was suspected to be of symbiotic origin in *T. solidum*.²⁰⁹ While there is to our knowledge no direct evidence supporting a cyanobacterial source of the didemnins in *T. solidum*, the recent discovery of a plasmid-localized didemnin biosynthetic gene cluster in the free-living α -Proteobacteria *Tistrella mobilis* and *T. bauzanensis*^{219,220} raises the possibility that *S. trididemni* has acquired the potential for didemnin biosynthesis via horizontal gene transfer.



In analogy to the didemnins, it was long suspected that another group of cyclic peptides in ascidians, the cyanobactins (including the patellamides 45, trunkamide 46, lissoclinamides 47, patellins 48, and many others), are produced by cyanobacterial symbionts. This hypothesis was based on the co-occurrence of *Prochloron* symbionts and cyanobactins in several didemnid ascidians, particularly those of the genus *Lissoclinum*.²²¹ Indeed, more recent studies identified the *Prochloron* gene cluster responsible for patellamide production (*pat*) and demonstrated its activity by heterologous expression in *Escherichia coli*.^{222–224} Notably, the discovery of the *pat* gene cluster²²⁴ represents one of the first examples to elucidate the biosynthetic pathway for the production of a symbiont-produced defensive metabolite in a marine system by whole genome sequencing. Interestingly, the *pat* cluster is highly conserved across *Prochloron* symbionts of diverse hosts, but hypervariable cassettes in the precursor peptide result in the large diversity of cyclic peptides.²²² Analogously, the *tru* cluster is responsible for the synthesis of diverse patellins, including trunkamide, and it shares a high degree of similarity with the *pat* genes, except for the region that is likely involved in the prenylation of the patellins.²²⁵ Thus, the variability of the cyanobactin gene clusters confers the metabolic versatility to the ascidian symbiosis as well as to free-living cyanobacterial relatives.²²⁵ Even though the fitness benefits of symbiont-mediated cyanobactin



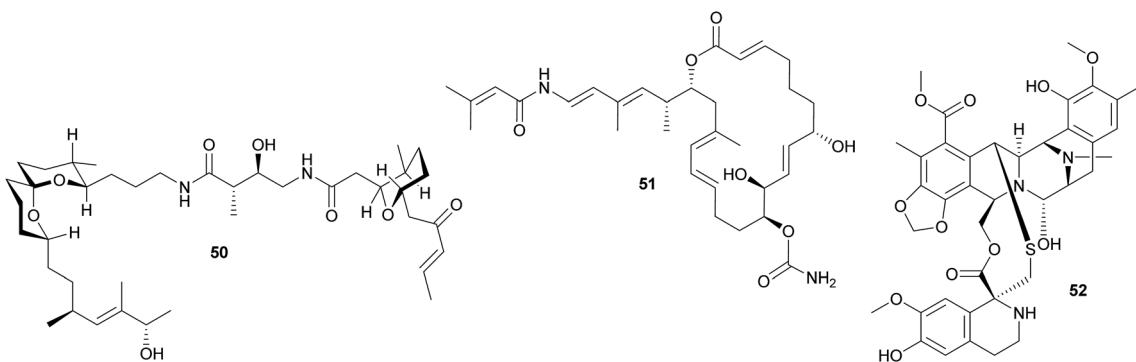


production for the host have not been demonstrated, their abundance in ascidian tissues and toxicity against eukaryotic cells strongly imply a protective function.^{11,209}

In addition to the cyanobactins, individuals of the ascidian *Lissoclinum patella* are occasionally found to contain the toxic patellazoles **49**, a group of thiazole-containing polyketides.^{226,227} Metagenomic approaches towards the identification of the patellazole-producing organisms excluded the *Prochloron* symbionts as possible candidates and rather pointed to a proteobacterial origin of these secondary metabolites.²²⁸ Subsequent studies verified this by identifying the patellazole gene cluster (*ptz*) in the intracellular α -proteobacterial symbiont '*Candidatus Endolissoclinum faulkneri*'.²²⁹ Interestingly, apart from the *trans*-AT PKS gene cluster responsible for patellazole synthesis, the genome of '*Ca. E. faulkneri*' shows clear signs of erosion, with a strongly reduced size and coding density, an AT-biased nucleotide composition, and the loss of regulatory genes

involved in DNA replication and cell division.²²⁹ Thus, '*Ca. E. faulkneri*' appears to be an obligate defensive mutualist of *L. patella*, similar to the recently discovered '*Candidatus Proftella armatura*' in the asian citrus psyllid, which retained the complete pathway for the putatively defensive compound dia-phorin in an otherwise eroded genome²³⁰ (see 2.2.2). As for the cyanobactins, the role of the patellazoles in the defense of the symbiosis against antagonists still needs to be established.

Other polyketides in didemnid ascidians include the bis-tramides **50** of *Lissoclinum bistratum*,^{231–234} and the palmerolides **51** of *Synoicum adareanum*.²³⁵ While the former were localized to the *Prochloron* symbionts by cell fractionation,²³¹ the evidence for a microbial origin of the latter is limited to the sequencing of bacterial *trans*-AT PKS ketosynthase domain fragments putatively involved in palmerolide synthesis.²³⁵ Finally, it should be noted that metagenomic analyses of *Prochloron* symbionts in *L. patella* revealed further secondary



metabolite gene clusters, which may be involved in the synthesis of as yet unknown bioactive compounds for protection against antagonists.²³⁶

Other ascidians. The intracellular γ -proteobacterial symbiont '*Candidatus Endoecteinascidia frumentensis*' was identified in the mangrove ascidian *Ecteinascidia turbinata* (Perophoridae).^{237,238} The bacteria are probably vertically transmitted, and recent studies identified the core of an NRPS biosynthetic gene cluster that could be tied to the intracellular symbiont through analyses of the codon usage.²³⁹ This cluster is likely responsible for the synthesis of the secondary metabolite ecteinascidin 743 52 (ET-743),²³⁹ a promising anti-cancer agent that is highly toxic to eukaryotic cells and may therefore serve as an anti-predator defense in the ascidian symbiosis.

2.2 Terrestrial invertebrates

2.2.1 Entomopathogenic nematodes. Among the chemically best-studied defensive symbioses are those between entomopathogenic nematodes and their bacterial partners.^{20,240-243} Following the speculation of bioactive compounds produced by bacterial symbionts of *Steinernema* nematodes in 1959,²⁴⁴ and the first identification of symbiont-produced compounds in 1981,²⁴⁵ a steady flow of reports has resulted in the description of more than 40 bioactive metabolites from nematode symbionts.

The two entomopathogenic nematode families *Steinermetidae* and *Heterorhabditidae* are characterized by their obligate association with bacteria in the γ -proteobacterial genera *Xenorhabdus* and *Photobacterium*, respectively. Although some of these symbionts can occur in multiple hosts, most strains are species-specific and essential for growth and reproduction of their nematode hosts.²⁰ Specifically, they assist the nematode in overcoming the immune system of the insect prey, killing it, and protecting the cadaver against microbial and animal competitors.²⁴⁶ To this end, an arsenal of diverse bacterial metabolites do not only repel insect scavengers like ants, but are also active against viruses, con- and hetero-specific bacteria, saprobic fungi, protozoa and nematode competitors. Their defensive chemistry enables the bacteria to essentially monopolize the insect for 1–2 weeks after colonization, which ensures optimal resource use by the nematode-symbiont consortium as well as successful acquisition of the symbiont by the host offspring.²⁰ Here we review the protection of the insect cadaver through defensive chemical compounds synthesized by the bacteria, but do not discuss the chemistry involved in killing the insect host, which is an offensive rather than defensive symbiont-provided benefit and has been reviewed extensively elsewhere.^{20,241,243}

All *Steinernema* and *Heterorhabditis* nematodes go through an infective free-living juvenile phase, during which they carry the bacterial symbionts in their intestinal tract. After location of a suitable prey by active search or ambushing, the nematode enters the insect through the respiratory or digestive system, penetrates the hemocoel and releases the bacterial symbionts.²⁴⁷ The host insect is typically killed 24–48 hours after infection, which is when the bacteria reach high abundances.

Most defensive compounds are produced by the bacteria during the following post-exponential phase of growth. For both nematodes and their symbionts, successful colonization of the insect host is crucial, as nematodes cannot re-emerge from an insect after infection and thus have only a single chance to colonize a host.²⁴⁷ This may explain why both *Xenorhabdus* and *Photobacterium* independently evolved extraordinarily effective insect-killing and carcass-defending abilities.²⁴⁸ However, although functionally similar by conferring protection against the same enemies, the defensive metabolites of both groups are structurally very different.

Broad-spectrum antibiotic activity of metabolites from the bacterial symbionts of nematodes against Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi was already demonstrated in the 1980's, long before the chemical nature of most of the antibiotic substances was known.²⁴⁹⁻²⁵¹ Subsequently, researchers attributed these antimicrobial effects to a series of (i) highly specific proteinaceous bacteriocins, *i.e.* lumicins²⁵² and photorhabdins²⁵³ from *Photobacterium* spp., and xenorhabdins²⁵⁴⁻²⁵⁶ from *Xenorhabdus* spp.; (ii) broad-spectrum antibacterial and antifungal compounds, including isopropylstilbenes,^{245,257,258} antraquinones,^{242,259} and a carbapenem²⁶⁰ from *Photobacterium* spp., as well as fabclavines,²⁶¹ xenorhabdins,^{262,263} xenorxides,²⁴² and nematophins,^{245,264} from *Xenorhabdus* spp.; and (iii) narrow-spectrum anti-Gram-positive xenobactin,²⁶⁵ xenematinide²⁶⁶ and xenocoumacins,²⁶⁷ and anti-Gram-negative benzylideneacetone²⁶⁸ from *Xenorhabdus* spp. Additionally, several other metabolites including xenoamicin,²⁶⁵ taxillaids,²⁶⁹ cyclohexandione,²⁷⁰ chaiyaphumine²⁷¹ and szen-tiamide^{272,273} with activity against human-disease causing protozoa (*Plasmodium falciparum*, *Trypanosoma brucei*) have been identified. While it is possible that these compounds defend the nematodes against competitors within the insect cadaver, direct evidence for their ecological role is thus far lacking. Finally, both *Photobacterium* and *Xenorhabdus* spp. produce rhabduscin 53 and chitinases, both with a dual function – the promotion of prey killing/digestion and defense against fungal competitors²⁷⁴⁻²⁷⁶ – and small molecules that deter animal scavengers.²⁰ In the following paragraphs, we will expand on the nature and function of the defensive compounds, with a focus on the bacteriocins and the anti-bacterial small molecules.

Bacteriocins are killer proteins used by bacteria to defend themselves against closely related competitors.^{20,253} In *Photobacterium* and *Xenorhabdus*, three kinds of bacteriocins, the lumicins, photorhabdins and xenorhabdins, have been described. Normally detectable in low quantities, their production is strongly induced when bacterial cells are lysed.²⁵⁵ Xenorhabdins were first described from *X. nematophila* and shown to be active against strains of *Xenorhabdus*, *Photobacterium*, and related sister taxa.²⁵⁶ Likewise, *Photobacterium* spp. synthesize photorhabdins and lumicins.²⁵² The biosynthetic genes for lumicins have been shown to be co-localized with the respective resistance genes, which together are highly diverse between symbiont strains.²⁵³ This likely ensures specificity of the bacteria-nematode partnership, if multiple founder nematodes colonize the same insect. Indeed, assays with different

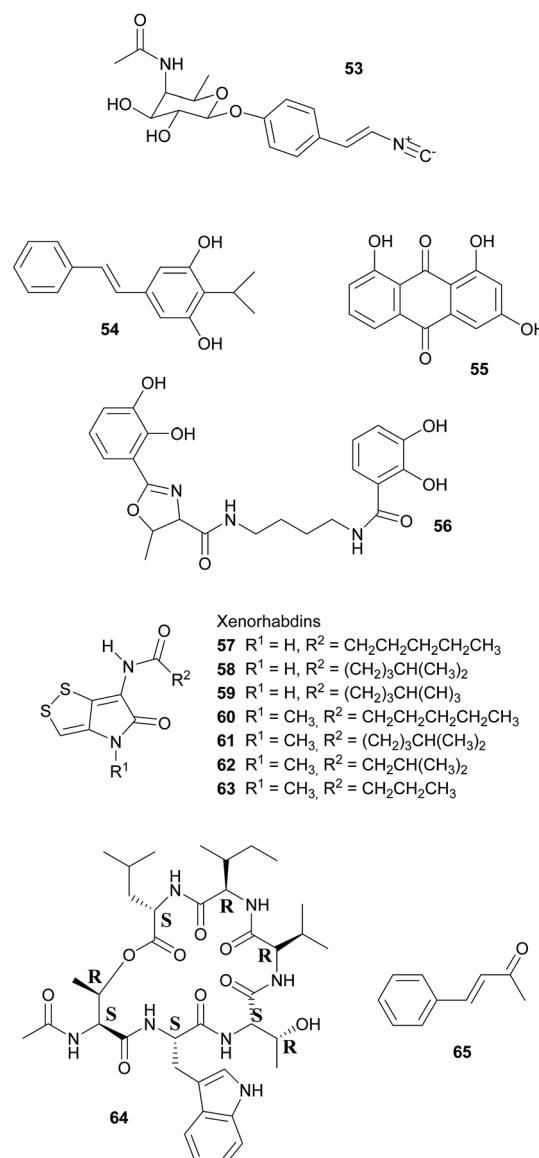


Xenorhabdus strains showed that their bacteriocins are primarily active against conspecific rather than heterospecific strains.²⁵⁴

Defense against unrelated bacterial competitors (e.g. the insect's gut community), fungi and animals is mediated by extracellular, non-proteinaceous small molecules with variable narrow- to broad-spectrum activity.^{20,242,250} Together, these compounds assure that the insect carcass does not putrefy for several weeks until the nematodes disperse.²⁷⁷ In *Photobacteroides*, carbapenem-like molecules, as well as isopropylstilbenes and anthraquinone pigments are mainly responsible for this effect.^{245,258,259,277,278} Carbapenems are β -lactam antibiotics that are best known from Enterobacteria. In *P. luminescens*, a gene cluster responsible for the production of a carbapenem-like molecule with specific activity against Gram-negative bacteria has been identified.²⁷⁷ This strain also synthesizes isopropylstilbene antibiotics that generally suppress bacterial growth by inhibiting RNA synthesis, of which one, 3,5-dihydroxy-4-isopropylstilbene **54**, is also strongly fungicidal, nematicidal and insecticidal.^{20,251,258,279} This compound is probably of crucial importance for defense, as large amounts are synthesized by the symbionts from days 2–5 after colonization of the insect prey throughout the following weeks until the cadaver is abandoned.^{257,280} Anthraquinone pigments **55** produced by a type II PKS²⁸¹ are responsible for the red color of insects killed by *Photobacteroides*.²⁷⁸ Several of these pigments have been isolated from the bacterial symbionts, which is remarkable as these compounds normally occur only in higher plants, lichens and fungi.^{242,258,259,282} Anthraquinone derivatives have antibiotic and nematicidal properties, thus indicating a defensive function.^{241,259} This is also assumed for photobactin **56**, a catechol siderophore from *P. luminescens*, although its exact function remains to be determined.²⁶⁰

Xenorhabdus spp. synthesize a different array of bioactive small molecules, including xenorhabdins, xenorxides, fabclavines, indole derivatives, xenocoumacins, xenemotide, xenobactin, and benzylideneacetone.^{20,241,251,261} Xenorhabdins **57–63**, the largest group among these, are dithiopyrrolone derivatives (compounds also known from *Streptomyces*) with suppression of Gram-positive bacteria and fungi by inhibition of RNA and protein synthesis.^{251,262,283} In many cases, several xenorhabdins are produced by the same bacterial strain, and as some are also insecticidal, they fulfill a double function by killing the insect and preserving/protecting the carcass against competitors.²⁴² Oxidized xenorhabdins, the so-called xenorxides, are broad-spectrum defensive metabolites against both Gram-positive and Gram-negative bacteria as well as fungi.²⁴² Four types of fabclavines have been identified from *X. budapestensis* and *X. szentirmaii* and are active against a broad spectrum of bacteria, fungi and protozoa.²⁶¹ Indole derivatives, like nematophin from *X. nematophilus*,²⁶⁴ likewise have a broad activity spectrum and are comparable to isopropylstilbenes in terms of their mode of action.^{245,251} By contrast, xenocoumacins, xenemotide and xenobactin inhibit Gram-positive bacteria,^{241,266,267} with xenobactin **64**, a hexapeptide, also being active against protozoa.²⁶⁵

Complementary to xenobactin, benzylideneacetone (*trans*-4-phenyl-3-buten-2-one) **65** specifically suppresses growth of Gram-negative bacteria.²⁶⁸



Ant-deterrent factors (ADFs) are small extracellular molecules that protect insect cadavers infected by both the *Heterorhabditis-Photobacteroides* and the *Steinernema-Xenorhabdus* symbiotic complexes against scavenging arthropods, particularly ants.²⁸⁴ ADF repellency depends on the strain and age of the bacteria and the ant species tested,²⁸⁵ with the *Heterorhabditis-Photobacteroides* association being the better protected complex.²⁸⁴ To date, however, the chemicals responsible for ant-deterrent effects have not been identified.

2.2.2 Insects. The exploration of insect-microbe interactions has shed light on many important aspects of the ecology and evolution of symbiotic associations.²⁸⁶ While research in this area has traditionally focused on interactions with a nutritional basis, more and more defensive symbioses are being discovered that significantly expand our understanding of the



prevalence, diversity, relevance, and mechanistic basis of protective symbioses in general.^{15–17,19}

Symbiotic antipredator defense. Natural enemies of insects include predators, parasitoids, and microbial pathogens, as well as nematodes and viruses. Examples of symbiont-conferred protection have been discovered against all of these antagonists. However, an anti-predator function has so far only been demonstrated for the association between rove beetles (*Paederus* spp.) and a close relative of *Pseudomonas aeruginosa*.²⁸⁷ These γ -Proteobacteria are capable of producing pederin 14, a potent toxin that is synthesized using enzymes of the *trans*-AT PKS family and resembles onnamide-type natural products found in sponges (see 2.1.1).¹⁰ The ecological relevance of this defensive compound is supported by the observation that beetle larvae hatching from pederin-containing eggs experience reduced predation from wolf spiders as compared to pederin-free larvae.²⁸⁸ Interestingly, there is evidence that the symbionts have horizontally acquired the genes required for the production of pederin, suggesting that mobile genetic elements may explain the widespread capability of producing highly similar bioactive metabolites in a range of phylogenetically distant symbiotic partners.²⁸⁹ In fact, a recently described case of a probable defensive symbiosis between the asian citrus psyllid and the β -Proteobacterium '*Candidatus Profftella armatura*' further supports this hypothesis.²³⁰ The highly reduced genome of the bacterial symbiont encodes the complete gene cluster for the synthesis of diaphorin 66, a toxin that is structurally very similar to onnamides and pederin. Thus, the gene cluster might have been transferred to or from the rove beetle symbiont. Notably, '*Ca. P. armatura*' and the production of diaphorin are observed without exception among individuals within and across geographically distant psyllid populations. This high prevalence suggests an obligate mutualistic association and diverges from the usually intermediate infection frequencies described for the majority of defensive symbioses.²³⁰

Symbiont-mediated protection against parasitoids, fungi, and nematodes in aphids and fruit flies. One of the earliest known cases of symbiont-mediated defense in insects involves the protection against parasitoid wasps in aphids. In the aphid *Acyrtosiphon pisum*, *Hamiltonella defensa* bacteria confer protection against the wasp *Aphidius ervi*.²⁹⁰ However, this defensive action depends on the presence of the bacteriophage APSE (*A. pisum* secondary endosymbiont) in the symbiont, which encodes toxins that are likely candidates for the defensive activity. Concordantly, three APSE variants that confer different degrees of protection carry distinct toxin genes, encoding for the production of shiga toxin, cytolethal distending toxin, and YD-repeat toxin, respectively.¹⁹ *H. defensa* also protects other aphid species against parasitoids, *i.e.* *Aphis fabae* and likely also *Aphis craccivora*,¹⁹ although the same defense mechanism might not operate in other host species like the grain aphid (*Sitobion avenae*). Interestingly, however, an alternative strategy for protection by this secondary symbiont in *S. avenae* is still likely, as parasitoid wasps preferentially oviposit in *H. defensa*-free eggs.²⁹¹ In addition to *Hamiltonella*, the secondary symbionts

Regiella insecticola and *Serratia symbiotica* can provide resistance against parasitic wasps in aphids. These cases, however, are not bacteriophage-mediated, suggesting alternative strategies for protection.¹⁹ Symbiont-conferred protection against parasitoids has also been reported in other insects, *e.g.* *Drosophila hydei*, in which *Spiroplasma* can defend the larvae against the wasp *Leptopilina heteroma*.²⁹² Additionally, some studies suggested that *Arsenophonus* in psyllids²⁹³ and *Wolbachia* in the weevil *Hypera postica*²⁹⁴ can similarly enhance the resistance of the host against parasitoids. In both cases, however, further experimental evidence is required to confirm the existence of a defensive symbiosis and to elucidate the mechanistic basis of protection.

The role of facultative symbionts in the defense against pathogenic fungi has also been studied in aphids. While *Hamiltonella* appears to have no effect on aphid susceptibility to fungal pathogens, at least four other secondary symbionts of the pea aphid (*Rickettsia*, *Rickettsiella*, *Regiella* and *Spiroplasma*) are capable of increasing survival chances of aphids exposed to the entomopathogen *Pandora neoaphidis*.^{295,296} In addition, the presence of these symbionts also reduces sporulation efficiency of the fungus in those cases where the pathogen kills the aphid. This may be adaptive for the aphids by reducing the spread of infection among groups of clonal aphids, thereby enhancing the inclusive fitness of the clone.^{295,296} However, the mechanistic basis of the symbiont-mediated protection against pathogenic fungi in aphids remains to be elucidated.

Little is known about symbiont-mediated defense against nematodes, with only one reported case in *Drosophila neotestacea*, in which *Spiroplasma* symbionts significantly enhance the reproductive output of flies that are parasitized by the nematode *Howardula aoronymphium* both in laboratory and wild populations.^{297,298} The presence of *Spiroplasma* results in reduced growth of the adult female nematodes within the host and ultimately in impaired fertility of the parasite as well as a reduced virulence against the host.²⁹⁸ Although the mechanistic basis of *Spiroplasma*'s protective activity is not yet fully known, transcriptional profiling suggests the production of toxins that may inactivate the ribosomes of parasitic nematodes.²⁹⁷

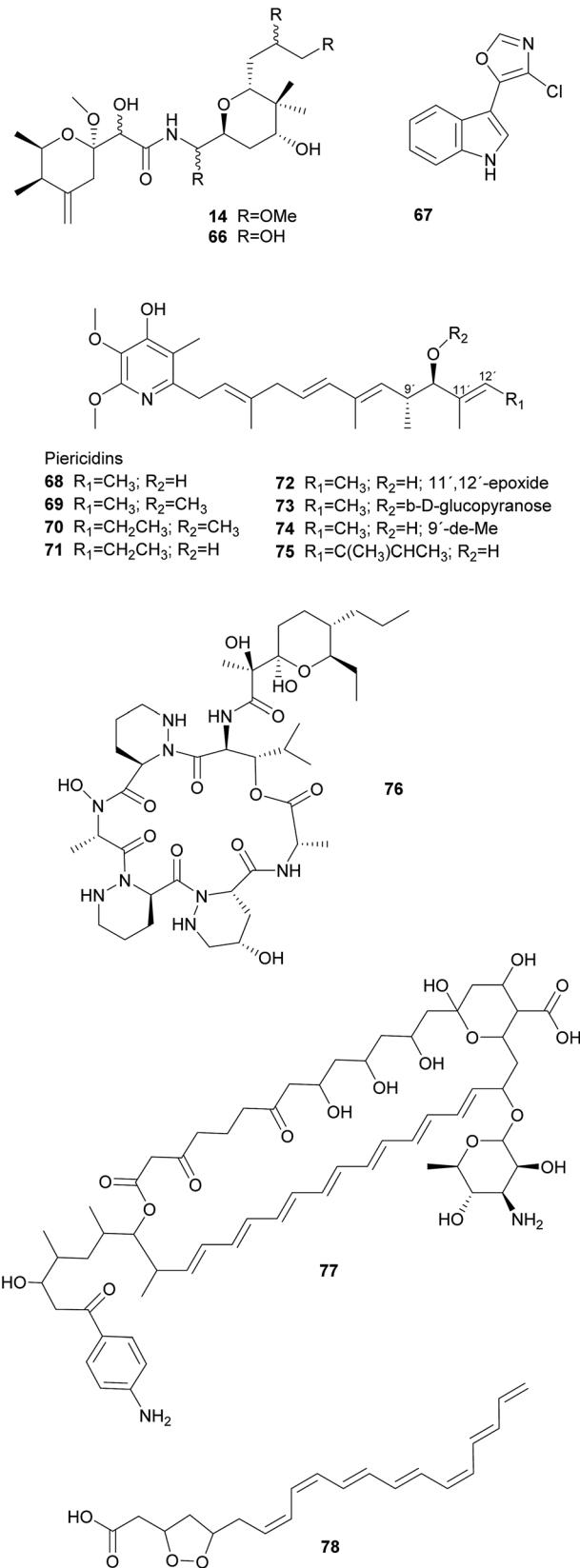
Protection against pathogens: Actinobacteria as defensive symbionts. Actinobacteria are of great importance for humans – most of our antibiotics today originate from these bacteria, specifically from members in the genus *Streptomyces*. But also other organisms make use of Actinobacteria and their defensive capabilities through protective symbioses.¹⁶ Interestingly, however, it remains a matter of debate whether antibiotics primarily evolved to defend their producers in nature. Instead, their immense diversity and occurrence in often sub-inhibitory concentrations in nature suggest that they may be used as signaling molecules, which modulate gene expression in the recipient organisms at low dosage.²⁹⁹ Thus, an increase in antibiotic production may have evolved secondarily in interactions with other organisms.³⁰⁰ Independent of their original function, antibiotics of Actinobacteria play a crucial role for the protection of several animals against pathogens. In insects, their roles are best understood in beewolf digger wasps and fungus-growing ants.



Solitary wasps of the tribe Philanthini within the Crabronidae ("beewolves") dig underground nests in soil, mass provision individual progeny in brood cells with insect prey and engage in a defensive symbiosis with '*Candidatus Streptomyces philanthi*' bacteria to protect their larvae against mold fungi from the surrounding soil.^{301–305} Uniquely, bacterial symbionts are applied to the brood chambers from antennal reservoirs of the females.³⁰⁶ *S. philanthi* strains display their protective abilities after incorporation into the cocoon by the larvae.³⁰² For the following two weeks, the symbionts produce a cocktail of streptochlorin **67** and eight piericidin **68–75** derivatives, which are distributed all over the surface of the cocoon and protect the immature wasp against opportunistic pathogens until its emergence several months later.^{302,307–309}

Like beewolves, fungus-farming ants nest in the soil and are confronted with environmental pathogens that threaten their brood and the fungal cultivars. Moreover, leaf-cutter ant gardens are challenged by specialized *Escovopsis* fungal pathogens and endophytic fungi, brought in by the ants with the plant substrate supplying the cultivars with nutrition.³¹⁰ To counteract these threats, ant workers combine continuous fungus-weeding and -tending behavior with the application of antimicrobial secretions from their metapleural glands³¹¹ as well as antimicrobials produced by symbiotic Actinobacteria.^{312,313} These Actinobacteria comprise vertically and occasionally horizontally transmitted *Pseudonocardia* symbionts^{310,314–316} as well as environmentally acquired members of the genera *Streptomyces* and *Amycolatopsis*.^{317–319} The *Pseudonocardia* symbionts defend the fungus garden against the specialized *Escovopsis* cultivar pathogens, by producing dentigerumycin **76** and five angucyclines (in a *Pseudonocardia* isolate from *Apterostigma dentigerum*),^{320,321} or a nystatin-like compound (in a *Pseudonocardia* isolate from *Acromyrmex octospinosus*), respectively.³¹⁷ *Streptomyces* and *Amycolatopsis*, on the other hand, produce candidin **77** and antimycin with broad-spectrum activities against fungal competitors of the cultivars (e.g. endophytic fungi in the leaf substrate).^{322,323} Furthermore, *Streptomyces* in small crypts on the body surface of adult ants may also protect the ants themselves against pathogens by producing actinomycins and valinomycin.^{317,323,324}

Apart from leaf-cutter ants, other Myrmicinae ants in the genus *Allomerus* possibly make use of *Streptomyces* and *Amycolatopsis* as defensive symbionts. These ants farm *Chaetothyriales* mould fungi within their ant-plant nests, but instead of food, these fungi give structure to the ant galleries.³²⁵ The galleries are used to trap and catch insect prey for nutrition.³²⁶ Several Actinobacteria showing antifungal activities were isolated from the cuticle of *Allomerus* ants, and these bacteria were hypothesized to play a role in the defense of the galleries against fungal pathogens and competitors.³²⁷ The examples of attine and *Allomerus* ants indicate that defensive secondary metabolites of Actinobacteria can play an important role in ant fungiculture. Given that only a handful of ant symbionts has been studied, it is likely that many more antibiotics may be isolated from such symbioses.³²⁸



Compared to fungus-growing ants, much less is known about the role of defensive bacterial symbionts in the gardens of the other fungus-farming insect groups: termites and bark/ambrosia beetles.³²⁹ Fungus-farming termites occupy the same

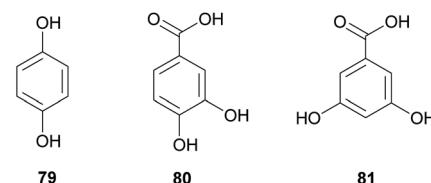


ecological niche in the Paleotropics as leaf-cutter ants in the New World. As in leaf-cutter ants, Actinobacteria have been isolated from termite nests, but *in vitro* assays showed anti-fungal activity against *Pseudoxylaria* and *Trichoderma* fungal competitors as well as the termites' *Termitomyces* cultivar.³³⁰ This indicates that antifungals are either applied in a targeted fashion by the termites, or unspecific Actinobacteria were isolated that do not act as defensive symbionts in fungus-farming termites. The activity of the two microtermolides A and B that were identified from termite-associated *Streptomyces* spp. was not tested.³³¹ Instead, it is possible that fungus-farming termites are associated with a *Bacillus* sp. as a defensive symbiont. This strain produces bacillaene A, which specifically inhibits several cultivar competitors *in vitro*.³³²

As in termites, comparatively little is known about the possible role of Actinobacteria in the defense of bark and ambrosia beetle nests. These beetles bore tunnels in the phloem (bark beetles) or xylem (ambrosia beetles), on the walls of which they cultivate food fungi in the orders *Microascales* and *Ophiostomatales*.³³³ Females transmit spores of their cultivars to new nests in highly specialized organs called mycetangia.³³⁴ As beetles typically nest in recently dead trees, cultivars are usually confronted with competition from other wood-colonizing fungi. Actinobacterial symbionts are typically isolated in very low abundance from beetles and their nests. In a study on *Dendroctonus frontalis* bark beetles, however, Scott *et al.*³³⁵ found *Streptomyces thermosacchari* to be present in the beetle's mycetangia as well as on the cultivars. These bacteria specifically inhibited the growth of *Ophiostoma minus*, a prevalent antagonist of the beetles, by producing the antifungal metabolite mycangimycin as well as other compounds that were not identified. *In vitro*, mycangimycin 78 turned out to be 20 times more effective against *O. minus* than against the beetle's cultivar *Entomocorticium* sp. A.^{335,336} Another *Streptomyces* strain displayed no activity in competition assays with associates of *D. frontalis*, but produces frontalamides A and B under certain culture conditions.³³⁷ However, *Streptomyces* are not consistently present in *D. frontalis* nests and are generally isolated at very low frequencies from other North American bark and ambrosia beetles.³³⁸ This underlines the importance of further *in vivo* studies to investigate the relevance of Actinobacteria for bark beetle defense in nature.

Protection against pathogens: gut and nutritional resources. Gut bacteria can play an important role in defense against invading microbial pathogens. In the locust *Schistocerca gregaria*, members of the intestinal microbiota can produce phenolic compounds with antimicrobial properties that have been suggested to derive from the conversion of plant secondary metabolites by microbes. Hydroquinone 79, as well as 3,4-hydroxybenzoic and 3,5-hydroxybenzoic acids 80 and 81, are usually present in the guts and feces of locusts, while absent in insects lacking their normal gut microbiota.³³⁹ Interestingly, the entomopathogenic fungus *Metarrhizium anisopliae* is inhibited by these compounds and fails to invade locust guts when the symbiotic microbiota is present.³⁴⁰ While *Pantoea agglomerans* appears to be responsible for producing at least one of the three antimicrobial phenols found in the locust gut,³³⁹ there is

evidence from *in vitro* experiments that *Klebsiella pneumoniae* and *Enterococcus cloacae* may also contribute to the production of defensive compounds.³⁴⁰ Furthermore, a greater diversity of the bacterial community in the locust gut is associated with improved resistance against pathogens, suggesting that multiple players contribute to the efficient defense.³⁴¹



Besides protecting against direct pathogen colonization in or on the insect body, gut-associated microbes can also contribute to the preservation of nutritional resources, as is the case for bacteria in honeybees and stingless bees, and for yeasts in drosophilids (see below, *Defensive symbioses with fungi*). In bees, a number of lactic acid bacteria including *Lactobacillus* and *Bifidobacterium* frequently occur in propolis and in the honey crop, both of which exhibit antimicrobial properties. These lactic acid bacteria participate in the fermentation and preservation of an essential food source, the beebread. In addition, the bees line their hive with a layer of propolis, which serves as a sterilization mechanism protecting the brood against pathogens.¹⁷ *In vitro*, a set of compounds with antimicrobial properties were produced by lactic acid bacteria isolated from the honey crop of the honeybee *Apis mellifera*, including organic acids (lactic, formic, and acetic acid), hydrogen peroxide, different volatiles (benzene, toluene, octane, ethylbenzene and nonane), 3-OH fatty acids, 2-heptanone and various peptides.³⁴² These substances inhibit a number of bacteria and fungi that are commonly found on bee-visited flowers. However, the strongest inhibitory effects were observed when several different lactic acid bacteria were co-cultivated with the potential pathogens, suggesting a synergistic activity of the microbial consortium.³⁴² Along with the sterilizing effects on resources and the hive, lactic acid bacteria can also enhance survival of honeybee larvae by conferring protection against the American and European foulbrood diseases, caused by *Paenibacillus larvae*³⁴³ and *Melisococcus plutonius*,³⁴⁴ respectively.

In addition to lactic acid bacteria, there are other gut-associated microbes that can play important protective roles in bees, particularly bumblebees. By experimentally manipulating the gut microbiota of the bumblebee *Bombus terrestris*, Koch and colleagues provided evidence that the bacterial community plays a role in reducing infection rates by the trypanosomatid parasite *Crithidia bombi*.³⁴⁵ Furthermore, the abundance of this parasite was shown to correlate negatively with the presence of the gut symbiont *Gilliamella apicola* (γ -Proteobacteria) in natural bumblebee populations.³⁴⁶ These and other studies on the bacterial community in different bees indicate that a balanced and stable microbiota plays a substantial role in bee health by reducing pathogen susceptibility.¹⁷

Similar to the aforementioned gut microbes, bacteria present on the egg surface of house flies are also involved in the

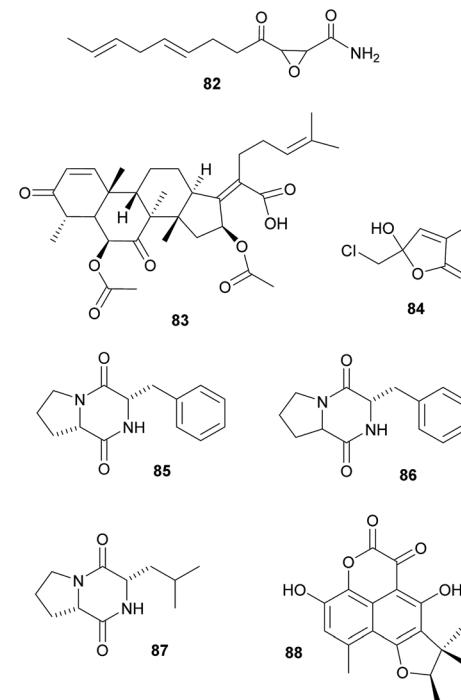


preservation of nutrient provisions. As house flies lay their eggs on manure that their offspring will use for nutrition, the larvae will most likely encounter fungal competitors that have been shown to reduce their chances of reaching adulthood. However, the bacterial community on the surface of the fly eggs can suppress the growth of these fungi on the manure and thereby play an important protective role for the developing larvae.³⁴⁷

Antiviral protection. Viruses can also pose a significant threat to many different insect species. The *Drosophila* C virus (DCV) is common in natural populations of *Drosophila melanogaster* and causes high mortality under laboratory conditions. However, *D. melanogaster* frequently carries the α -Proteobacterium *Wolbachia pipiensis*, which can reduce host susceptibility to DCV and other RNA viruses.^{348,349} These findings have stimulated investigation of other insect–symbiont–virus systems, particularly those involving vectors of human pathogenic viruses.³⁵⁰ In *Culex quinquefasciatus*, the natural occurrence of *Wolbachia* resulted in reduced titers and impaired transmission capacity of West Nile virus.³⁵¹ Although the mechanistic basis underlying this effect is not yet completely understood, significant progress in this area has been made in non-naturally infected vectors of arboviruses and other human parasites. In *Aedes aegypti* mosquitoes, infections with dengue and chikungunya viruses, as well as the malaria-causing protozoan parasite *Plasmodium*, are restrained when the insect is artificially infected with a *Wolbachia* strain from *D. melanogaster*.³⁵² This protective effect is achieved through activation of the host's immune system, which involves stimulating the expression of several Toll-pathway genes as well as defensins and cecropins.⁸ In addition, the presence of symbiont genes potentially involved in the production of antimicrobial compounds might also play a role in inhibiting mosquito pathogens.^{352,353}

Defensive symbioses with fungi: protection of food or the nesting environment. Most of the defensive fungal symbionts of animals have been described from fungus-farming insects, specifically from leaf-cutter ants, fungus-growing termites and bark and ambrosia beetles. All three groups farm their fungi in social societies and show behavioral adaptations to protect their fungi against fungal competitors and pathogens.³²⁹ Furthermore, in addition to defensive actinobacterial symbionts (see above), several studies implicated fungi in the protection of the host or its fungal cultivar against pathogens. Specifically, 'killer yeasts' were shown to inhibit the growth of *Escovopsis* cultivar pathogens within the gardens of *Atta* ants,^{354,355} and *Ogataea pini*, a yeast associated with fungus-growing *Dendroctonus* bark beetles, produces volatiles (ethanol, carbon disulfide and delta-3-carene) that inhibit the growth of *Beauvaria bassiana* entomopathogens.³⁵⁶ Additionally, in some cases the cultivar fungi themselves produce defensive secondary metabolites. Among ambrosia beetles, *Euwallacea validus* is associated with a cultivar (likely an unidentified *Fusarium* sp.) that produces cerulenin 82 and helvolic acid 83 – antibiotics that inhibit the growth of mould fungi *in vitro* and likely also suppress bacterial contaminations.³⁵⁷ Similarly, the *Lepiota* and *Tyridiomyces* cultivars of *Cyphomyrmex* fungus-growing ants produce lepioclinorin 84^{358,359} and several diketopiperazines 85–87,³⁶⁰ respectively, which may be active against bacterial and fungal

pathogens. Likewise, *Leucocoprinus* cultivars of *Atta* ants show *in vitro* suppression of fungi endophytic to the leaves that the ants provision as substrate for the cultivar.³⁶¹ The active secondary metabolites, however, have not been identified yet. The importance of host protection by the cultivars of leaf-cutter ants is supported by the observation that almost all species cover their broods with the cultivar fungus.^{362,363} In fungus-growing termites, unknown myocins produced by the *Termitomyces* cultivars suppress the growth of related strains *in vitro*³⁶⁴ – thereby reducing competition and ensuring the specificity of the symbiosis, analogous to the bacteriocins inhibiting close relatives in the bacterial symbionts of nematodes (see 2.2.1).

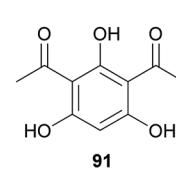
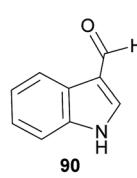
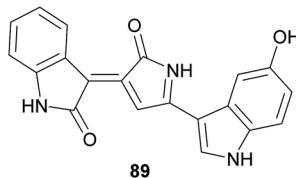


Beyond fungus-farming insects, leaf-rolling weevils in the genus *Euops* (Attelabidae) are associated with polysaccharide-degrading *Penicillium* symbionts that are planted on leaves in which eggs and larvae are rolled. *Penicillium herquei*, the associate of *Euops chinensis*, has been shown to produce (+)-scleroderolide 88 *in vivo*.³⁶⁵ This antibiotic inhibits the growth of several bacterial and fungal pathogens in competition assays on plates and keeps larval cradles free of other microbes^{365,367} In honeybees, *Penicillium* spp., *Aspergillus* spp. and several Mucorales have been shown to decrease colony failure due to chalkbrood disease caused by the fungus *Ascospaera apis*, by competitive exclusion due to the production of antimycotic substances.³⁶⁸ Several mold fungi that are typically regarded as insect pathogens are also potent producers of antimycotic substances³⁶⁹ and are potentially more common defensive symbionts than currently apparent. Finally, *Drosophila melanogaster* fruit flies strongly benefit from their association with yeasts that – in addition to their nutritional role – also inhibit the growth of fungal food competitors, like the noxious mold *Aspergillus nidulans*, by producing as yet unknown secondary metabolites.³⁷⁰

2.3 Vertebrates

In contrast to invertebrates, only a limited number of specific defensive symbioses with microorganisms have been discovered in vertebrates. The relatively complex nature of the vertebrate microbiota as well as the difficulty of manipulative experimentation in this group of organisms severely restricts our current understanding of potential key symbiotic relationships with specific bacteria and fungi. There are, however, a few examples suggesting that different vertebrate groups including fish, amphibians, birds and humans, engage in associations with microbial partners that can reduce their susceptibility to pathogens and predators.

Antipredator defense. In addition to the presence of tetrodotoxin (TTX) 37 in marine invertebrates like nemerteans and mollusks, this highly potent neurotoxin also occurs in fish as well as in amphibians. While the case of bacteria-mediated TTX production seems to be strongly supported in some marine organisms, there is little evidence for a bacterial origin of TTX in frogs, newts and salamanders. Several of these species possess unique analogs of TTX, which are absent or only present in very low quantities in marine animals or in bacteria.¹⁶⁵ Despite the lack of conclusive evidence for the source organism, there are clear indications of its antipredator functions. For example,



some amphibians actively secrete the toxin upon predator encounter.¹⁶⁵ Also, coevolutionary signatures are observed between newts of the genus *Taricha* and garter snakes (*Thamnophis* spp.), where the spatial dynamics of TTX levels in the newt and the corresponding resistance in the predator are suggestive of an evolutionary arms race.^{164,165}

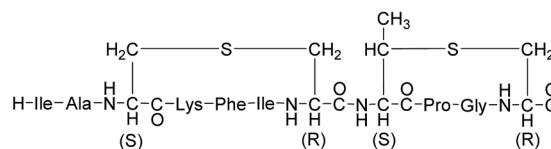
Protection against microbial pathogens on the skin and in the gut. Besides predators, microbial pathogens are a major threat to vertebrates and exert a strong selective pressure on evolving efficient defense mechanisms. Amphibians, which are particularly vulnerable to infectious diseases,³⁷¹ have recently begun to suffer devastating effects from chytridiomycosis caused by the fungus *Batrachochytrium dendrobatidis*. Populations from Australia as well as North, Central and South America have been affected,³⁷² but resistance to the chytrid fungus infection varies both within and across species.^{373,374} One of the hypotheses that have been proposed to explain this variability in resistance is the presence of symbiotic bacteria, mainly on the skin, that can produce compounds capable of inhibiting the pathogen. Concordantly, the β -Proteobacterium *Janthinobacterium lividum*, isolated from the skin of the red-back salamander *Plethodon cinereus*, inhibits the growth of the chytrid fungus *in vitro* by producing indole-3-carboxaldehyde 89 and violacein 90.³⁷³ From the same salamander species, an isolate of *Lysobacter*

gummosus was shown to produce 2,4-diacetylphloroglucinol 91, which also exhibits *in vitro* activity against *B. dendrobatidis*.³⁷⁵ Further work on protective bacteria in *P. cinereus* revealed that 63% of field collected individuals harbor *J. lividum* or other bacteria capable of violacein production.³⁷⁶ Such frequencies are in line with the often facultative nature of defensive symbioses. In addition to its *in vitro* activity, the presence of violacein was shown to be associated with increased survival in *P. cinereus*³⁷⁶ as well as in the frog *Rana muscosa*.^{377,378} These results open the possibility for applying bacterial violacein-producers directly on infected amphibians or their natural environment in order to mitigate the effects of the pathogen.^{373,374} An interesting aspect of this defensive symbiosis is the potential synergy between compounds produced by the bacteria and AMPs from the host, as demonstrated *in vitro* for the inhibitory effect of 2,4-diacetylphloroglucinol and a mixture of AMPs from *R. muscosa* against *B. dendrobatidis*.³⁷² Furthermore, co-cultures of four different bacterial isolates from the red-back salamander including *Janthinobacterium* sp. resulted in synergistic inhibition of *B. dendrobatidis*.³⁷⁹ Thus, the pathogen defense of amphibians likely relies on a combined protective effect of the bacterial community and the host's immune response.

As in amphibians, the skin is also a potential entry gate for pathogens in other vertebrates including humans. In fact, human skin is one of the main habitats accommodating microbial partners. The variety of physicochemical conditions on the skin in terms of temperature, humidity, oiliness, and oxygen availability contribute to the high bacterial diversity on its surface. One of the main constituents on the healthy human skin is *Staphylococcus epidermidis*, a bacterium capable of producing a number of AMPs, including epidermin 92, Pep5 and epilancin K7, which are classified as lantibiotics owing to the presence of lanthionine and/or methyllanthionine in their structures.^{380,381} Both are unusual thioether amino acids,³⁸² which account for the multiple rings in the structure of lantibiotics and are considered essential for their antibacterial activity.³⁸¹ However, evidence for the efficacy of lantibiotics against pathogens is limited to *in vitro* studies. Phenol-soluble modulins (PSMs) are a second group of AMPs produced by *S. epidermidis*. In contrast to the lantibiotics, there is *in vivo* evidence for a role of PSMs in the protection of the skin surface, as their inoculation on mouse skin resulted in a significant reduction of the commonly pathogenic group A *Streptococcus*, while it does not affect the presence of *S. epidermidis*.^{381,383} In addition to the direct antimicrobial action, PSMs can also support the host's immune system. Specifically, the application

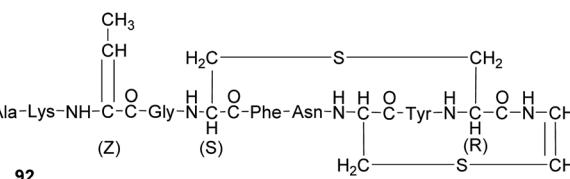


of PSMs to isolated neutrophils resulted in increased eradication of pathogenic bacteria, co-localization with host AMPs and enhancement of extracellular trap formation by the neutrophils.^{381,384} Other mechanisms for pathogen inhibition by *S. epidermidis* include blocking of *quorum sensing* via a thiolactone-containing peptide and its derivatives,^{381,385} as well as inhibition of biofilm formation in the nasal cavity by the production of serine proteases.^{381,386} Despite the numerous examples for a mutualistic potential of *S. epidermidis* on human skin, this symbiosis provides a good example for a context-dependent host–microbe association, as there are also clear indications of the potential for pathogenicity by *S. epidermidis*: an unbalanced microbiota composition possibly associated with an impairment of the host's innate immune response can allow for *S. epidermidis*' access to internal tissues and result in pathogenesis, which is often reflected in severe nosocomial infections.³⁸⁷



among fish-associated LAB has only been identified in *Carnobacterium* strains, which synthesize carnocin UI49, piscicocin V1, and divercin V41. However, the microbes' inhibitory activity has also been attributed to the production of additional compounds such as organic acids, hydrogen peroxide and siderophores.³⁹⁵ In addition to the synthesis of bioactive compounds, there is evidence for the stimulation of the host's innate immune response caused by the LAB, as demonstrated *in vivo* in gilthead seabream individuals infected with *Lactobacillus delbrueckii*.³⁹⁶

Protection against microbial pathogens on bird eggs. Perhaps the most remarkable defensive symbiosis between microbes and vertebrates is that of *Enterococcus* bacteria inhabiting the uropygial gland secretions of hoopoe birds (*Upupa epops*). This system is particularly interesting in terms of the elaborate set of behavioral and morphological adaptations underlying the



Similarly, while *Bacteroides fragilis* is frequently isolated from clinical samples and can be involved in human disease,³⁸⁸ it has also been recognized as a native member of the human gut microbiome and can be beneficial for the host. Its potential for a mutualistic role has been extensively investigated in mice, where there is strong evidence that the production of polysaccharide A by *B. fragilis* prevents intestinal inflammatory disease caused by the opportunistic pathogen *Helicobacter hepaticus*. Although the mechanistic details of this protective effect are not yet fully understood, the abundances of both symbiont and pathogen do not differ between healthy and diseased mice, so an immunomodulatory effect suppressing disease development (*i.e.* absence of detrimental consequences for the host), rather than pathogen clearance, appears to be the key to this bacteria-mediated protection.³⁸⁹ Concordantly, there is a growing body of evidence in humans suggesting that the microbial community, including both bacteria and fungi, plays a crucial role in immunoregulatory processes.^{390–392} In fact, the presence of a healthy microbiota cannot only regulate inflammatory responses but also train the host's immune system and thus confer an indirect defense to the action of pathogens.³⁹¹

Gut pathogens are also a frequent threat for fish populations, which is particularly problematic in the case of intensive fish culture that facilitates the emergence of pathogens as well as the spread of antibiotic resistant bacterial strains.³⁹³ However, several lactic acid bacteria (LAB) such as *Carnobacterium* and *Lactobacillus* strains isolated from different fish species are capable of inhibiting pathogenic bacteria *in vitro*, in particular *Aeromonas salmononicida* and *Vibrio anguillarum*.^{394,395} The production of specific inhibitory substances

evolution and maintenance of the partnership. While male and non-breeding female hoopoes produce a white and odorless uropygial gland secretion with only the occasional presence of few bacteria, secretions from breeding females and nestlings are brown, emit a strong smell and contain high numbers of Firmicutes in the genus *Enterococcus*, mainly *E. faecalis*.³⁹⁷ Interestingly, female birds actively collect the secretions from the uropygial gland and deposit them on both feathers and eggs, the latter of which contain specialized structures to harbor the symbiont-containing secretions.³⁹⁸ Recent studies provide evidence that the bacteria in the secretions play a protective role by preventing the growth of detrimental microbes on the eggs. Notably, there is a positive correlation between hatching success and *Enterococcus* loads in the uropygial secretions and on the egg shells.³⁹⁸ Additionally, *E. faecalis* can inhibit the keratin-degrading action of pathogenic *Bacillus licheniformis*, thus playing a protective role on the feathers of adult hoopoes.³⁹⁹ When first isolated from the gland secretions, *E. faecalis* was shown to produce enterocins, later specified as enterocins MR10 and AS-48, which present *in vitro* inhibitory activity against a range of Gram-positive and Gram-negative bacteria.^{400,401} Later on, it was discovered that also the volatile fraction of the brown secretions contained bacteria-produced compounds with antimicrobial activity, primarily butanoic acid, 2-methyl butanoic acid, 4-methyl pentanoic acid, indole, 3-phenyl propanoic acid and 4-chloroindole.⁴⁰² While individual volatile compounds showed differential efficacy against various bacteria, a mixture resembling the composition of the brown secretions consistently inhibited a broad spectrum of microbes.⁴⁰²



3 Ecological and evolutionary implications

The previous sections illustrate the expanding body of literature describing symbiont-mediated chemical defense across diverse animal taxa inhabiting a broad range of habitats. In this section, we aim to draw some general conclusions on the key ecological and evolutionary factors shaping this diversity and point to novel directions for future research.

3.1 Implications of host ecology and lifestyle

Protective associations between microorganisms and animals are present across a range of phylogenetically distant metazoan

taxa (Fig. 2 and Table S1†). This ubiquity not only reflects the general potential to evolve defensive symbioses but also its occurrence in organisms with markedly different natural histories. However, the identity and diversity of an organism's antagonists strongly depends on its environment, lifestyle and life history, so these factors are likely to have a major impact on the evolution of defensive symbioses. In marine environments, many organisms have a sessile life-stage that must be especially well defended due to its often conspicuous nature, predictability in space and time, and incapability to escape from predators. Thus, chemical protection is widespread among marine invertebrates, especially in taxa with soft bodies that lack structural protection. Concordantly, sponges, bryozoans,

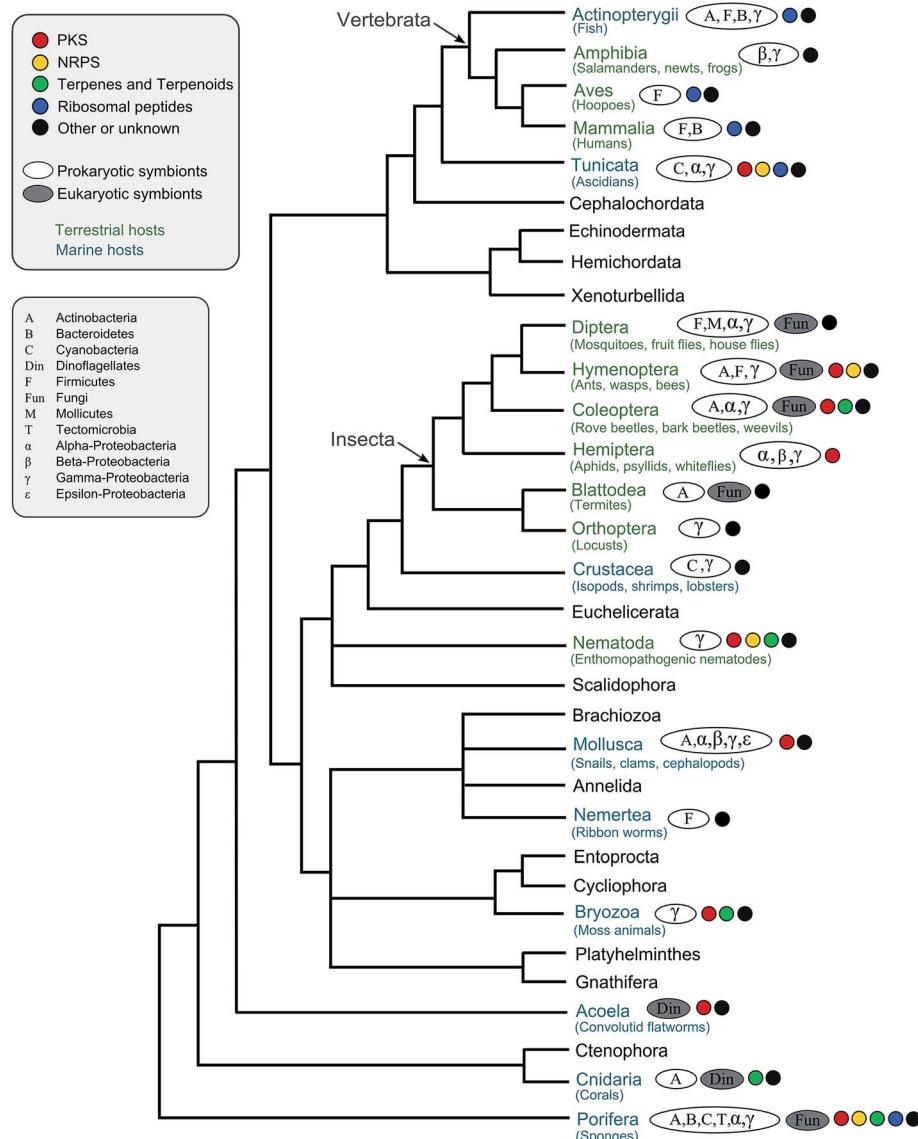


Fig. 2 Cladogram of selected animal groups highlighting those with described defensive microbial symbionts and the corresponding symbiont taxa. Colored circles represent the major biosynthetic pathways reported for symbiont-produced compounds. Branch lengths are not to scale and branch order is adapted from previous phylogenetic analyses for the deep branches,⁴⁰³ hexapods,⁴⁰⁴ and all other bilaterians.^{405,406} Common names in brackets denote selected groups within the respective taxon that harbor known defensive symbionts.



tunicates, some corals and mollusks exhibit an arsenal of defensive chemicals, more and more of which are being discovered to be of symbiotic origin (Table S1†).^{9–11,208} As the sessile lifestyle often goes along with filter feeding, these animals have a high probability to get in contact with microbes, thereby increasing the chances to acquire a beneficial symbiont, while at the same time risking exposure to pathogens.¹² In terrestrial environments, by contrast, sessile animals are exceptionally rare. However, many terrestrial organisms have either a valuable, but immobile resource (nest, food) or immobile developmental stages (eggs, pupae) that are vulnerable to threats from predators, pathogens and parasites due to their predictability in time and space. Food resources that are available *en masse*, like fungus gardens of insects, insect cadavers killed by nematodes or mass-provisioned insect nests, for example, and eggs or brood that are exposed to a hostile environment for a long time (*e.g.* in nests within soil) run a high risk of pathogen infection.^{16,17} While there are a few known examples of a symbiotic protection in these immobile life stages of terrestrial animals,^{302,397} the abundance of such associations in the marine environment suggests that there are likely many more to be discovered.

The transition between mobile and immobile stages during the life cycle of an animal can incur a trade-off between chemical and mechanical defenses, resulting in life-stage specific benefits of defensive symbioses. In bryozoans, for example, the larvae and early post-settlement stages are associated with higher mortality given the increased vulnerability to predation, competition and disease, as well as desiccation, temperature stress, and radiation.¹² Concordantly, the concentration of symbiont-produced defensive bryostatins is particularly high in bryozoan larvae, while adults switch to a predominantly structural defense, with only reproductive tissues being chemically defended by the bryostatins.¹²⁵ Analogously, in a terrestrial system, high concentrations of symbiont-provided antibiotics are only produced during the cocoon stage of the European beewolf and provide protection during the immobile phase of hibernation.³⁰² Thus, the symbionts' investment in chemical defense can vary during the host's life cycle, being complemented by alternative defenses of the host.¹²

Another important aspect of an organism's exposure to pathogens and thus its benefit to engage in defensive microbial symbiosis is its degree of sociality. Social or gregarious behavior in combination with an often high relatedness and low genetic variability among group members makes colonies and aggregations of members of an individual species particularly exploitable by pathogenic microbes.^{407,408} On the other hand, group-living also confers the benefit of social immunity⁴⁰⁷ and can facilitate the transmission and maintenance of beneficial microorganisms, *e.g.* such that provide protection against the colony's pathogens.⁴⁰⁹ Hence, it is not surprising that defensive symbionts are commonly found in social insect colonies and clonal groups of organisms (*e.g.* aphid colonies, fungal monocultures of farming insects), in which social behaviors commonly ensure the spreading of defensive symbionts.^{344,345,410,411}

3.2 Diversity of defensive symbionts and protective chemicals

Across animals, a wide diversity of microbial partners have been identified as defensive symbionts (Fig. 2 and Table S1†). However, particular groups of microbial taxa stand out among these partnerships. Non-surprisingly, the high biosynthetic potential of Actinobacteria renders them effective defenders for a number of animals including sponges,⁵⁴ corals,⁴¹² mollusks,^{176,177,180} and insects.¹⁶ However, other bacterial groups like Proteobacteria and Firmicutes, as well as eukaryotic organisms like dinoflagellates, are also important and widespread players in defensive partnerships (Fig. 2 and Table S1†). Likewise, despite the predominance of PKS- and NRPS-derived compounds in protective symbioses across many taxa, an array of other chemical classes also appear repeatedly, particularly ribosomal peptides and terpenes, but also β -lactam and oligosaccharide antibiotics (Fig. 2 and Table S1†).

3.3 Implications of symbiont localization

For cases in which symbiont-mediated defense relies on the production of a bioactive compound, it has been suggested that an external localization of the microbial partner, *i.e.* on the body surface or in the lining of body cavities of the host, is more effective than an endosymbiotic localization, since the protective substances are readily exposed to potential enemies.¹² In fact, a majority of the microbial symbionts described to play an anti-pathogenic role are located directly on the body surface of the host,^{373,375,381} on specific superficial structures,^{202,302,310,398} within the gut,^{341,343,344,396} or externally on food provisions or the nesting environment.³⁶⁷ In such localizations, defensive symbionts can exert their protective activity before antagonists breach the host's surfaces, thereby reducing detrimental effects to a minimum. In addition, the localization outside of the host's body may reduce potentially harmful side effects that the noxious defensive chemicals may have on the host itself. There are, however, a number of symbionts providing chemical defense that are located within the host's body,^{47,287,298} sometimes even within the host's cells.^{105,229,230} Interestingly, for many of these endosymbionts, the relevant antagonists identified so far are predators, so various types of antagonists may exert different selective pressures on symbiont localization. Yet, a correspondence between symbiont localization and type of enemy remains speculative, given the often limited information on the complete range of relevant antagonists.

3.4 Evolutionary dynamics

As opposed to many intracellular symbionts conferring nutritional benefits, those playing a defensive role are often found at intermediate infection frequencies in host populations.^{47,76,229,287,288,290,296,298,348,349,413} The underlying reasons are probably multiple, a primary one being the context-dependent nature of protective functions. In the absence of relevant antagonists, the host still pays a cost for harboring the symbiotic partners,⁴¹⁴ which can outweigh the benefits and shift the selective balance in favor of symbiont loss. A similar situation



can occur when only certain life stages are protected and thus the relative benefit of carrying the symbionts changes during the life cycle of the host. In the long run, however, balancing selection is most likely responsible for maintaining the partnerships.^{19,290}

Another cause for intermediate infection rates may be the comparatively low degree of intimacy and stability observed in many – but not all²³⁰ – defensive symbioses, which stands in stark contrast to the known intracellular nutritional mutualists that have been associated with some groups of invertebrates for hundreds of millions of years.⁴¹⁵ The often exposed localization of ecto- and extracellular symbionts increases the chances of environmental acquisition of other microbial strains and thus symbiont replacement, resulting in the general lack of strict co-cladogenesis in many systems.⁴¹⁶ Although a vertical transmission route does exist in several examples of defensive animal–bacteria symbioses, occasional horizontal transmission often occurs, *e.g.* in antibiotic-producing actinomycetes of fungus-growing ants^{314,315,417} and beewolves,³⁰³ in the secondary symbionts of aphids,⁴¹⁸ and in the defensive symbionts of bryozoans.¹⁴⁴ Also, in many other marine symbioses, intraspecific variation in both the associated microbial communities and the symbiont-provided defensive chemistry indicate a high probability of symbiont exchange by occasional horizontal transfer or environmental determination.^{47,76,413} The flexible acquisition of defensive symbionts might represent a fast and versatile adaptive process for defense against coevolving antagonists, but also requires sophisticated partner choice mechanisms to ensure the evolutionary stability of the symbiotic partnership.⁴¹⁹

The acquisition of genetic material from unrelated microbes through horizontal gene transfer (HGT) can also mediate rapid chemical changes and thereby facilitate adaptations in defense against coevolving antagonists. While rare in nutritional symbioses, there are several examples of defensive traits that were likely acquired *via* HGT. The *Pseudomonas* symbiont of rove beetles shows strong indications of HGT of the genes for the defense toxin pederin. The striking similarity of the biosynthetic genes of pederin and diaphorin, the toxin produced by the intracellular symbiont of the asian citrus psyllid, '*Ca. Proftella armatura*', suggests that a horizontal transmission event, led to the convergent characteristics in distant lineages.²³⁰ The horizontal acquisition in the *Paederus* symbiont is also supported by the localization of the gene on a genomic island,²⁸⁹ and the occurrence of a similar gene cluster for onnamide biosynthesis in the marine sponge *Theonella swinhonis*.^{73,76,420} Although still lacking direct evidence, cases of potential HGT have also been suspected in several marine symbioses, particularly those of ascidians and bryozoans. The recent discovery of a plasmid-localized didemnin biosynthetic gene cluster in the free-living α -proteobacteria *Tistrella mobilis* and *T. bauzanensis*^{219,220} raises the possibility that the *Synechocystis trididemni* cyanobacterial symbiont of didemnid ascidians has acquired genes for didemnin biosynthesis *via* HGT.¹² Furthermore, the synthesis of bioactive tambjamines by microbial partners of the distant marine groups of ascidians and bryozoans might be explained by horizontal gene transfer.¹²

Nevertheless, our understanding of the prevalence of HGT in defensive symbioses and its impact on the evolutionary dynamics of the host's interaction with antagonists remains rudimentary.

An interesting feature found repeatedly across different animal–microbe protective associations is the simultaneous employment of multiple defensive chemicals, produced by either a single or several symbiotic partners. In the beewolf–*Streptomyces* symbiosis, for example, a “cocktail” of compounds produced by a single symbiotic strain per host species is capable of providing an efficient protection against an array of opportunistic bacterial and fungal pathogens.³⁰⁹ In a similar fashion, animal hosts with strikingly different life history strategies including hoopoe birds,^{400–402} locusts,³³⁹ entomopathogenic nematodes,²⁰ didemnid ascidians,^{222,225} salamanders,³⁷³ and bryozoans,¹²⁴ are associated with a mixture of symbiont-derived compounds likely involved in defense. While in some cases, individual symbionts produce a range of different chemicals, in others – like the didemnid ascidians²²⁸ – multiple bacterial partners are responsible for the production of the defensive compounds. In both cases, effects are often not only complementary, but also synergistic.^{309,342} These combined strategies are in line with the aforementioned versatility, as they are more likely effective against a range of antagonists. Additionally, co-application of several antibiotic substances at the same time is known to strongly hamper the evolution of resistance in the targeted antagonists.⁴²¹

3.5 Outlook: current status, challenges and opportunities of defensive symbiosis research

Compared to nutritional symbioses, defensive ones are generally more difficult to detect,¹⁷ because they are often of facultative nature and their effects are only perceived in the presence of the relevant antagonists, which are in many cases not known, not available under laboratory conditions, and/or not reliably detectable in short-term or site-restricted observations.¹⁹ Furthermore, defensive symbiont localization can be varied and unexpected, including occurrence of the symbionts on the surface of the host, within the food resource or the nesting environment, which makes distinction of symbionts from environmental contaminants challenging.¹⁷

Additional challenges of characterizing defensive symbioses are habitat-specific. In fact, defensive symbioses in marine and terrestrial animals have been explored from evidently different perspectives. A majority of the studies on marine associations has been motivated by the prospect to discover novel bioactive compounds, while the recognition of their bacterial origin has come much later. Hence, with few exceptions, research on marine defensive symbioses is characterized by a strong background on the chemical basis of defense, whereas the link to the producing microorganisms and the fitness consequences of the symbiosis for the host often remain enigmatic. Obviously, this is also due to the limitations for experimental manipulation in marine habitats, specifically the assessment of fitness benefits by artificially generating aposymbiotic hosts. On the other hand, terrestrial systems – represented to a great extent by



insects and nematodes – have been most often approached from an ecological perspective and usually first described based on the identification of the key partners. However, there is often less information about the mode of action of protective symbionts in terrestrial animals and – with a few notable exceptions – on the chemistry involved in defense (Table S1†). Thus, while gaps in the ecological knowledge of many of the marine symbioses remain to be filled, terrestrial studies could take advantage of the advances in natural product discovery accomplished in the marine world. Certainly, an interdisciplinary approach integrating mechanistic and ecological studies, molecular characterization and natural product research is and will remain to be of utmost importance for the field.

In this context, current technological developments will continue to play an important role for the progress in defensive symbiosis research. Molecular biology tools, particularly next-generation-sequencing of microbial communities, RNAseq, and single-cell genomics can rapidly provide strong links between natural products and their producers, especially in systems not amenable to manipulative experimentation. Additionally, increasing sensitivity and resolution in mass spectrometry (MS) as well as improvements in MS-imaging (like nanoSIMS, MALDI imaging, and DESI imaging^{422,423}) allow for the detection and quantification of bioactive compounds *in situ*, which is currently missing for most (but not all^{309,323}) terrestrial defensive symbioses.

In the search for novel defensive symbioses, special attention should be directed towards systems that exhibit ecological and evolutionary conditions predisposing them towards defensive alliances with microbes. For example, sessile or ground-nesting animals, those that have developed food domestication habits, or have gregarious or social lifestyles in combination with high relatedness, stand out as promising candidates. Beyond associations with bacteria, defensive symbiotic partnerships between animals and fungi remain heavily understudied.^{25,83,424} Fungal partners are common nutritional symbionts of various animal groups, but have been rarely screened for their bioactive potential. This is surprising as fungi have a vast biosynthetic potential and are a rich source of antibiotics.^{25,83,424–426} Likewise, only few cases of defensive symbiotic viruses have been described. Polydnaviruses in *Microplitis demolitor* and other parasitoid wasps (families Braconidae and Ichneumonidae) aid in suppressing the immune response of the parasitized host and thereby confer protection to the wasp.^{427,428} In another intriguing example, an RNA virus of the parasitoid wasp *Dinocampus coccinellae* manipulates the behavior of the wasp's coccinellid beetle host, inducing it to protect the wasp pupa from predation until it emerges from the cocoon.⁴²⁹ Finally, bacteriophages have been shown to adhere to metazoan mucosal surfaces and limit bacterial infections to their own and the host's benefit.⁴³⁰ The potentially high degree of specificity of the interaction between viruses/phages and bacteria, along with the simplicity of acquiring viruses and the low cost of their maintenance make them appear as ideal defensive symbionts. In summary, it is quite possible that other cases of symbiotic

relationships with fungi and viruses await discovery for those who venture to look beyond bacterial symbionts.

In conclusion, animal–microbe defensive symbioses are widespread, ecologically diverse and evolutionarily dynamic. They are a promising research target for the field of natural products discovery, due to their immense chemical potential and the advantages of studying the microbial producers directly embedded in an ecological context (*i.e.* fulfilling a role for their eukaryotic host), as opposed to free-living microorganisms.^{29,431} Furthermore, discovered natural products are more likely to be applicable in medical contexts, since they have been naturally tested for side effects on, at least some, eukaryotes. Although significant gaps in our understanding of symbiont-mediated defenses remain, the fast pace of technological advances and the momentum currently experienced by symbiosis research promise to quickly deepen our insights into these fascinating and promising associations.

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