Burkholderia as bacterial symbionts of Lagriinae beetles

Symbiont transmission, prevalence and ecological significance in *Lagria villosa* and *Lagria hirta* (Coleoptera: Tenebrionidae)

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“It's life that matters, nothing but life—the process of discovering, the everlasting and perpetual process, not the discovery itself, at all.”

Fyodor Dostoyevsky, The Idiot

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CHAPTER 1

General Introduction

1.1. The significance of microorganisms in eukaryote biology

The awareness that microbes can persist in intimate association with macroorganisms without causing disease first came in the late nineteenth century, along with the coining of the term symbiosis as "the living together of two organisms of different species" by Heinrich Anton de Bary (1). This seemingly clear-cut definition actually embraces a remarkably diverse, ubiquitous and complex range of associations. Symbiosis indeed seems to be more the rule than the exception in nature, and our vision on the relationships of eukaryotes with microbes is changing drastically (2–4). Importantly, we now acknowledge the immense relevance of symbiotic interactions including both mutualism and pathogenicity, including cases in which the net effect on the host depends on specific conditions (5). Along with this realization comes the great challenge to discover the real diversity of symbiotic partnerships between microbes and eukaryotes, assess their impact on levels ranging from specific organisms to whole ecosystems, and understand how and why symbiosis became such a recurrent evolutionary phenomenon.

Especially in animals, various biological aspects including development, metabolism and behavior had been traditionally studied without considering the influence of microorganisms (4). Yet, animals, plants and fungi evolved in the presence of and interacting with microbes, having important implications for host traits (6). Similarly, a host-associated lifestyle can affect symbiont physiology and genomics, implying that symbiosis has substantial consequences on the ecology and evolution of both partners (7,8). In certain circumstances, living in symbiosis leads to the alignment of fitness benefits or selective overlap (9), and depending on the degree of integration over generations, it can result in little or no chance of returning to independent living (7). In many other cases, however, autonomous evolutionary interests are maintained, and ecological and molecular mechanisms play a role in sustaining a persistent interaction despite potential conflict. As will be expanded on in the following sections, these non-obligate symbioses can also have significant effects on the ecology and evolution of the partners involved (10,11).

1.1.1. Insect-bacteria associations as models in symbiosis

The study of insect-microbe associations has played a fundamental role in building and experimentally substantiating current knowledge on symbiosis. Paul Buchner's seminal work on compiling an extraordinary collection of examples (12) has served as an invaluable reference for further exploration - now at the molecular level- of many of the existing insect-microbe symbioses, one being the focus of this dissertation.

Notably, systems involving intracellularly localized bacteria and highly specific associations with an insect host, as is the case of several γ-Proteobacteria and Bacteroidetes in various sap-feeding hemipterans (13,14), carpenter ants (15) or tsetse flies (16,17), have been pivotal for the study of symbiosis. In these, a highly specialized localization and a mechanism for strict vertical transmission has resulted in obligate and specific partnerships in which the bacteria cannot survive outside the host and the host depends on the bacteria, often for nutritional provisions in the form of vitamins and/or amino-acids (13,18,19). The prolongation of these highly interdependent associations across generations of strict vertical transmission generally reflects on clear co-diversification patterns recognized in host and symbiont phylogenies. Additionally, although not directly due to the intracellular localization of the symbionts, small effective population sizes during vertical transmission and relaxed selection on genes that are not essential for a host-associated lifestyle result in markedly reduced symbiont genomes (7,20,21).

Like other plant and animal groups, insects also associate with facultative symbionts which are not required for host development and reproduction. While many of these are heritable, horizontal transmission can also occur at variable

rates and has important consequences for symbiont population structure and genome composition (11). The genomes of these symbionts are markedly different from those with a strictly vertical transmission route; they are larger, often have many repetitive regions, high numbers of mobile elements, and contain phage and phage-derived genes (8).

Despite lower infection rates compared to obligate symbionts, several facultative symbionts have been shown to have a mutualistic effect or manipulate host reproduction, allowing them to spread over generations and persist in host populations (11,22). Yet, the identification of facultative symbionts in animals has gone at a faster pace than the corresponding elucidation of their effects on host survivorship or fitness (11,23). Usually, functional roles in these symbionts are more difficult to recognize, as they might be comprehensible only in a complex natural context (8,10). Notably, the most recurrent and best described examples of facultative symbionts and their effects on insect hosts are (i) *Wolbachia*, likely owing to its prevalence in various arthropod hosts, and (ii) several γ-Proteobacteria associated to aphids (11,21,22,24,25). As facultative symbionts, *Wolbachia* strains have been shown to facilitate host herbivory in leaf-miners (26) and hinder effects of antagonistic viruses in dipterans (27–29) in addition to their ability for reproductive manipulation in numerous other arthropods (30). In aphids, facultative symbionts can protect against fungi and parasitoids, aid in heat tolerance or affect host plant suitability (11). Despite these enlightening descriptions, the diversity of facultative symbionts and their functional roles most certainly goes far beyond and is thus worth investigating.

1.1.2. Plant-microbe-insect interactions

Recently, several microbe-eukaryote associations have been explored at the community level, where niche overlap and three-way interactions involving both animal and plant hosts reveal a more complex ecological dimension of symbiosis (31–33). For example, symbionts that supplement their host insect's diet can promote feeding on plants or specific plant parts (34), and plant-associated microbes can facilitate insect herbivory by affecting plant physiology (32,33,35). In other cases, microbes can directly interact with both plant and insect hosts (33). The ability of the microbial symbiont for niche expansion, however, generally relies on specific adaptations or preexisting genetic and physiological traits that facilitate a close interaction with different types of organisms (31,36–38). Since facultative symbionts usually do not depend on a specific host, these microbes might be more prone to a multihost lifestyle.

Insect-vectored plant pathogens provide examples of tripartite interactions which are known to occur in nature. Bacteria within the genera *Phytoplasma* and *Spiroplasma*, which are agriculturally relevant plant pathogens, are often vectored by sap-feeding insects like planthoppers, leafhoppers, and psyllids (36,39–42). The effect of these bacteria on the insect can be detrimental, neutral, or beneficial, although the impact remains largely elusive in most cases (36). In the leafhopper *Macrosteles quadrilineatus*, feeding on plants infected with aster yellow phytoplasma increases lifespan and fecundity. However, there is no evidence that these effects are directly caused by the presence of the bacteria in the insect (39). Rather, it has been demonstrated that phytoplasma infection in the plant impairs jasmonic acid production, thereby affecting anti-herbivory defense and ultimately favoring vector reproduction (41). Indirect mutualisms with a herbivorous insect are additionally known to occur in *Wolbachia*, which can aid leafminer moths to maintain metabolically active and nutrient-rich areas in otherwise yellow senescent leaves (i.e. the green-island phenotype). This effect, which is mediated by the action of cytokinins, retains a nutrient-rich resource on which the moths feed (26,43). Bacteria within the genus *Arsenophonus* are another interesting case of insect-associated microbes which can be plant pathogenic (38,44). Although these bacteria have been found in a broad range of arthropods, including phytophagous hemipterans (44–46), their impact on host fitness is less clear. For example, the geographical correlation in frequencies between *Arsenophonus* infection and parasitism pressure is in line with a protective role, although direct evidence is lacking (47). In whiteflies, secondary symbionts including *Arsenophonus* were found to be associated only to specific insect biotypes and thus potentially linked to characteristics like host plant range, speciation or insecticide resistance (48). However, no direct evidence is available and negative effects have also been found for *Arsenophonus* in whiteflies (49).

Similar cases of plant pathogen vectoring have been described as well for certain insect-associated viruses and fungi. For example, a Begomovirus vectored by whiteflies can benefit the insect by altering plant defenses (50). In thrips, the Tomato spotted wilt virus confers an advantage to the host by increasing insect growth rate and thereby reducing vulnerability to predation (51). In some fungus-bark beetle associations, the fungus can benefit the insect host either directly as a nutrient source and/or indirectly by negatively affecting the tree and thereby supporting tree colonization. The latter are usually facultative associates which compete with the beetles to some degree for the phloem substrate, so their benefit for the insect depends very much on the beetle population density and the condition of the host tree (52,53). Fungi can also be transmitted between plants by aphids and play a potentially beneficial role for the insect, as has been suggested for the rust fungus *Puccinia punctiformis* and the large thistle aphid *Uroleucon cirsii*. The aphids vector the fungal spores and form larger colonies on fungus-infected plants (54). However, evidence for a direct mutualistic effect of the fungus on the aphid is lacking.

From an alternative perspective, plants can also represent a source of bacterial symbionts for the insect. In fact, plant-mediated horizontal transmission of insect symbionts has been documented for *Rickettsia bellii*, a secondary symbiont of the whitefly *Bemisia tabaci*. In this case, the plants do not suffer from any apparent disease symptoms, but the bacteria can spread in the plant through the phloem (55). As to the impact of *Rickettsia* on the whitefly, it is known that the insects experience increased fecundity and survival until adulthood when infected, but the precise mechanism underlying these effects remain elusive and the potential influence of the environment on these observations is also unclear (56,57). In laboratory experiments, it was shown that *Rickettsia*-carrying individuals are more resistant against infection by *Pseudomonas syringae* (58). However, whether this is a natural enemy of whiteflies and the relevance of this protection in field conditions has not been evaluated.

While these tripartite interactions are likely widespread in nature and are now being documented from an integrated perspective, the ecological and evolutionary relevance for all partners is often difficult to discern and in many cases has not been elucidated (32,38,42). Importantly, these interactions are relevant for understanding multispecies interactions in natural communities, as well as for agricultural purposes.

1.2. The versatile lifestyles of *Burkholderia* bacteria

Burkholderia bacteria were initially designated as *Pseudomonas*, and only until 1992 they were assigned to a separate genus (59). Since then, more than 90 different species have been described, which occupy a broad diversity of ecological niches involving a range of metabolic capabilities and lifestyles like plant and animal pathogenicity, nitrogen fixation, biodegradation of aromatic compounds, plant growth promotion, as well as mutualistic associations with fungi and insects (60). The next sections provide a brief overview of well-characterized associations between different *Burkholderia* taxa and insect, plant, or fungal hosts.

1.2.1. Burkholderia-insect symbioses

Burkholderia have been found as members of the gut community in several insects, including ants (61,62), beetles (63,64), bugs, and solitary bees (65). However, evidence for a consistent symbiosis between *Burkholderia* and an insect host is only available for stinkbugs from the Lygaeoidea and Coreoidea superfamilies (66–69) as well as the Largidae family (70). These bugs acquire *Burkholderia* from the environment every generation, which colonize specialized crypts associated with the insect gut (66,70,71).

In the bean bug *Riptortus pedestris*, the symbiotic crypts develop in the posterior midgut during the second nymphal instar. Only then, the insects can acquire the symbionts (72), with around 80 bacterial cells being enough for initiating the association (73). Despite relying on the environment for symbiont acquisition and lacking a clear pattern of co-diversification between symbiont and host (66), the infection rates in natural populations of *R. pedestris* are as high as 95-100% (71). The beneficial effect of the symbionts has been supported experimentally, with

Burkholderia-free bugs suffering significantly impaired growth (71). Furthermore, the symbionts can degrade the insecticide fenitrothion, and thereby confer resistance to the insect host (74). In the field, application of this insecticide can lead to the enrichment of fenitrothion-resistant *Burkholderia* strains in the soil, and thus the bugs can readily acquire these (75), as has been also observed in field conditions in another stinkbug, the sugarcane pest *Cavelerius saccharivorus* (76). In a more recent study, Kim and coauthors demonstrated that *R. pedestris* bugs that carry *Burkholderia* show more pronounced upregulation of antimicrobial peptide production when challenged with other bacteria in comparison to aposymbiotic bugs, suggesting that the insect immune system is also enhanced in the symbiotic condition (77).

The association between *Burkholderia* and *R. pedestris* has become an important model system among insect-bacteria symbioses, especially due to the experimental and genetic tractability of the system. In recent years there has been significant progress in understanding the molecular mechanisms in both host and microbe that regulate the establishment and maintenance of the association (78,79). Specifically, it has been demonstrated that *Burkholderia* genes involved in supporting peptidoglycan integrity in the cell membrane are essential for the initial establishment of the association with the insect (80). In later stages of host development, purine biosynthesis by the symbionts is involved in both accommodation and persistence in the insect (81,82). Additionally, biofilm formation and the production of polyhydroxyalkanoate (PHA) granules, both associated to stress tolerance in the *Burkholderia* symbionts, have proved to be crucial for their maintenance in the midgut crypts and thereby for host fitness (82,83). As to the insect, there are several mechanisms for regulating the symbiont population during development. While *Burkholderia* titers increase within nymphal instars, in the pre-molting stage lysozyme and antimicrobial peptide production in the gut is upregulated, causing a decrease in bacterial cell numbers, which is thought to control the *Burkholderia* population size (84). As an additional mechanism for control, symbiotic *Burkholderia* are more vulnerable to host-produced AMPs due to modifications in the cell wall that arise only when associated to the insect. However, the overall lower production of AMPs in the midgut region where symbionts are located in comparison to other body parts, like the fat body, suggests a sophisticated and fine-tuned mechanism allowing for the persistence of the symbiosis with *Burkholderia* (85).

1.2.2. *Burkholderia* as plant pathogens

Multiple *Burkholderia* have been shown to closely interact with a variety of plants, either causing disease (86), enhanced plant growth (87), or fixing nitrogen in root nodules of legumes (88). Interestingly, *Burkholderia* are also vertically transmitted symbionts of *Sphagnum* mosses (89) and several species within the Rubiaceae and Primulaceae plant families (90), yet their functional role in these is currently unclear. While there is extensive literature on this broad range of plant-*Burkholderia* interactions, for the purpose of this work I will focus on those involving *Burkholderia gladioli*, as these are the main symbionts of Lagriinae beetles.

B. gladioli is mostly known for its pathogenic effects on plants, causing disease in onions, gladiolus and iris (91), as well as in orchids (92), corn (93) and rice (94–96). Like *B. cepacia*, *B. gladioli* are known to produce extracellular factors that affect cell membranes and the structural integrity of plant tissues (86). Most research on *B. gladioli* plant pathogenicity has been carried out in strains isolated from rice, where similar to *B. glumae*, they cause panicle blight rot affecting rice production in Asia (96), North- (95) Central- (94), and South America (97). Upon infection, the production of toxoflavin is associated with virulence in pathogenic strains of *B. gladioli* (96,98). This phytotoxin has been shown to cause chlorosis, as well as impaired growth of leaves and roots in several plants including hot pepper, eggplant, tomato, perilla and sesame (99), and can also be toxic to animals and other microorganisms (100). As in *B. glumae*, the production of toxoflavin in *B. gladioli* is regulated by the quorum-sensing system, and the absence of QS genes correlates with the inability to produce toxoflavin in several *B. gladioli* strains (98). Further genomic analyses on twelve strains including plant pathogenic *B. gladioli*, as well as the closely related *B. plantarii* and *B. glumae* reveal the presence of a highly conserved type III secretion system across the strains (101), which might play an important role in pathogenesis.

Although first described for its pathogenic effects on plants, *B. gladioli* has also been found as an opportunistic human pathogen, and is an occasional cause of acute infections in immunosuppressed patients as well as in those suffering from cystic fibrosis and chronic granulomatous disease (102,103). It has also been involved in food poisoning by its interaction with a fungus, as mentioned in the next section.

1.2.3. *Burkholderia*-fungi symbioses

Several fungi can also host *Burkholderia*. Particularly, *B. gladioli* pathovar *cocovenenans* can infect the fermentation fungus *Rhizopus oligosporus*, which is used in the preparation of the south-east Asian specialty tempeh bongkrek. By producing bongkrekic acid, this pathovar has been the cause of severe food poisoning in humans (104,105). Another pathovar, namely *B. gladioli* pv. *agaricicola*, causes cavity disease in white button mushrooms which can result in severe losses for the mushroom industry (106). This pathovar also shows broad *in vitro* antifungal effects (107), and additional *B. gladioli* strains have been proposed as potential biocontrol agents, as these inhibit a variety of mold fungi in harvested fruits (108). Other members of the genus *Burkholderia* are predominant bacterial partners associated to arbuscular mycorrhizae and ectomycorrhizal fungi (109). Also, a fascinating example of a *Burkholderia*-fungus symbiosis was discovered in the causative agent of rice seedling blight, *Rhizopus microsporus*. This fungus harbors the intracellular bacteria *B. rhizoxinica* and *B. endofungorum* (110), which are necessary for host vegetative reproduction (111). Interestingly, the phytopathogenic activity of the fungus relies on the production of the macrocyclic polyketide rhizoxin by symbiotic *B. rhizoxinica* (112,113). An additional toxin in *R. microsporus*, the cyclopeptide rhizonin, was also found to be of symbiotic origin, yet the ecological value in this case is less clear than for rhizoxin (114). Detailed molecular and biochemical descriptions in the *Rhizopus-Burkholderia* system have revealed the specific mechanism by which the bacteria manage to enter the fungal cells for symbiosis establishment. The bacterial type II secretion system is responsible for the release of a combination of chitinolytic enzymes which loosen the fungal cell wall, allowing the symbionts to penetrate the hyphae without detrimental effects for the fungus (115). Thus, both host and symbiont have evolved sophisticated molecular mechanisms that support this mutualistic interaction, and thereby tailor a remarkable ecological strategy for phytopathogenicity.

1.3. Lagriinae beetles and their unexplored symbiosis with bacteria

1.3.1. The biology of *L. villosa* and *L. hirta*

The Lagriinae, previously assigned to a separate taxonomical family (Lagriidae), are now generally recognized as a subfamily within the darkling beetles or Tenebrionidae. While there are worldwide reports on the occurrence of Lagriinae beetles and numerous taxonomic descriptions (116–122), thorough characterizations of their biology are scarce and phylogenetic associations have not been elucidated at the molecular level. In particular, the genus *Lagria* Fabricius, 1775, is distributed throughout Europe, Asia, Africa, New Guinea and Australia (117). Here I will focus on the two species within this genus on which we carried out the major part of experimental work, i.e. *Lagria villosa* Fabricius, 1781, and *Lagria hirta* (Linnaeus, 1758).

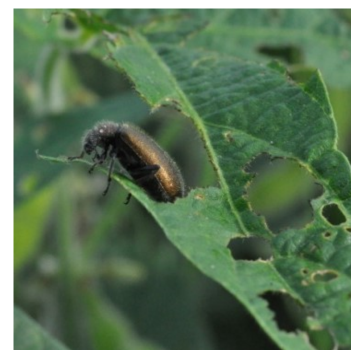

Figure 1.1. *Lagria villosa* female feeding on soybean leaves in Brazil. Photograph credits: Martin Kaltenpoth.

L. villosa (Fig.1.1.) is native to Africa and has been found in rice plantations (123), and as defoliators of rubber trees (124). However, reports on its distribution and occurrence across the African continent are limited. In the 1970's, this beetle was first reported as an introduced species in Brazil (125) and since then has been found on a broad range of plants of agricultural importance in Brazil and the north of Argentina, including soybean (126–128), potato (129), rape (130), cotton (128), common bean, corn, banana, pineapple and coffee (131). Its feeding habits, together with its capacity to reproduce in high numbers, drove the attention of a number of Brazilian farmers and researchers towards *L. villosa* as a potential agricultural pest (126,128,129,131,132). In fact, damage caused by both adults and larvae have been described for rape (130) and strawberry (133). In the field, adults occur in plantations between January and March and lay their eggs on the soil or on the leaf litter (128). Abundant larvae and pupae can also be found during this period, although detailed reports on their life cycle and phenology in natural conditions are lacking. Under laboratory conditions, eggs hatch five to six days after being laid, develop as larvae for approximately 40 days and remain as pupae for five to six days. After emergence, adults survive for variable periods which can reach up to two additional months for mating and oviposition. The duration of the complete life cycle thus ranges between three and four months in the laboratory based on our observations and those reported in the literature (134).

Figure 1.2. *Lagria hirta* female on grass leaf in Ammerbach, Germany.

L. hirta (Fig 1.2.) is known to be univoltine, occurring as adults from June to August and overwintering as larvae (135), while the duration of both the egg and pupal stages is similar to *L. villosa*. Notably, the synchronization between larval development and seasonal changes has been shown to be finely tuned in *L. hirta*, as the initiation of diapause must occur within a specific developmental window (fourth to fifth instar) in order to reach adulthood (136). This species occurs throughout the European continent, and is the most common member of the Lagriinae in Central Europe (137). The adults feed on fresh and dry leaves of a wide variety of plants, and the larvae usually consume leaf litter. As in *L. villosa*, eggs clutches are laid on the soil and in the leaf litter.

1.3.2. Morphology of the symbiont bearing structures in Lagriinae

Almost a century ago, Stammer (138) published a detailed morphological description of bacteria-bearing structures associated to the reproductive system of *L. hirta* females. In this species, there are two sac-like glandular structures on each side of the ovipositor which develop from the cuticle between the caudal-most segments of the insect abdomen, as it folds towards the body cavity (Fig 1.3). Additionally, *L.hirta* females have two elongated structures which prolong on both sides of the oviduct, within the ovipositor, and also contain the symbiotic bacteria (Fig 1.3c) (138). Throughout the dissertation I will refer to the former organs as accessory glands and to the latter as ovipositor-associated structures. According to Stammer, both similar structures and the symbionts are absent in adult males.

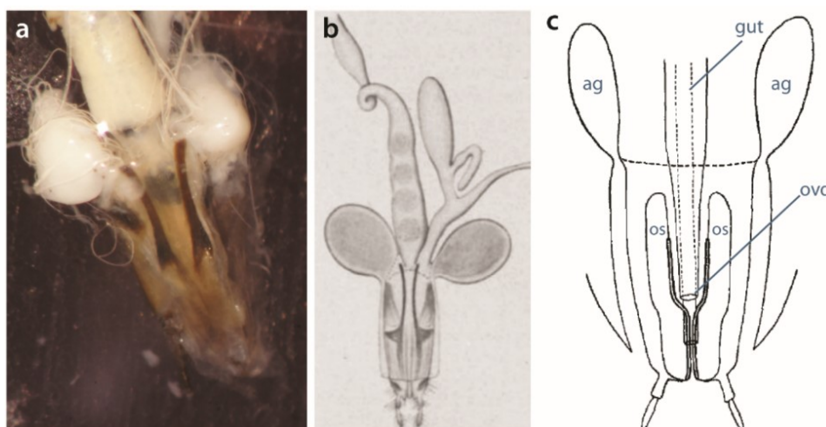

Figure 1.3. Symbiotic organs in *L. hirta* adult females. (a) Freshly dissected ovipositor with the pair of sac-like accessory glands (dorsal view). (b) Illustration of the ovipositor and associated glands relative to the gut, bursa copulatrix and oviduct (ventral view). (c) Schematic representation of the ovipositor and accessory glands (ag) including the ovipositor-associated structures (os), the oviduct (ovd) and the caudal-most region of the gut. Both (b) and (c) are adapted from (138).

Stammer also characterized the histology and formation of three specialized compartments located dorsally in the larvae, which form as invaginations of the intersegmental cuticle between the first four body segments (Fig. 1.4.) (138). According to Stammer's observations, the symbiotic bacteria are deposited by the adult females on the egg surface during oviposition and from there migrate into the dorsal invaginations during the last day of embryonic development. The invaginations finally close to form the three compartments, as observed in a longitudinal section of an *L. hirta* larva (Fig. 1.4.) (138). Several cerambycid, anobiid and curculionid beetles harbor yeast or bacterial symbionts within structures similar to those in adult *Lagria* females. However, Stammer, and later Buchner, referred to the dorsal compartments of the larvae as especially unusual organs for bearing symbiotic bacteria in an insect (12,138).

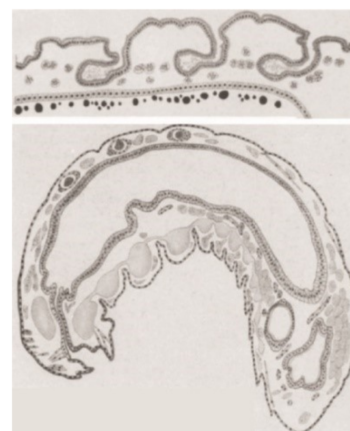

Figure 1.4. Illustrations of longitudinal sections on the dorsal region of a developing *L. hirta* embryo (upper) and second instar larva (lower) showing the three cuticular invaginations which are colonized by the bacterial symbionts. Illustrations from (138).

Stammer additionally investigated preserved adult specimens from 94 different beetle species that were at the time assigned to the "Lagriidae" family (now Lagriinae). He found comparable structures in 83 of these, yet he discovered a remarkable morphological diversity in the organs across different species, including tubular, branched or lobed structures of varying length (Fig. 1.5.) (138). In contrast to the paired sac-like glands of *L. hirta*, *L. villosa* exhibits two sets of four tubular organs as accessory glands and lack elongated structures associated with the ovipositor (Fig 1.6.).

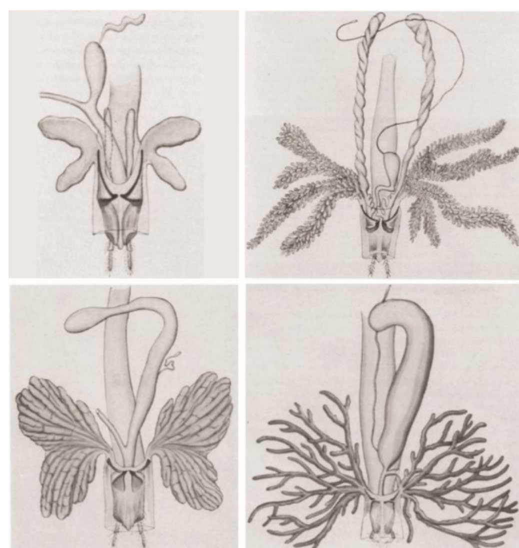

Figure 1.5. Selected illustrations representing a subset of the symbiotic structures in Lagriinae beetles described by Stammer, 1929. Paired structures to each side of the ovipositor represent the accessory glands in diverse morphologies. In the two upper illustrations, the ovipositor-associated structures are significantly elongated, extending anteriorly. All illustrations are from (138).

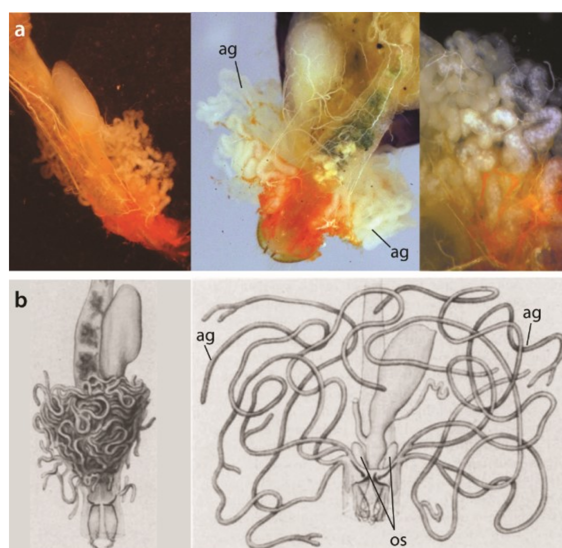

Figure 1.6. Symbiotic organs with tubular-shaped accessory glands in Lagriinae females. (a) Freshly dissected ovipositor of an *L. villosa* adult female with two sets of tubular accessory glands (ag) displayed from a lateral and dorsal view, also showing the relative position of the bursa copulatrix and gut. The inset shows a detailed view of the tubular glands. (b) Illustration of the symbiotic structures of *Ecnolagria grandis*, with similar morphological characteristics to *L. villosa* from (138). In contrast to *L. villosa*, additional ovipositor-associated structures (os) are present.

1.4. Thesis outline

There is a growing body of evidence supporting the substantial impact of symbiotic associations with microbes in insect, animal and eukaryote biology. It is thus pertinent, and also fascinating, to explore poorly understood symbioses with potentially novel functional roles. This not only expands our knowledge on the existing diversity, but can also deepen our understanding on the mechanistic basis of microbial symbiosis. While the comprehensive morphological characterization on the Lagriinae-bacteria symbiosis by Stammer (138) provides valuable insights into this system, current technologies offer tools to investigate biological interactions at a molecular level and use this knowledge to tackle evolutionary and ecological questions. Several characteristics of this insect-bacteria association pointed to a promising system for the discovery of potentially novel functions and particular ecological foundations of microbial symbiosis. The localization and formation of the symbiotic organs in the larval stage of the host is completely different to any other known in insects, and despite the widespread occurrence of the beetles -some in agriculturally relevant crops-, research on their bacterial symbiosis was so far extremely limited. Furthermore, indications from our preliminary work on this system revealed *Burkholderia* as the main bacterial symbiont in *L. hirta*, which additionally motivated us to investigate this association, given the interesting metabolic and ecological characteristics of this bacterial genus. With this background, we set out to molecularly characterize the *Burkholderia* symbionts and their transmission route, assess if the symbiosis is present in multiple Lagriinae hosts and is thus evolutionarily ancient, and investigate the functional and ecological significance of *Burkholderia* in these beetles.

In Chapters 2 and 3, a conceptual framework is given on two essential aspects for symbiosis in general, and for the *Lagria-Burkholderia* system in particular: symbiont-mediated defense and extracellular symbiont transmission mechanisms. Chapter 2 reviews the known associations with a defensive basis between microorganisms and animals, providing examples of protective symbiosis in a wide range of metazoans. While those that rely on chemical defense are highlighted, defensive symbiosis is discussed in a broad sense. We propose ecological features that unify or are recurrent in these interactions, and comment on the dynamic evolutionary signatures observed. This is a highly relevant background for understanding the *Lagria-Burkhlolderia* interaction, as there is strong evidence that the symbionts play a defensive role (Chapter 4). Chapter 3 provides a review of the different types of extracellular transmission routes and corresponding examples in insects. It also discusses symbiont transmission in an evolutionary context, including the transition from a free-living life style to symbiosis, co-evolutionary patterns between host and symbiont in the light of different transmission mechanisms, and the consequences of the transmission route on the genomic characteristics of the symbionts. These considerations are relevant for the *Burkholderia* symbionts in Lagriinae, as they are extracellularly located and transmitted, and exhibit relatively complex transmission dynamics (Chapter 5).

Chapter 4 addresses the question of symbiont functional role in a particular ecological context, and provides an extended view on the evolutionary origin and ecological significance of the bacterial symbionts when considering their interaction with a plant, which serves as a food source for the beetle. We provide evidence that the *Burkholderia* symbionts of Lagriinae evolved from plant pathogenic ancestors and, using *L. villosa* as a model system, we show that a symbiotic strain can still successfully infect a plant. Importantly, we demonstrate that the symbionts are also mutualistic to the beetle, as they protect the insect eggs from antagonistic fungi. The chemical basis of defense is also described in this chapter.

In Chapter 5, we investigate the symbiont dynamics during the life cycle of *L. hirta* and assess the prevalence and consistency of *Burkholderia* symbionts across different host populations. Importantly, we reveal a regular pattern of coinfecting symbiotic strains within single host individuals, providing further insight into the complexity of the association. Additionally, we evaluate the impact of a natural vs. laboratory environment on the bacterial community and *Burkholderia* strain composition within the symbiotic organs.

Bearing in mind the defensive role of the *B. gladioli* in *Lagria* beetles, the experimental work presented in Chapter 6 aims at assessing whether the bacterial symbionts have an impact on the beetle host in the absence of antagonists. Specifically, I evaluate the effects of the symbiots on survival and overall performance of *L. hirta* and *L. villosa*. The observations are discussed in the context of additional functional roles (e.g. nutritional) and potential metabolic costs imposed by the symbionts, considering the ecological background described in the previous two chapters.

Finally, chapter 7 presents preliminary work on the genomes of two symbiotic *Burkholderia* strains, one isolated from *L. hirta* and one from *L. villosa*. I summarize general genomic features and highlight noteworthy gene clusters associated to the production of secondary metabolites with potential ecological relevance.

An integrated discussion of all the above mentioned results is provided in Chapter 8.

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CHAPTER 2

Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms

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Summary: 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.

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 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 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 (Appendix: 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 symbiosis.

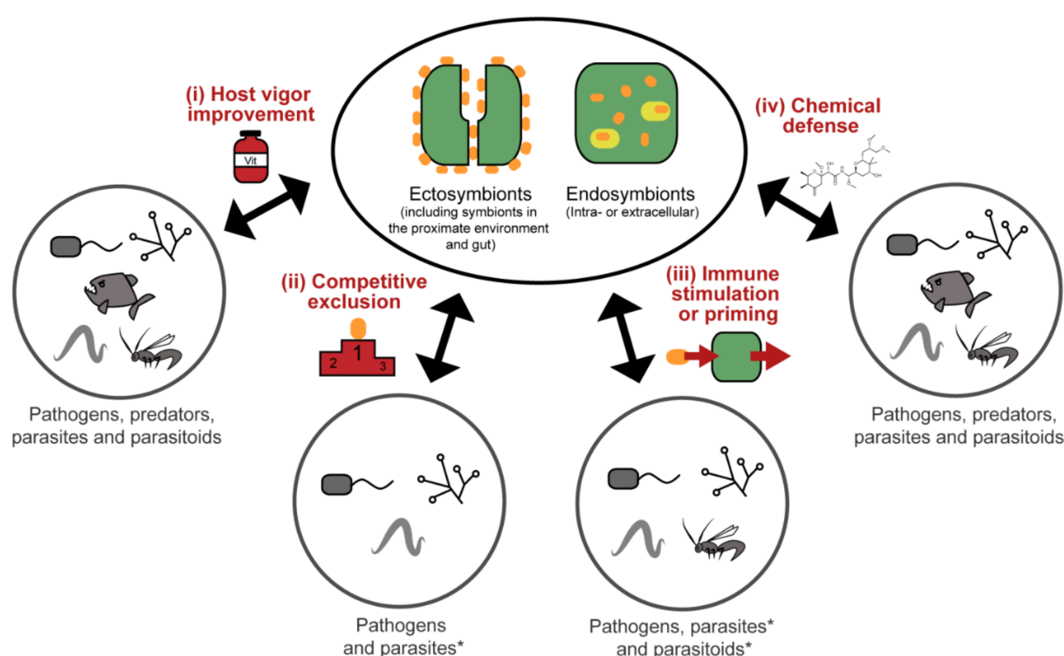


Figure 1. Types of defense mechanisms in animals mediated by ectosymbionts (including those in the gut and in the proximate environment of the host) or endosymbionts (intra- or extracellular) against different possible antagonists (described or likely effective against).

2. Defensive animal-microbe symbioses

2.1 Marine invertebrates

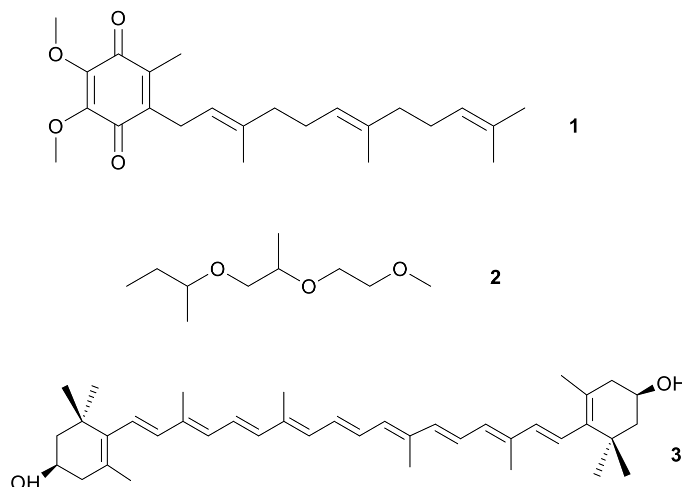
2.1.1 Sponges

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 unculturable 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 sponges[33] 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

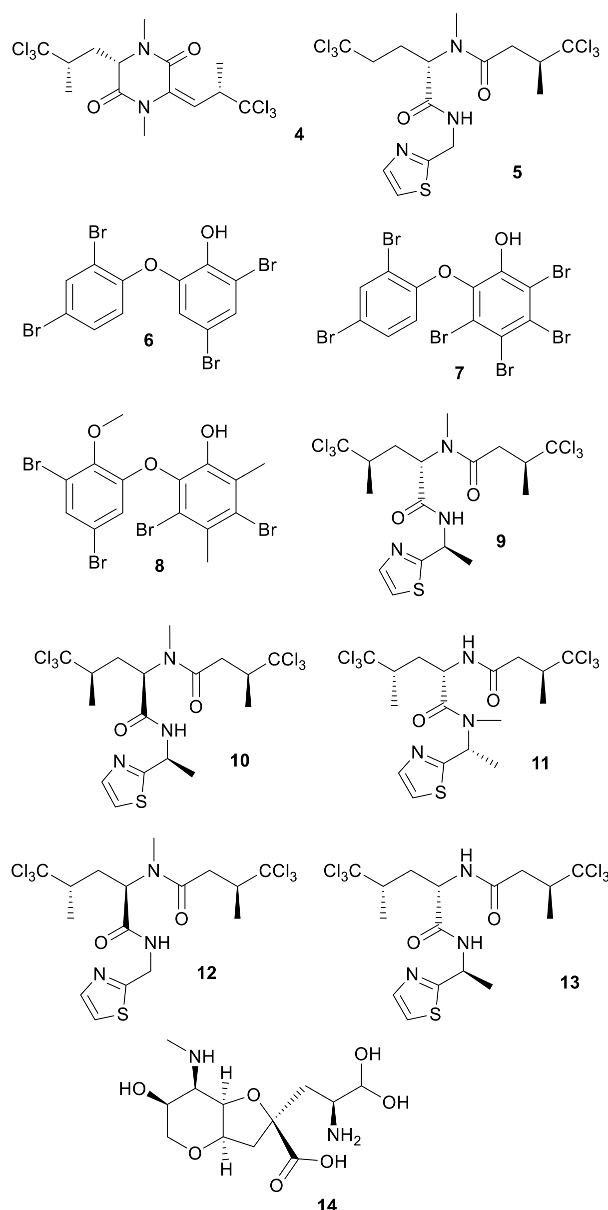
Some of the first insights in sponge defensive symbioses were gained by culture-dependent approaches. Konya *et al.*[37] followed the reports of surface-associated compounds influencing the settlement of invertebrate larvae causing fouling, and the idea that bacteria might produce these compounds. Concordantly, they succeeded in isolating an *Alteromonas* strain from the sponge *Halichondria okadai* that inhibited the settlement of *Balanus amphitite* cyprides. 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. amphitite* 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 diketopiperazides dihydrodysamide C **4** and demethyl-dihydrodysamide C, as well as 13-demethylisodysidenin **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 a single symbiont species, 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-demethylisodysidenin **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 Dysideidae[48] 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 *Salinospora* 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 carribbean 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], plakortethers[60]), or in regulating its microbial community by modulating the host's immune system (plakosides[61], simplexides[62]). An attempt to isolate the plakortin biosynthesis genes failed, but yielded an unusual polyketide-fatty acid synthase

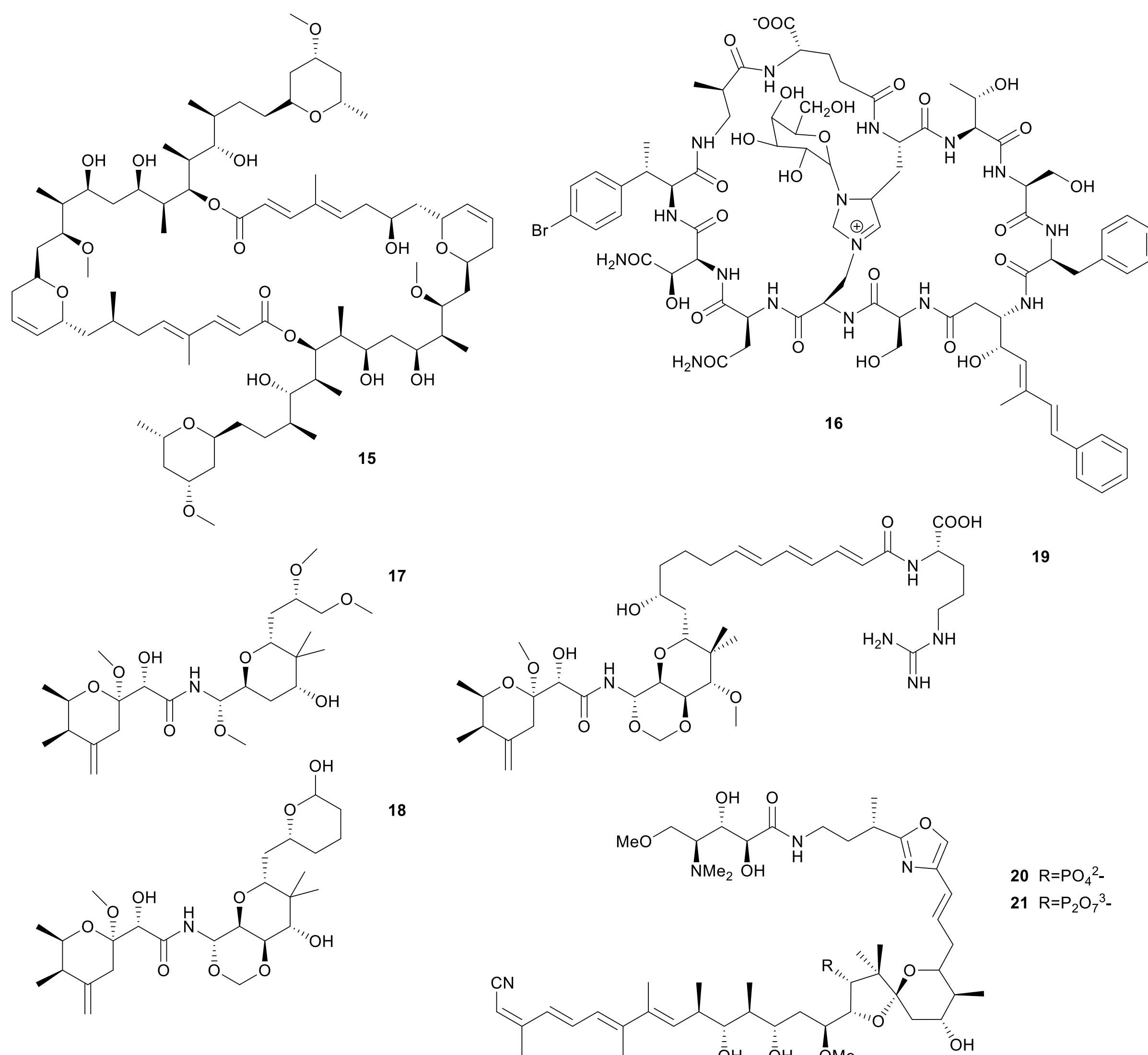
hybrid that supposedly synthesizes an acyl chain with various functional groups, probably containing a sulfate group.[63] Fisch *et al*. also exploited the conserved sequence of the ketosynthase (KS) domain to screen metagenomic fosmid libraries from the sponges *Psammocini*a aff. *bulbosa* and *Mycale 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 antiviral[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 cytotoxic[69] 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 theonegramid[70] 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, theonegramide 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 **18**[74] 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 yielded 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 channels[78] 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 released host 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 sponges[84] 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 18S rRNA sequences in sponge databases.[24] Furthermore, sponge-associated fungi were found to contain a large diversity of PKS and NRPS genes,[89] although their possible roles in the defense of the host remain enigmatic.

2.1.2 Cnidarians

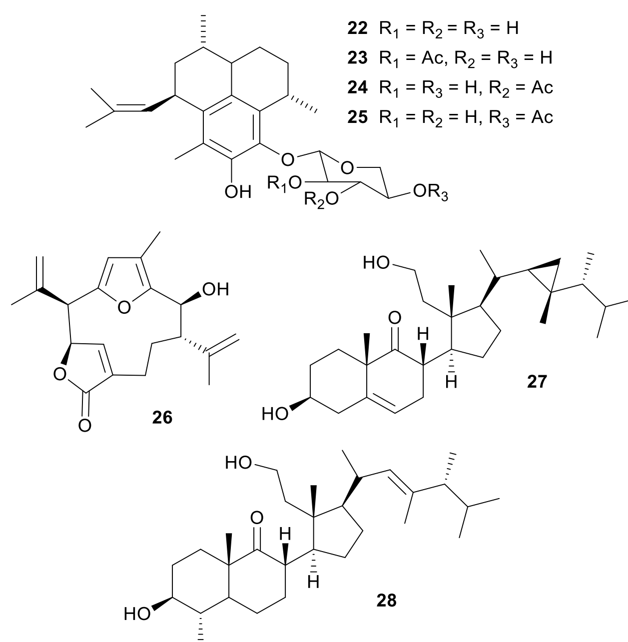
Many corals are intimately associated with algal symbionts as well as a diverse community of bacteria. In particular, dinoflagellate symbionts of the genus *Symbiodinium* are well-known for their important contributions to the coral hosts' metabolism by providing photosynthetically derived nutrients,[90] 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 $NaH^{14}CO_3$ or tritiated geranylgeranyl diphosphate (^{3}H-GGDP) resulted in labeled pseudopterosins.[105] A similar strategy of symbiont cell enrichment and subsequent radioactive labeling with ^{3}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 6,000 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, Diaperoecia 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.

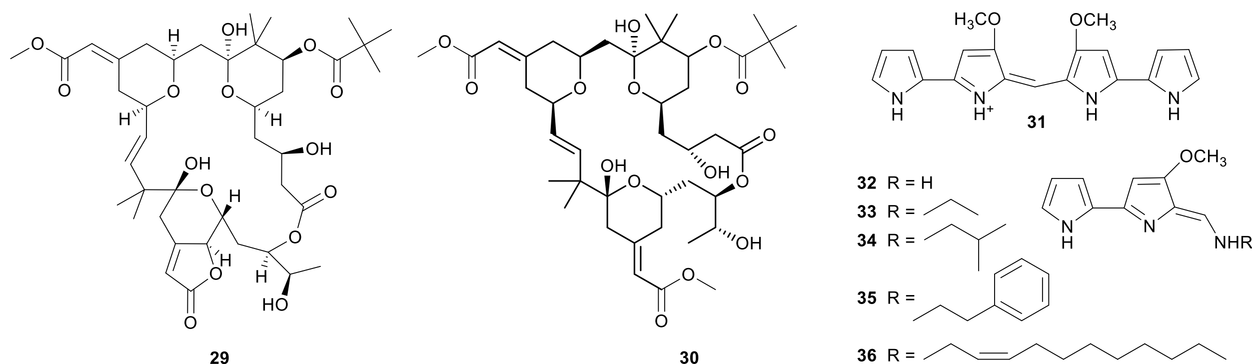
Bryostatins

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]

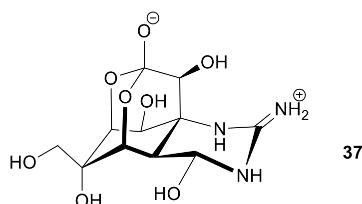


Tambjamines

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 animals[153] – including bryozoans, mussels, ascidians, fish, corals, and sponges[153-155] – support this hypothesis. Recently, the molecular basis of tambjamine production in *Pseudoalteromonas 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 harbor 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]

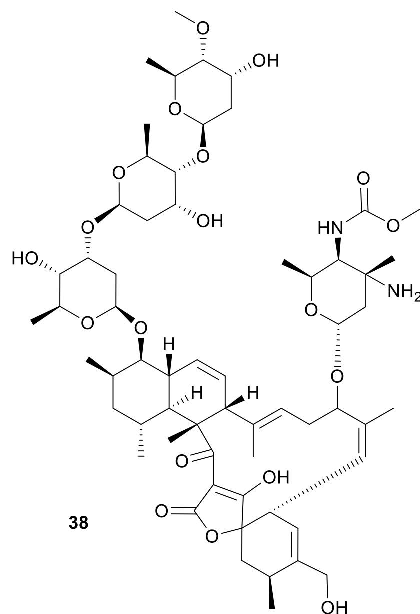
Gastropods

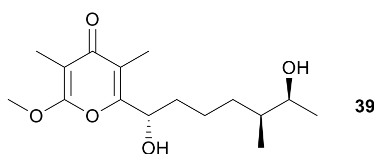
An interesting case of symbiont-mediated structural protection has been described in the scaly snail *Crysomallon squamiferum,* a gastropod occurring at hydrothermal vents.[171] The snail's foot is covered in hardened scales of multiple layers that likely confer protection against predation.[171, 172] The outer layer is composed of pyrite (FeS_2) and greigite (Fe_3S_4), 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.

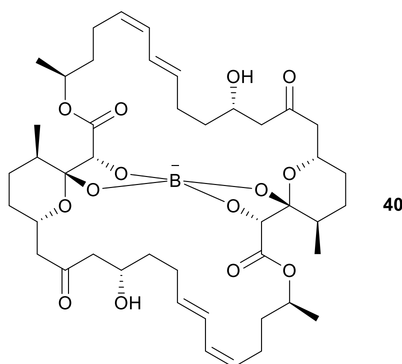
As cone snails are well-known for their arsenal of protective peptide toxins, further microbe-derived defensive compounds were not expected. Surprisingly, however, Peraud *et al.* found a diverse actinomycete community associated with different cone snails of the genus *Conus* that displayed bioactive 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-inflamatory 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 circumcin derivatives that show neuroactivity or broad antimicrobial bioactivity.[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, nocapyrones **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 gills[181] 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]







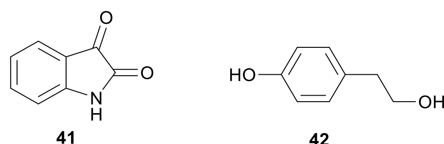
Cephalopods

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 'counterillumination'.[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

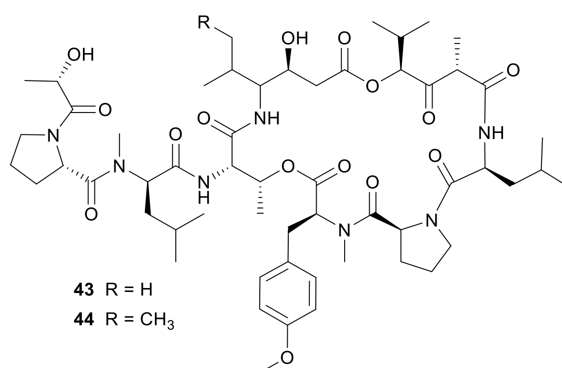
Tunicates are sessile or pelagic filter feeders that occur worldwide in marine environments. Among tunicates, the colonial ascidians are an especially rich source of secondary metabolites, many of which are believed to originate from microbial associates. Comprehensive reviews on ascidian natural products 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.

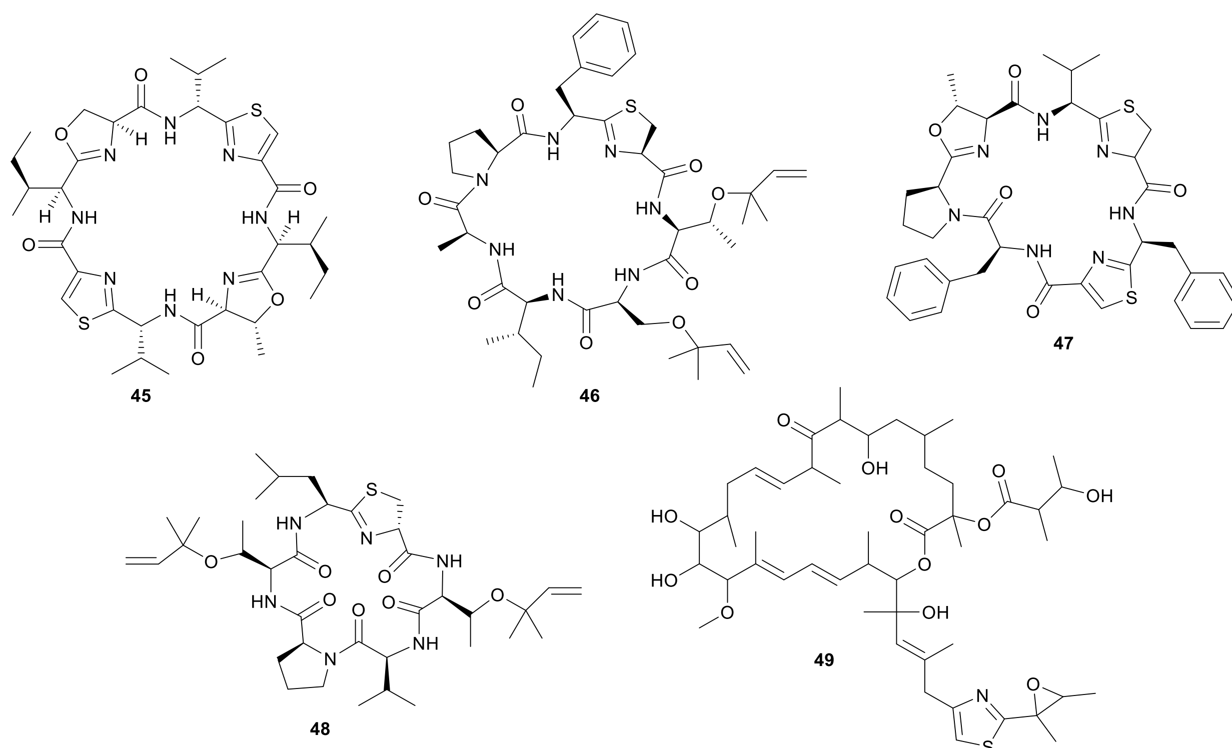
Many didemnid ascidians live in an obligate symbiosis with vertically transmitted cyanobacteria of the genera *Prochloron* or *Synechocystis*.[211-213] *Prochloron* symbionts have been found on the surface and/or in the common cloacal cavity of colonial didemnids such as *Lissoclinum patella, L. bistratum, 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 nordidemnin 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.

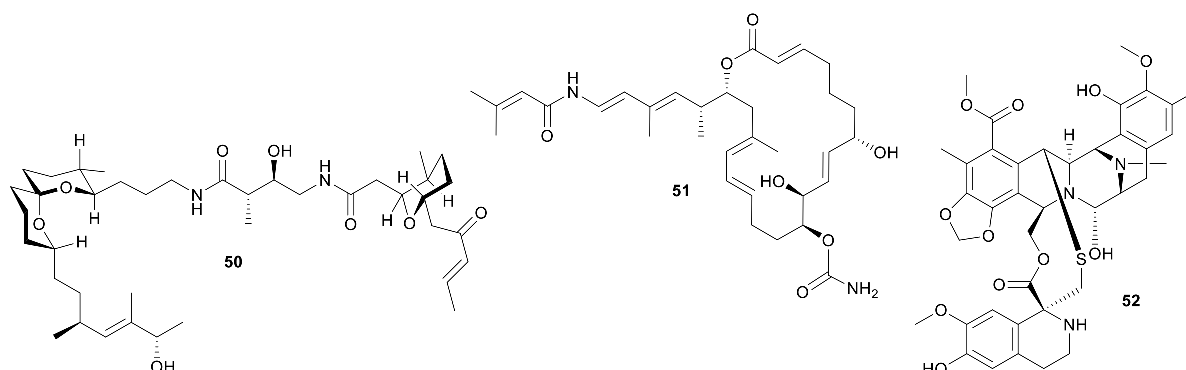


In analogy to the didemnins, it was long suspected that another group of cyclic peptides in ascidians, the cyanobactins (including the patellamides **45**, trunkamide **46**, lissoclinamides **47**, patellins **48**, and many others), are produced by cyanobacterial symbionts. This hypothesis was based 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 cluster[224] 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* Profftella armatura' in the asian citrus psyllid, which retained the complete pathway for the putatively defensive compound diaphorin in an otherwise eroded genome[230] (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 bistramides **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.

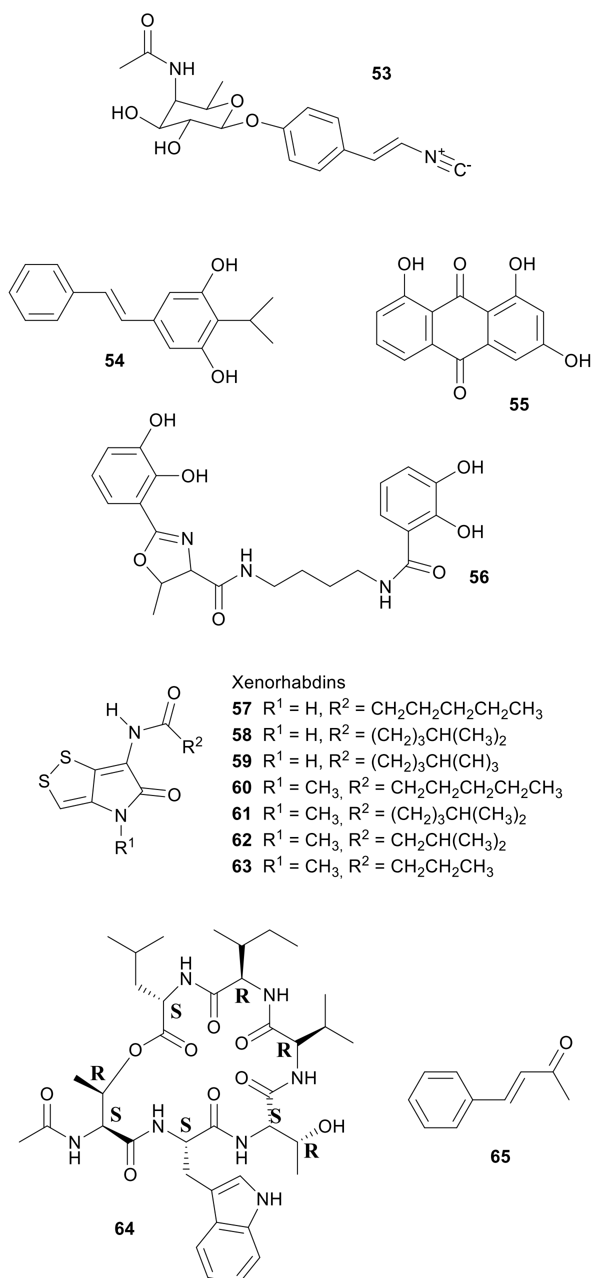
The two entomopathogenic nematode families *Steinernematidae* and *Heterorhabditidae* are characterized by their obligate association with bacteria in the γ-proteobacterial genera *Xenorhabdus* and *Photorhabdus,* respectively. Although some of these symbionts can occur in multiple hosts, most strains 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 *Photorhabdus* 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 (1) highly specific proteinaceous bacteriocins, i.e. lumicins[252] and photorhabdicins[253] from *Photorhabdus* spp., and xenorhabdicins[254-256] from *Xenorhabdus* spp; (2) broad-spectrum, antibacterial and antifungal compounds, including isopropylstilbenes,[245, 257, 258] antraquinones,[242, 259] and a carbapenem[260] from *Photorhabdus* spp., as well as fabclavines,[261] xenorhabdins,[262, 263] xenorxides,[242] and nematophins,[245, 264] from *Xenorhabdus* spp.; and (3) narrow-spectrum anti-Gram-positive xenobactin,[265] xenematide[266] and xenocoumacins,[267] and anti-Gram-negative benzylideneacetone[268] from *Xenorhabdus* spp. Additionally, several other metabolites including xenoamicin,[265] taxlllaids,[269] cyclohexandione,[270] chaiyaphumine[271] and szentiamide[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 *Photorhabdus* and *Xenorhabdus* spp. produce rhabduscin **53** and chitinases, both with a dual function – the promotion of prey killing/digestion and defense against fungal competitors[274-276] – 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 *Photorhabdus* and *Xenorhabdus,* three kinds of bacteriocins, the lumicins, photorhabdicins and xenorhabdicins, have been described. Normally detectable in low quantities, their production is strongly induced when bacterial cells are lysed.[255] Xenorhabdicins were first described from *X. nematophila* and shown to be active against strains of *Xenorhabdus*, *Photorhabdus*, and related sister taxa.[256] Likewise, *Photorhabdus* spp. synthesize photorhabdicins 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 *Photorhabdus*, carbapenem-like molecules, as well as isopropylstilbenes and anthraquinone pigments are mainly responsible for this effect.[245, 258, 259, 277, 278] Carbapenems are ß-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 species 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 PKS [281] are responsible for the red color of insects killed by *Photorhabdus*.[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, xenematide, xenobactin, and benzylideneacetone.[20, 241, 251, 261] Xenorhabdins **57-63**, the largest group among these, are dithiolopyrrolone derivatives (compounds 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, xenematide and xenobactin inhibit Gram-positive bacteria,[241, 266, 267] with xenobactin **64**, a hexadepsipeptide, 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-Photorhabdus* 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-Photorhabdus* 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 strong 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 *Acyrthosiphon 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* sp. can defend the larvae against the wasp *Leptopilina heteroma.*[292] Additionally, some studies suggested that *Arsenophonus* sp. in psyllids[293] 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 actinomycetes 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 defensive symbioses 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]

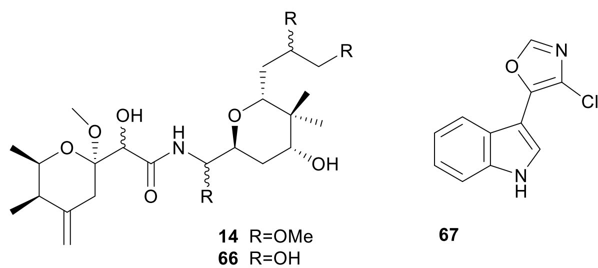
Like beewolves, fungus-farming ants nest in the soil and are confronted with environmental pathogens that threaten their brood and the fungal cultivars. Moreover, leaf-cutter ant gardens are challenged by specialized *Escovopsis* fungal pathogens and endophytic fungi, brought in by the ants with the 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 glands[311] as well as antimicrobials produced by symbiotic Actinobacteria.[312, 313] These Actinobacteria comprise vertically and occasionally horizontally

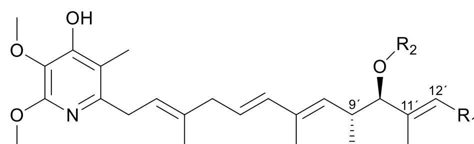
transmitted *Pseudonocardia* symbionts[310, 314-316] as well as environmentally acquired members of the genera *Streptomyces* and *Amycolatopsis*.[317-319] The *Pseudonocardia* symbionts defend the fungus garden against the specialized *Escovopsis* cultivar pathogens, by producing dentigerumycin **76** 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 candicidin **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 also protect the ants themselves against pathogens by producing actinomycins and antibacterial 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 antifungal 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.





Piericidins

68 $R_1=CH_3$; $R_2=H$

69 $R_1=CH_3$; $R_2=CH_3$

70 $R_1=CH_2CH_3$; $R_2=CH_3$

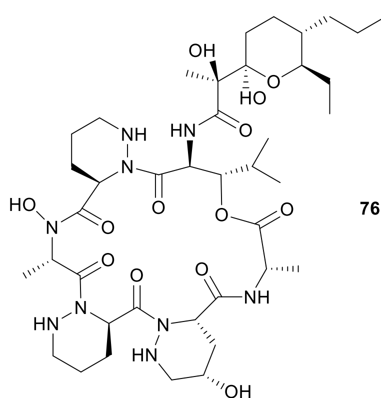
71 $R_1=CH_2CH_3$; $R_2=H$

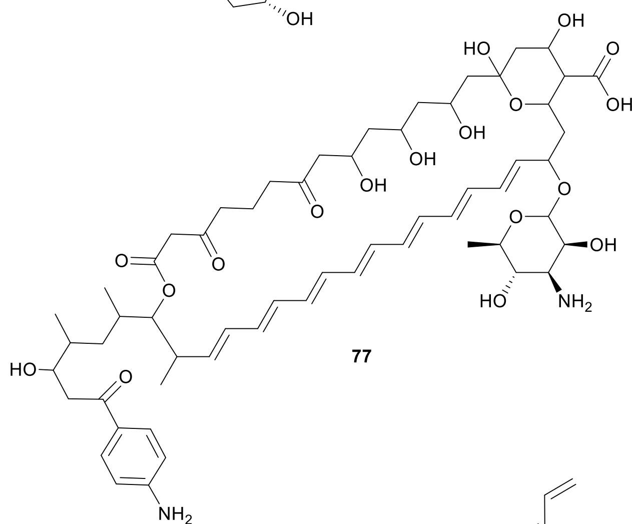
72 $R_1=CH_3$; $R_2=H$; 11´,12´-epoxide

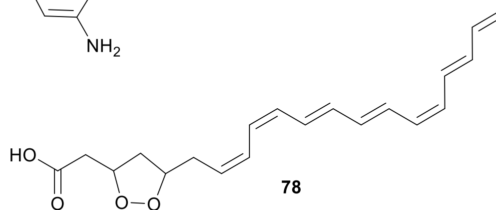
73 $R_1=CH_3$; R_2=b-D-glucopyranose

74 $R_1=CH_3$; $R_2=H$; 9´-de-Me

75 $R_1=C(CH_3)CHCH_3$; $R_2=H$

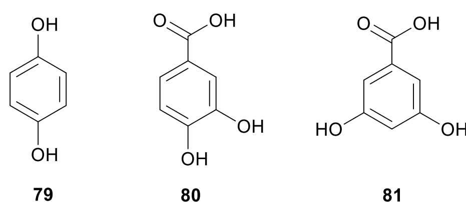






Protection against pathogens: gut and nutritional resources

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 **80-81**, are usually present in the guts and feces of locusts, while absent in insects lacking their normal gut microbiota.[339] Interestingly, the entomopathogenic fungus *Metarhizium 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 honey bee *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 bumble bees. By experimentally manipulating the gut microbiota of the bumble bee (*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 bumble bee 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]

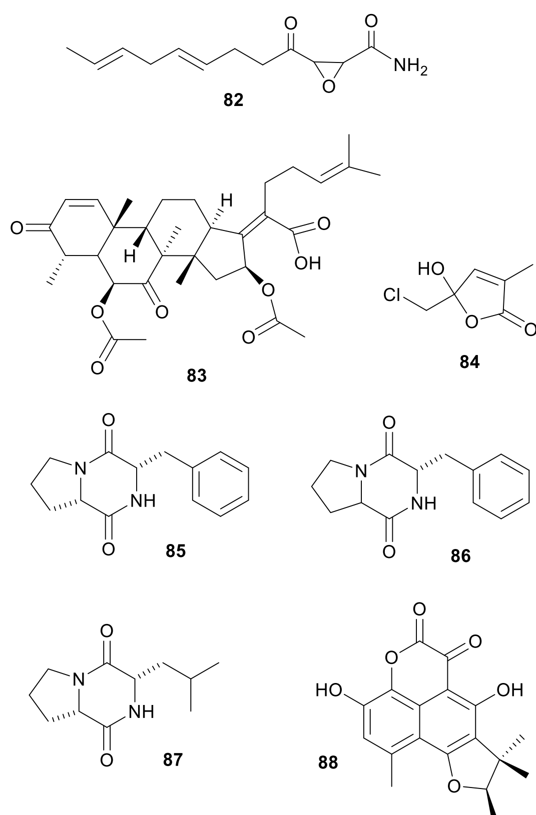
Similar to the aforementioned gut microbes, bacteria present on the egg surface of house flies are also involved in the preservation of nutrient provisions. As house flies lay their eggs on manure that their offspring will use for nutrition, the larvae will most likely encounter fungal competitors that 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 pipientis*, 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, *Euwalecea 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 lepiochlorin **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 *Ascosphaera 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 substances[369] 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

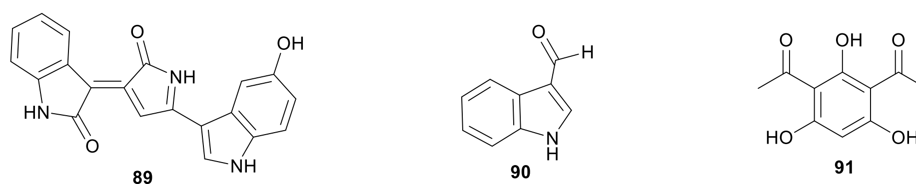
In contrast to invertebrates, only a limited number of specific defensive symbioses with microorganisms have been discovered in vertebrates. The relatively complex nature of the vertebrate microbiota as well as the difficulty of manipulative experimentation in this group of organisms severely restricts 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) 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 began 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 symbiosis. 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 synergism 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. epidermis* 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]

H-Ile-Ala-N-C-C-Lys-Phe-Ile-N-C-C-N-C-C-Pro-Gly-N-C-C-Ala-Lys-NH-C-C-Gly-N-C-C-Phe-Asn-N-C-C-Tyr-N-C-C-N-CH

92

Similarly, while *Bacterioides 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 can not 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 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 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.

Implications of host ecology and lifestyle for defensive symbioses

Protective associations between microorganisms and animals are present across a range of phylogenetically distant metazoan taxa (Fig. 2 and Appendix: Table S1). This ubiquity not only reflects the general potential to evolve defensive symbiosis, 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, life style 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, tunicates, some corals and mollusks exhibit an arsenal of defensive chemicals, more and more of which are being discovered to be of symbiotic origin (Appendix: 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 (but also some marine ones) 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.

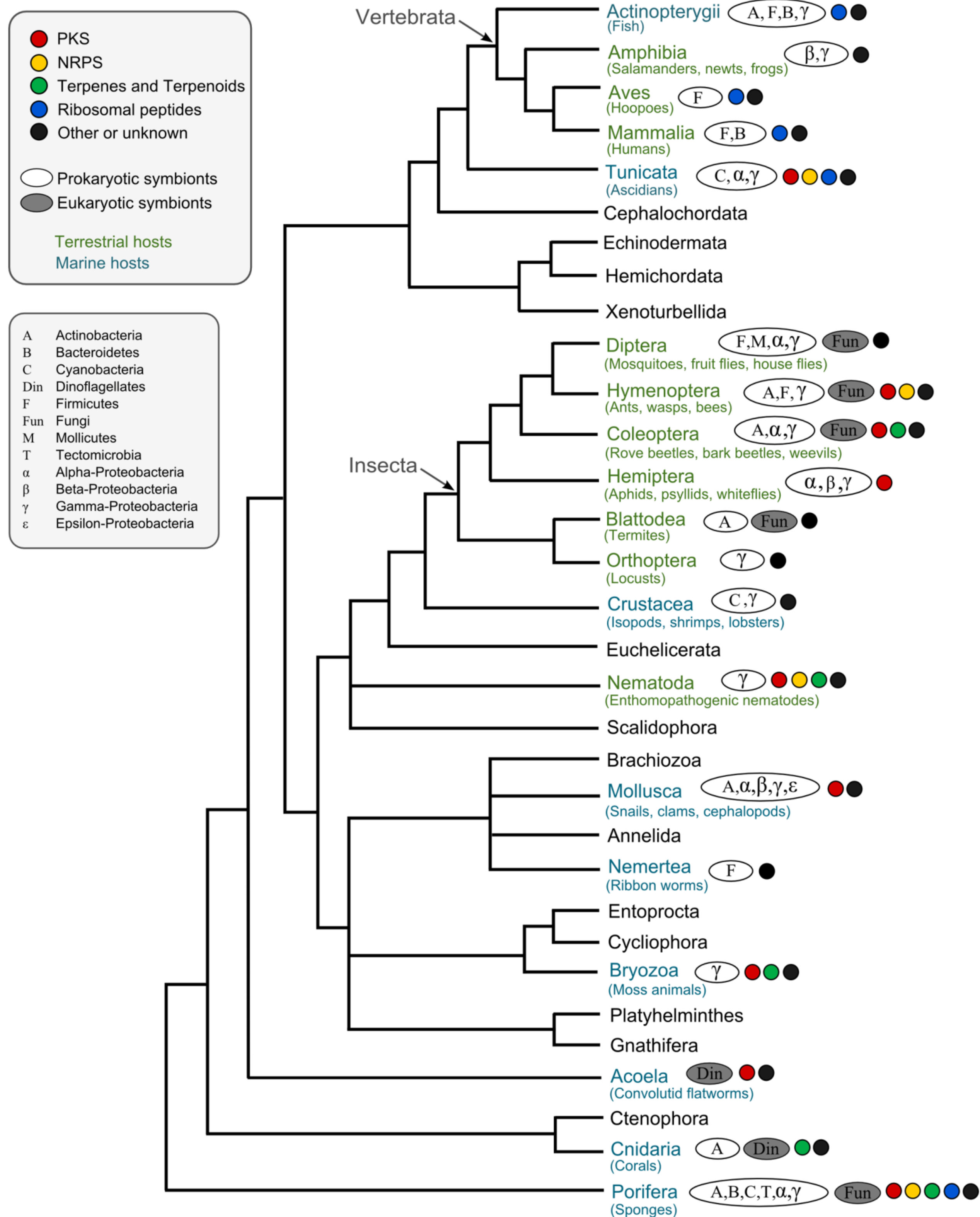


Figure 2. Cladogram of selected animal groups highlighting those with described defensive microbial symbionts and the corresponding symbiont taxa. Colored circles represent the major biosynthetic pathways reported for symbiont-produced compounds. Branch lengths are not to scale and branch order is adapted from previous phylogenetic analyses for the deep branches,[403] hexapods,[404] and all other bilaterians.[405, 406]

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 immunity[407] 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]

Diversity of defensive symbionts and protective chemicals

Across animals, a wide diversity of microbial partners have been identified as defensive symbionts (Fig. 2 and Appendix: 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 Appendix: 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 beta-lactam and oligosaccharide antibiotics (Fig. 2 and Appendix: Table S1).

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.

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 all[230] - 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 risk 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 intacellular symbiont of the asian citrus psyllid, '*Ca.* Profftella armatura', suggests that a horizontal transmission event, perhaps by ingestion of the psyllid by the beetle, 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 swinhoei*.[73, 76, 420] Although still lacking direct evidence, cases of potential HGT have 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-bacteria 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 ascidians[228] - 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]

4. 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 (Appendix: 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, phages 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 may 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.

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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CHAPTER 3

An out-of-body experience: The extracellular dimension for the transmission of mutualistic bacteria in insects

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3.1. Abstract

Across animals and plants, numerous metabolic and defensive adaptations are a direct consequence of symbiotic associations with beneficial microbes. Explaining how these partnerships are maintained through evolutionary time remains one of the central challenges within the field of symbiosis research. While genome erosion and co-cladogenesis with the host are well-established features of symbionts exhibiting intracellular localization and transmission, the ecological and evolutionary consequences of an extracellular lifestyle have received little attention, despite a demonstrated prevalence and functional importance across many host taxa. Using insect-bacteria symbioses as a model, we highlight the diverse routes of extracellular symbiont transfer. Extracellular transmission routes are unified by the common ability of the bacterial partners to survive outside their hosts, thereby imposing different genomic, metabolic and morphological constraints than would be expected from a strictly intracellular lifestyle. We emphasize that the evolutionary implications of symbiont transmission routes (intracellular vs. extracellular) do not necessarily correspond to those of the transmission mode (vertical vs. horizontal), a distinction of vital significance when addressing the genomic and physiological consequences for both host and symbiont.

3.2. Introduction

Through a variety of interactions, resident microorganisms have played a significant role in the origin and evolution of animals [1]. Among animals, insects serve as excellent models to elucidate the functional importance of these interactions, since they engage in a particularly wide range of mutualisms with bacteria and fungi [2].

The remarkable diversity in form and function of insect-microbial interactions, however, can only be rivaled by the variety of symbiont transmission structures and behaviors that contribute towards the fixation, persistence and evolution of such partnerships [2]. These adaptations ensure that beneficial microbes can be transferred across host generations directly from parent to offspring (vertical mode), indirectly from con- or heterospecific host individuals (horizontal mode), indirectly from the environment (horizontal mode), or through a combination of transmission mechanisms (mixed-mode) [3].

Some of the best-studied insect-bacterial mutualisms (e.g. aphids and *Buchnera*, carpenter ants and *Blochmannia*, tsetse flies and *Wigglesworthia*) have yielded extensive knowledge on the transmission ecology and the ensuing evolutionary consequences of mutualistic interactions between insects and intracellularly localized symbionts [4-12]. These obligate mutualists can be transmitted in a number of ways during oogenesis or embryogenesis [4]. For example, in carpenter ants, *Blochmannia* is vertically transmitted via an acute intracellular infection of the ovaries and subsequent incorporation into the eggs [9]. Similarly, in aphids, *Buchnera* is transovarially transferred to the developing eggs via a highly selective mechanism at the ovariole tips [10]. As for tsetse flies, their B vitamin-supplementing symbiont *Wigglesworthia* is transmitted via milk gland secretions as the larva develops *in utero* [11].

While many intracellular symbionts are maternally transmitted to the offspring [4], a recent study [12] has demonstrated both maternal and paternal vertical transmission of *Rickettsia* in leafhoppers.

The evolutionary implications of an intracellular lifestyle coupled with the strict vertical transmission mode of many insect symbionts have been the focus of considerable attention [5-8]. Nonetheless, research efforts of the past few decades have resulted in a steadily increasing body of knowledge on the diversity, function, and evolutionary history of extracellularly localized and transmitted symbionts in insects (Supplementary Table 1). Given this wealth of recent data, it is now feasible to assess the fundamental ecological and evolutionary implications of these types of transmission routes for both host and microbe.

In addition to providing an overview of extracellularly transmitted bacterial symbioses in insects, we discuss the evolutionary origin of such associations and the factors influencing their persistence. We also emphasize the impact of symbiont transmission on the co-evolutionary trajectory of the symbioses, and on the genomic and metabolic signatures of the bacterial partners. By illustrating that some extracellularly localized and transmitted bacterial symbionts can exhibit similar patterns of metabolic integration and host-microbe co-evolution as strictly intracellular mutualisms, we stress that the mode of symbiont transmission (vertical vs. horizontal) is a more accurate indicator of mutualism stability and integrative potential than the stage at which microbes are transmitted (prenatal vs. postnatal host development).

3.3. Overview of extracellular transmission routes of insect symbionts

Despite broad functional and taxonomic diversity (Figure 1; Supplementary Table 1), extracellularly transmitted symbionts can be unified by the ability to survive outside of their host for part, or all, of their lifetime. This feature markedly differentiates them from the majority of intracellular symbionts, where survival outside the host is no longer possible [5].

In insects, extracellular transmission routes for bacterial symbionts include environmental determination, coprophagy, smearing of brood cell or egg surface, social acquisition, capsule transmission, or infection via jelly-like secretions (Figure 2).

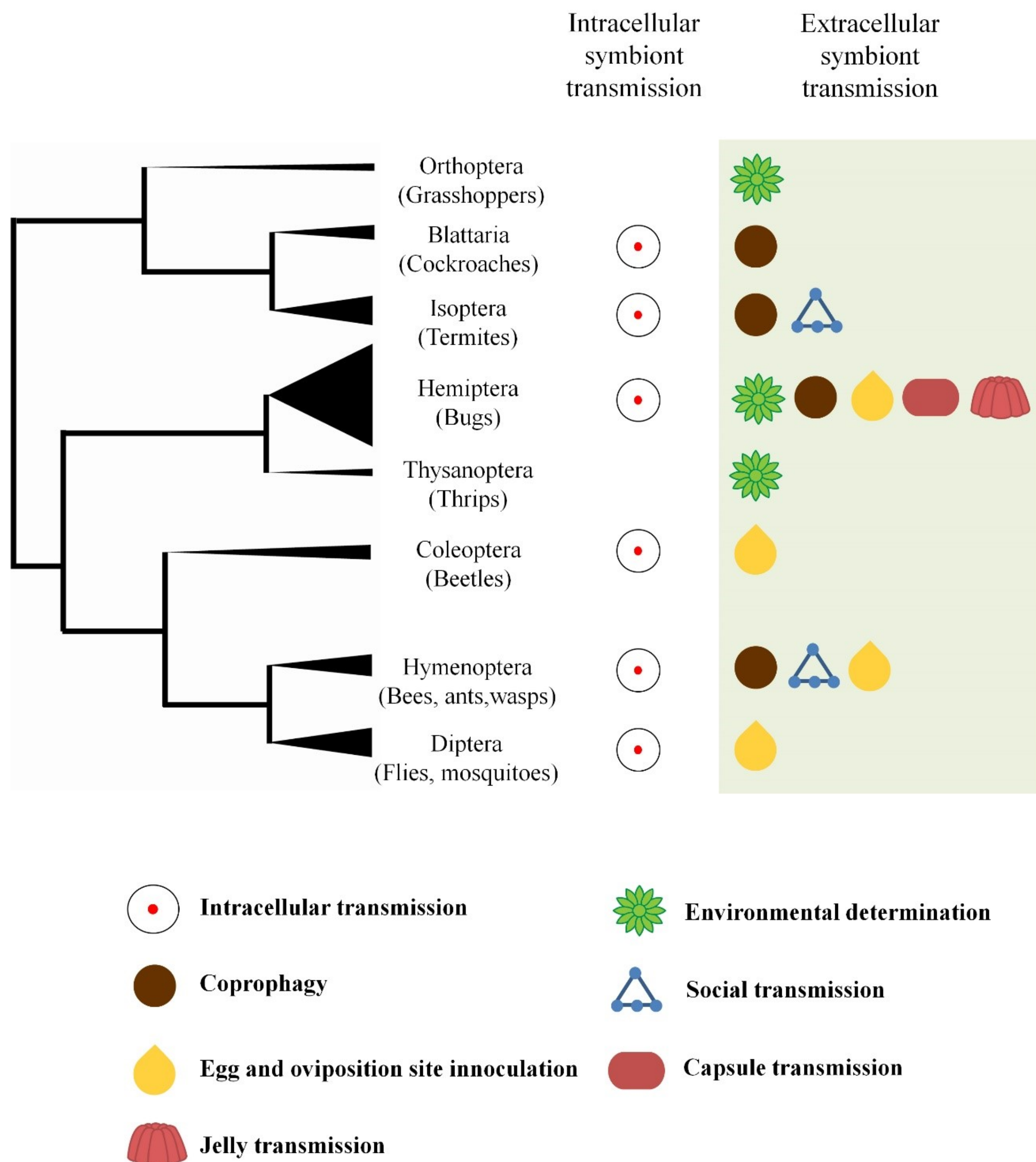

Figure 1. Cladogram depicting the diversity of insect orders with reported extracellularly transmitted bacterial symbionts (as listed in Supplementary Table 1). Symbols indicate extracellular transmission routes. Terminal branch thickness is proportional to the number of families within the order that have been reported to rely on an extracellular route for symbiont transfer. Orders featuring taxa with an intracellular symbiont transmission route have been designated with a symbol as well (per Supplementary Table 3).

Environmental determination

In animals, the acquisition of specific beneficial microbes from the environment is particularly prevalent in marine invertebrates including tubeworms and luminescent squids [13,14]. However, recent studies examining the microbial symbionts of several broad-headed bug species and whiteflies demonstrate that terrestrial environments can also be a suitable source for the acquisition of beneficial microbes by insect hosts [15,16].

Bean bugs (*Riptortus pedestris*), as well as many other species within the Lygaeoidea and Coreoidea superfamilies, harbor environmentally acquired *Burkholderia* symbionts that localize primarily within crypts along their posterior midgut section [11,17]. The environmental dimension of the symbionts' transmission route was first established following the inadvertent generation of developmentally regressed, aposymbiotic (symbiont-free) *R. pedestris* when the bugs were reared in sterile bottles. In fact, eggs laid in sterile laboratory settings by *Burkholderia*-infected individuals were also completely devoid of symbionts, strongly suggesting that the bugs acquired their free-living symbionts every generation from the environment, particularly the soil [15] – not unlike well-established plant-microbe partnerships involving rhizobia [18].

Environmental symbiont acquisition can also occur in associations with predominantly vertical transmission. In addition to their primary endosymbiont *Portiera*, whiteflies (*Bemisia tabaci*) also harbor a number of secondary symbionts, including a widely occurring *Rickettsia* sp. [19]. While *Rickettsia* has been demonstrated to be primarily transmitted vertically via the eggs during embryogenesis [19], significant inconsistencies were nonetheless observed between the phylogenies of hosts and symbionts, suggesting that the microbe likely undergoes substantial horizontal exchange between whiteflies [20]. Caspi-Fluger and colleagues [16] confirmed this by demonstrating that the symbiont can be transmitted among *B. tabaci* via the host plant, as demonstrated by the detection of *Rickettsia* in the phloem of cotton, basil and black nightshade plants following feeding by an infected whitefly. Additionally, *Rickettsia*-free individuals were successfully re-infected with the symbionts when allowed to feed on the same leaf (despite physical separation) as *Rickettsia*-infected *B. tabaci* [16]. However, the subsequent vertical transmission of horizontally acquired *Rickettsia* to the whitefly progeny has not yet been demonstrated. While the predominant route for symbiont acquisition in this system is vertical (via the egg), such findings suggest that plants may also serve as sources and sinks for symbiont inoculants in herbivorous insects.

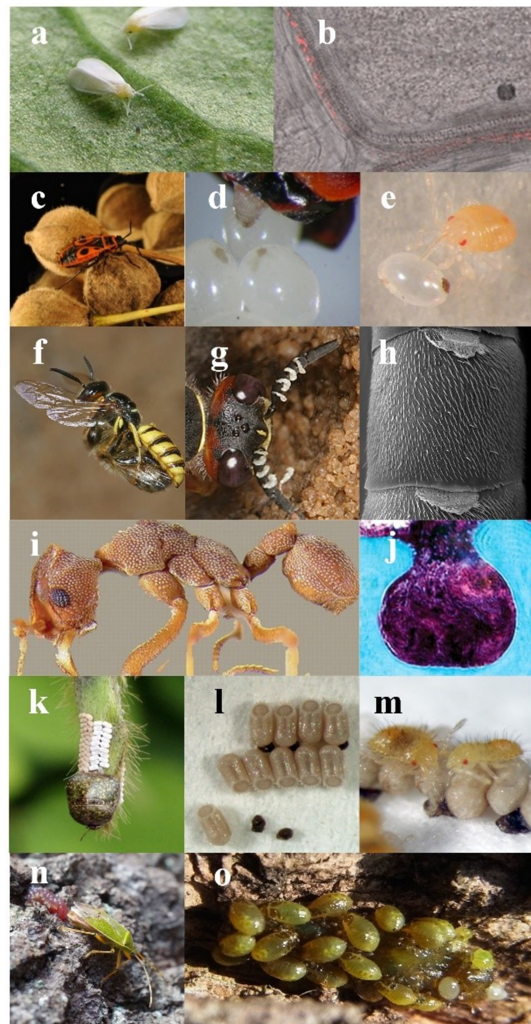

Figure 2. Extracellular symbiont transmission routes in insects. Horizontal transmission of *Rickettsia* among whiteflies (*Bemisia tabaci*) (**a**) involves the utilization of the insect's host plant [16] (**b**). Transmission of beneficial gut symbionts in the European firebug (**c**) relies on secretions that are smeared over the egg surface following oviposition (**d**, **e**) [38]. Beewolves (**f**) cultivate the defensive symbiont '*Candidatus* Streptomyces philanthi' in specialized antennal gland reservoirs (**g**, **h**) and transmit it via the brood cell [36]. Fungus-growing ants harbor defensive bacteria in specialized regions of their cuticle (**i**, **j**) that are transmitted via social behavior among nestmates [26]. Beneath their egg mass, plataspid stinkbugs (**k**) deposit brown symbiont-bearing capsules (**l**) that are ingested by newly hatched nymphs (**m**) to initiate infection with the gut symbiont. An adult female of *Urostylis westwoodii* depositing egg-encapsulating, symbiont-containing jelly (**n**) that is later ingested by newly hatched nymphs (**o**).

Coprophagy

Acquisition of beneficial bacteria through conspecific probing of feces has been described as a predominant route of symbiont transmission for several insect groups, including Hemiptera (true bugs) Blattaria (cockroaches) and Isoptera (termites) (Supplementary table 1). The symbionts usually reside in the insect gut, where they are shed

alongside the gut lumen and excreted in feces [21]. Symbiont acquisition by aposymbiotic individuals then requires direct contact with feces during or after excretion.

Interestingly, coprophagic symbiont transfer has been suggested to provide the opportunity for biological control of the reduviid bug *Rhodnius prolixus*, an important insect vector of the Chagas disease-causing parasite, *Trypanosoma cruzi* [22]. Despite near ubiquitous infection of adult *R. prolixus* with the actinobacterial symbiont *Rhodococcus rhodnii* in natural populations, newly hatched nymphs are aposymbiotic until they acquire the symbiont by probing conspecific feces [22]. The route of symbiont transfer, coupled with the bacterium's amenability for genetic transformation, could facilitate biological control via paratransgenesis, i.e. the introduction and expression of exogenous trypanocidal genes via the symbionts [22,23]. Such findings highlight the potential for manipulation of extracellularly-transmitted symbioses to control vector-borne diseases.

Social acquisition

Advanced social behavior in insects imposes different parameters for the transmission of microbial partners. A central feature of many social and subsocial insects is the intimate interaction of conspecifics through behaviors such as trophallaxis, the transfer of food or other fluids through mouth-to-mouth (stomodeal) or anus-to-mouth (proctodeal) feeding [24]. These behaviors can facilitate exchange of microbes among nest members, thereby contributing to maintenance of a beneficial microbiota, as has been demonstrated in ants [25-27], termites [28] and bees [29,30]. In fact, it has been speculated that the evolution of complex social forms could be reinforced, among other factors, by the convenience of acquiring beneficial microbes through recurring contact with conspecifics [31].

Recent examination of the gut microbiota of different bee species suggests that sociality plays an integral role in maintenance of the distinctive microbial communities within the Apoidea superfamily [29,32]. While the majority of solitary bee microbiota examined by Martinson *et al.* [33] seem to be indiscriminately dominated by *Burkholderia* or *Wolbachia*, the social corbiculate clade (including *Bombus* and *Apis*) carry a largely conserved microbiota that may have co-evolved with the hosts as a byproduct of eusocial behavior. In honeybees (*Apis mellifera*), workers lack this distinctive microbial community upon eclosion, and recent findings demonstrate that they acquire the most dominant members of the microbiota either through social contact with nest-mates (trophallaxis), specifically nurses, or via contact with the hive components (e.g. combs and honey) [30,34]. As for bumblebees, molecular analyses carried out across three host species (*B. sonorus, B. impatientis* and *Bombus* sp.) show that two of the most dominant bacterial strains (*Snodgrassella alvi* and *Gilliamella apicola*) are transmitted vertically from the mother colony to daughter queens, and that social contact among nestmates following pupal emergence is required for intra-colony transmission [34].

Social transmission of beneficial symbionts has also been described for fungus-farming ants (Attini: Formicidae) in their association with the defensive mutualist *Pseudonocardia* [25]. Most attine ant genera extracellularly harbor the symbiont in specialized cuticular crypts [26] (Figure 2i and j), and the presence of *Pseudonocardia* on foundress queens during their mating flight suggests a vertical transmission route linking parent and offspring colonies [25,26]. The singular association of each nest to individual *Pseudonocardia* strains further implies that the symbionts proliferate among nest members via social behavior [36,37], which was confirmed in a recent study by Marsh and colleagues [27]. Here, the ants were found to only acquire *Pseudonocardia* following contact with nest mates within the first two hours after emerging from their pupal cases.

Egg and oviposition site inoculation

Smearing bacteria over the surface of newly deposited eggs is one of the most commonly described routes of extracellular symbiont transfer and has been reported for various insect orders including Diptera, Coleoptera, Hymenoptera and Hemiptera (Fig. 1, Supplementary Table 1). Successful infection primarily depends on the ability of nymphs or larvae to acquire their bacterial symbionts shortly after hatching, usually through active probing of egg or brood cell surfaces.

Within the Hemiptera, numerous studies have reported that egg smearing by the mother leads to successful transmission of beneficial microbes, particularly among bugs of the Pentatomomorpha infraorder (*e.g.*, firebugs, stinkbugs, shield bugs, etc.) (Supplementary Table 1). For example, firebugs (Pyrrhocoridae) deposit excretion droplets that are later taken up from the egg surface by young nymphs, ensuring successful transfer of the two co-occurring actinobacterial symbionts *Coriobacterium glomerans* and *Gordonibacter* sp. [38] (Figure 2c-e). Similarly, in shield bugs of the family Acanthosomatidae, a γ-proteobacterial symbiont is harbored in cavities that are sealed off from the midgut main tract, as well as in a pair of lubricating organs associated with the female ovipositor [39]. It is through these specialized organs that the symbionts are vertically transmitted via egg surface contamination. Across both insect groups, disruption of the symbiont transmission route through the surface sterilization of newly laid eggs results in aposymbiotic individuals that suffer retarded growth, higher mortality, and lower reproductive success [39,40].

In constrast, transmission of defensive *Streptomyces* symbionts of solitary digger wasps (*Philanthus, Trachypus,* and *Philanthinus* sp.) relies not on surface contamination of eggs but rather of brood cells where eggs are deposited [41] (Figure 2f – h). Prior to oviposition, female wasps secrete a symbiont-containing white substance from their antennal glands and onto the ceiling of brood cells [41]. During cocoon spinning, larvae then take up the bacteria, which confer protection against pathogenic fungi in the brood through production of antibiotic substances on the cocoon [42].

Capsule and jelly transmission

Plataspid and urostylidid bugs utilize two of the most specialized mechanisms for extracellular symbiont transmission at the oviposition site [43-45]. In Plataspidae, adult females produce symbiont-enclosing “capsules”, which they deposit among their newly laid egg masses to ensure the successful vertical transmission of their γ-proteobacterial midgut mutualist ‘*Candidatus* Ishikawaella capsulata’ (Figure 2k – m) [43]. Infection of newly hatched nymphs is associated with capsule feeding [43]. In addition to the adverse fitness effects associated with aposymbiosis (*e.g.* high juvenile mortality and slow development), capsule removal causes bugs to wander from the egg masses rather than rest in aggregation - the typical behavior associated with capsule feeding [46]. This suggests that insect behavior may be linked to ensuring successful symbiont acquisition, as has been demonstrated in social insects.

In *Urostylis westwoodii* (Urostylididae), jelly-like secretions deposited by mothers over newly laid egg masses represent a remarkable adaptation with versatile biological roles [45]. While the sugar- and amino acid-rich jelly allows the newly-hatched nymphs to withstand the nutritional burdens of overwintering underground (in the absence of their natural food source of plant sap), Kaiwa and colleagues [45] have also implicated the gelatinous structure in ensuring the successful transmission of the gut symbiont ‘*Candidatus* Tachikaweaea gelatinosa’ following ingestion (Figure 2n, o).

3.4. Transition from a free-living state to symbiosis

Symbiont transmission mechanisms represent adaptations to ensure the maintenance of mutualisms. It is imperative to understand the factors that initially contributed towards the emergence of these mutualisms, and the evolutionary transitions that have shaped their history. Insects that rely on extracellular routes for transmission of their beneficial microbes present us with excellent systems to address such topics, given the diversity in evolutionary states of the symbioses, ranging from facultative and horizontally acquired, to obligate and vertically transmitted.

The origin of bacterial mutualisms, however, remains one of the most elusive questions within the field of symbiosis [47]. Traditionally, several hypotheses have been suggested for the initial evolution of microbial symbioses [48-50]. Recent phylogenetic analyses demonstrate the plausibility and occurrence of all these scenarios and suggest that the evolution of bacterial mutualists from environmental strains may represent a more common occurrence [51].

For extracellularly transmitted symbionts, there are several systems consistent with environmentally acquired bacteria providing immediate benefits to their hosts upon establishment. In leguminous plants, the nitrogen fixing ability of soil rhizobia coupled with the gain of a core set of symbiosis loci as selected for by access to a metabolically stable environment (*i.e.* the host), enabled the establishment and maintenance of a cosmopolitan mutualistic partnership [18]. Analogous mutualisms that exemplify this dynamic have also been described for insects. Within the bean bug's (*R. pedestris*) association with *Burkholderia*, recent findings by Kikuchi *et al.* [52] demonstrate that under certain conditions, the environment can select for the optimal symbiont; which, in turn, can inadvertently align the bilateral benefits of the host and symbiont without the prerequisite of strict vertical transmission.

However, for such mutualisms to be evolutionarily stable, the benefits of investing in a symbiont transmission mechanism by the host must outweigh a couple of significant costs: the risk of not acquiring the symbiont and/or acquiring an antagonistic partner (*e.g.* pathogens, parasites or cheaters). In other words, insects that rely on environmentally acquired symbionts [15,17] likely run a higher risk of aposymbiosis as well as pathogen exposure than mutualisms featuring adaptations that ensure faithful vertical transmission of the microbial partner [44,48,50].

For example, for *R. pedestris* and several other bug species, failure to pick up specific bacterial symbionts during a short development window significantly affects fitness [53]. Similarly, the uptake of a suboptimal symbiont also poses risks for the host in terms of competitively subsisting on resources in a specific niche [52]. Interestingly, across the true bugs, there are multiple independent origins of *Burkholderia* symbioses, many, and possibly all, of which are presumed to be dependent on environmental acquisition [17]. These bug-*Burkholderia* partnerships are interspersed amongst other systems that utilize vertical transmission through both internal and external mechanisms. One possibility is that environmental acquisition is selected for in fluctuating environments when the symbiont genotype that is most optimal varies significantly across space or time. Thus, being able to switch partners, and possibly to actively select the optimal partner or to allow potential partners to outcompete one another, may supersede the costs of risking the failure to obtain an optimal partner.

This raises interesting questions relating to the initial stages of symbiont colonization; specifically, what are the mechanisms that mediate the recognition and uptake of the right symbiont, and how does the host select for these microbes while eliminating less beneficial ones?

Mechanisms could be behavioral, in which insects actively seek out symbionts with certain traits, or physiological, in which insects take in a diversity of microbes and then actively winnow down associations to a narrow few. There has been little exploration of the former, but there is increasing evidence for the latter. As previously discussed, *R. pedestris* possess remarkably efficient symbiont detection and uptake mechanisms, where a mere 80 *Burkholderia* cells in a gram of soil are sufficient for successful infection [53] during a highly specific developmental window of acquisition [54]. The efficient establishment of only a small subset of the diverse microbes that these bugs encounter in soil is mediated, at least in part, through complex immunological processes that prevent the growth of other bacteria and may tightly regulate proliferation of the symbionts [54-58]. Antimicrobial peptides isolated from the

hemolymph of the *Burkholderia*-harboring coreoid *Alydus calcaratus,* for example, can suppress growth of some soil-dwelling Gram-negative bacteria (*e.g. Escherichia coli*), but not *Burkholderia* symbionts [58]. Comparative transcriptomic analyses of the midguts of symbiont-containing and aposymbiotic *R. pedestris* revealed an upregulation of cysteine-rich antimicrobial peptides, which could inhibit growth of some bacteria and also regulate symbiont populations [57]. These findings are complemented by evidence suggesting that *Burkholderia* symbiont establishment and proliferation requires the bacteria to have several genes necessary for combating host-induced stress [55,56]. This suggests that the host's physiology and immune system may be under selection to suppress proliferation of non-symbionts while allowing for regulated growth of symbiont populations. *Burkholderia* may have become the primary symbionts because they possessed the necessary features to facilitate establishment (*e.g.,* motility, resistance to host antimicrobial activity) prior to, and independent of, any host-mediated selection.

Another factor that could favor the evolution of environmental acquisition would be the ubiquity of the microbe in the environment. Common occurrence of beneficial symbionts in the environment could relax selection to maintain vertical transmission mechanisms. To date, we have little data on the prevalence of beneficial symbionts in environmental reservoirs in systems where environmental acquisition is known (but see [15,16,58,59]). Further insight into factors favoring the evolution of environmentally acquired mutualisms will require such environmental sampling as well as characterization of symbiosis mechanisms across groups of insects, such as has been started, to some extent, for the true bugs [17].

3.5. Evolutionary transitions among transmission routes

Given the diverse mechanisms of extracellular transmission and their varying ecological and evolutionary implications, it is tempting to speculate on likely scenarios of evolutionary transitions between transmission routes. Due to the high selective pressures of endowing beneficial symbionts efficiently to offspring, the majority of specific mutualistic insect-bacteria interactions appear to transition to vertical or mixed-mode transmission routes over the course of evolution.

Extracellular nutritional symbionts are usually localized within the gut, and significant numbers of cells are often shed and excreted along with fecal matter [21,38]. Thus, transmission via feces (*i.e.,* coprophagy and proctodeal trophallaxis) constitutes a simple transitory step from environmental acquisition to vertical transmission as it does not require any specialized morphological adaptations of the host [2]. In taxa with social interactions between parents and offspring or within groups of related or unrelated conspecifics, direct transfer of feces by proctodeal trophallaxis also ensures symbiont transmission along with the provisioning of enzymes that may facilitate digestive processes in immature individuals [27]. Non-social insects without direct contact between symbiotic and aposymbiotic individuals, on the other hand, can increase the probability of successful transmission to the offspring by applying symbiont-containing feces to locations that have high chances of being frequented and probed by the hatching larvae [2]. The egg surface is the most commonly used and reliable place of symbiont application and uptake (*e.g.* [2,39,60]), but in special cases with locally confined developmental conditions, the brood cell surface can be equally suitable [41].

Coincident with, or subsequent to, an increased fidelity in vertical symbiont transmission, several insects with extracellular symbionts have evolved specialized structures to house and transmit symbionts to the offspring (*e.g.* [43,45]). As discussed for the capsule and jelly transmitted symbionts of plataspid and urostylidid bugs, these structures can serve to protect the symbionts from abiotic stresses during egg development [45], as well as allow for enhanced control over the identity and number of symbiont cells allocated to offspring, thereby ensuring that the progeny are endowed with sufficient numbers of viable symbionts for successful colonization [61]. While speculative, such adaptations may also mitigate the chances of co-transmitting potentially detrimental microbes, which is consistent with theoretical predictions implicating the restriction of symbiont migration as a mechanism

adopted by the host in order to reduce virulent tendencies arising from competition between heterospecific symbiont lineages [62].

Finally, the highest level of integration between host and symbiont occurs when symbiont transmission is internalized within the host's body. Starting out with an extracellular symbiosis, however, this can only be achieved through a shift in the symbiont's lifestyle to intracellular maintenance and transmission to the developing oocyte or embryo (e.g. aphids-*Buchnera*, carpenter ants-*Blochmannia*) [4].

3.6. Implications of transmission ecology for symbiont genome evolution

Obligate mutualisms can have a strong effect on the evolution of the bacterial partner's genome. As exemplified by *Buchnera* and *Sulcia* – the primary intracellular endosymbionts of pea aphids and sharpshooters, respectively – as well as many others, symbiont genomes can undergo strikingly convergent patterns of degradation and reduction [5]. These small genomes exhibit extensive AT nucleotide enrichment and undergo accelerated molecular evolution [6,7]. Such features are presumed to be driven by gene loss resulting from a combination of strong genetic drift in small populations undergoing severe bottlenecks during transmission, and relaxed selection to no longer maintain genes necessary for an extracellular lifestyle [7].

As a result, many of the aforementioned genomic features became consequential hallmarks of an intracellular lifestyle within animal hosts [8]. Thus, it seemed unlikely that extracellularly transmitted and localized symbionts would undergo similar patterns of reductive genome evolution, considering that these microbes reside outside of the insect host for part, or all, of their life cycle, where they can readily undergo recombination to offset gene loss due to genetic drift, and generally need to retain a larger set of genes to survive in less stable environmental conditions and to move between various habitats (e.g. different host tissues and outside of hosts).

Nonetheless, studies examining genomic features of some extracellularly transmitted symbionts indicate that similar evolutionary processes can occur in these symbionts as those restricted to a strict intracellular lifestyle (Figure 3; Table 1).

Specifically, extracellularly transmitted symbionts of plataspid (capsule transmission), acanthosomatid (egg smearing) and urostylidid (jelly transmission) bugs possess reduced genomes (estimated sizes around 0.7 – 0.9 Mb) [39,44,45,63]. Additionally, *Ishikawaella* symbionts isolated from the plataspid *M. punctatissima* has other genomic features reminiscent of intracellular symbiotic bacteria, (i.e. AT nucleotide bias and few mobile elements) [63]. Examination of *Ishikawaella*'s metabolic potential reveals that despite significant gene loss, the bacterium retains the ability to synthesize almost all essential amino acids, in addition to some vitamins and cofactors [63]. This is consistent with the suggested benefit of the symbiont for *M. punctatissima*, whose plant diet is poor in essential amino acids and certain vitamins.

Despite exhibiting similar patterns of reductive genome evolution, pairwise comparisons of gene profiles between *Ishikawaella* and a range of intracellularly localized and transmitted symbionts revealed a number of important discrepancies that may reflect their different ecologies [63]. Most prominent was complete retention of genes involved in the TCA cycle, as well as many other genes underlying energy production and conversion. This was attributed to the more stringent metabolic requirements of an extracellular lifestyle, where access to metabolic intermediates in the host cytoplasm is not an option, unlike for many obligate intracellular symbionts. Additionally, *Ishikawaella* possesses a greater number of genes involved in the synthesis of amino acids and cofactors than *Buchnera* (aphids), *Blochmannia* (ants), or *Wigglesworthia* (tsetse flies), a condition that implies a broader metabolic repertoire for supplementation, and/or a younger co-evolutionary history with its host [63].

The genomic features of these symbionts provide insight into the evolutionary forces driving genome reduction in obligate microbial mutualists [39,44,45,63] by demonstrating that these convergent traits are not strictly a consequence of an intracellular lifestyle but rather are more likely due to increased impact of genetic drift associated with a host-restricted lifestyle. This highlights small population sizes and strong bottlenecks promoted by spatial isolation, prior to and/or during transmission, as important factors for genome evolutionary patterns in heritable symbionts.

These findings, coupled with analyses of a broad range of bacterial genomes demonstrating a clear inverse correlation linking genome size and the incidence of genetic drift [64] further support the concept that reductive genome evolution can be associated with intracellularity but is not necessarily derived from it.

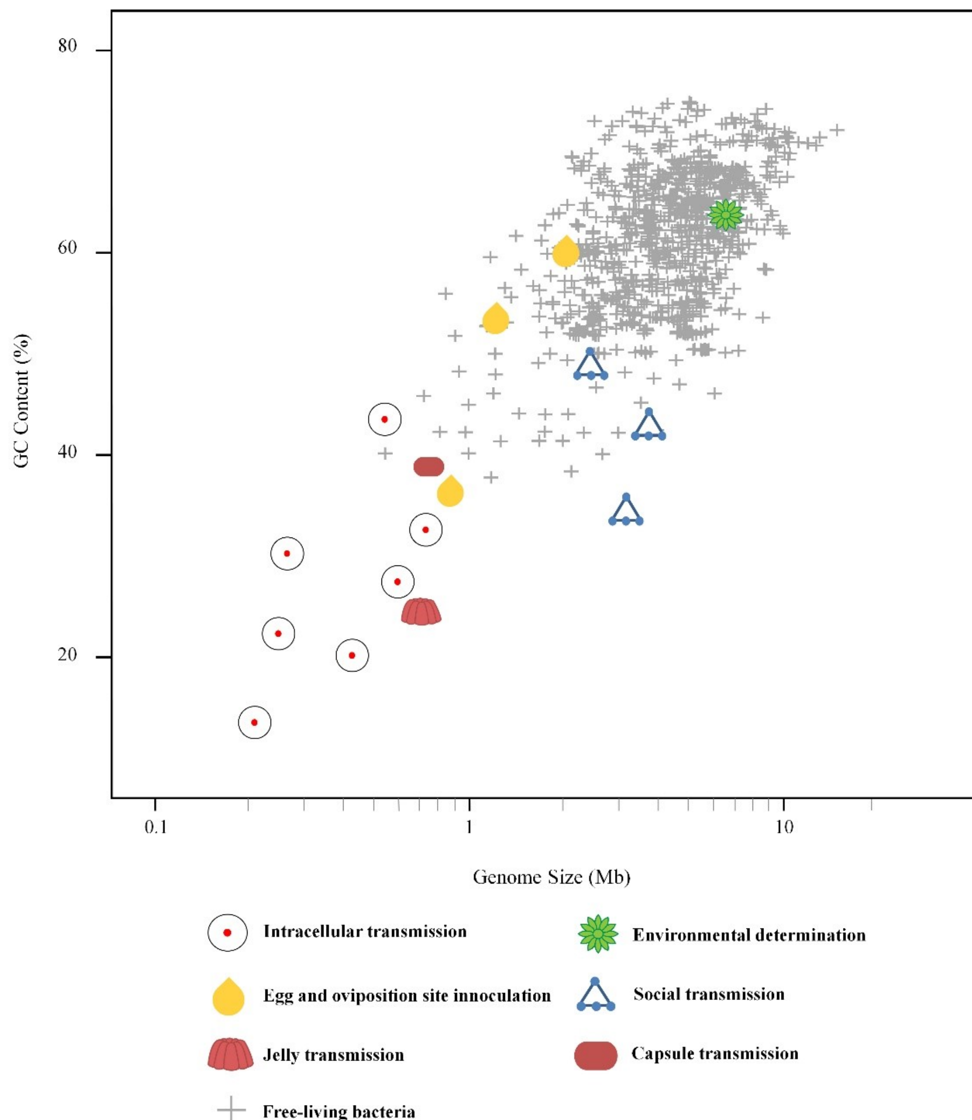

Figure 3. Relationship between genome size and GC content for a representative subset of intra- as well as all extracellularly transmitted bacterial symbionts in insects (per Supplementary Table 2), as compared to free-living bacteria. Symbols indicate symbiont transmission route and biotic condition (symbiotic vs. free-living).

3.7. Host-symbiont co-evolutionary dynamics

Acquisition of complex traits and adaptations by insects to ensure that their progeny are endowed with beneficial microbes often results in symbiotic systems that are evolutionarily stable and mutually obligatory. The high fidelity exhibited by these partnerships can be quantified (and visualized) through the congruent branching patterns of host and symbiont phylogenies in what is commonly referred to as co-cladogenesis [5]. Strict co-cladogenesis has been demonstrated in a number of insect-bacterial mutualisms featuring intracellular symbionts, for example among sap-feedings insects (e.g. aphids, sharpshooters, etc) [4].

With co-cladogenesis as a measure for mutualism fidelity, inferring phylogenetic relationships between symbiotic bacteria relative to their insect hosts can provide insights into the evolutionary implications of different routes of symbiont transmission. Bugs of the Pentatomomorpha infraorder – whose bacterial partners colonize similar gut regions but utilize different transmission routes and modes to initiate infection – provide a point of comparison for consequences of transmission routes on host-symbiont evolution (Figure 4). In members of this group that acquire beneficial bacteria via the environment (*e.g.* soil), there are numerous discrepancies between symbiont and host phylogenies [17] (Figure 4a). Some host species harbor different symbiont genotypes, while others share a single identical symbiont [17,58]. This lack of fidelity is driven by the bugs acquiring symbionts from a potentially diverse, shared environmental reservoir. Similarly, there are instances where vertical transmission of symbionts via egg smearing does not result in clear co-cladogenesis between host and microbe, as has been demonstrated in pentatomid [65] and pyrrhocorid bugs (Figure 4b) [66]. In such instances, it is presumed that while a mechanism exists for the microbes to be transmitted directly from mother to offspring, significant exchange of symbionts within and between host species is also taking place.

Conversely, in instances where gut symbionts are transferred directly from mother to progeny in a monoclonal manner, aided by symbiont-bearing structures and/or intimate behavioral responses, a remarkably convergent evolutionary history is often observed between host and microbe, irrespective of whether the symbionts are transmitted intra- or extracellularly [39,44,45]. For example, symbionts of Acanthosomatidae, Plataspidae and Urostylididae bugs, which are transmitted via egg smearing, symbiont capsules and jelly secretions, respectively, exhibit near strict co-cladogenesis with their hosts [39,44,45] (Fig. 4c-e), as has been shown for a multitude of intracellular symbionts [4].

Thus, despite the consistent localization of symbionts in similar midgut environments across many bugs, differences in extracellular transmission mechanism alter patterns of co-cladogenesis and, more broadly, likely have important consequences for co-evolution. Specifically, in cases of strict vertical transmission, fitness of host and symbiont are aligned, even in the absence of intracellular maintenance and transmission. However, when symbionts are either occasionally or frequently environmentally acquired, there is reduced host-symbiont fidelity and reduced alignment of host and bacterial interests. In the former case, we can expect that host and symbionts are both evolving in response to one another. In the latter case, much of the bacteria's adaptation may be shaped by forces external to a given host.

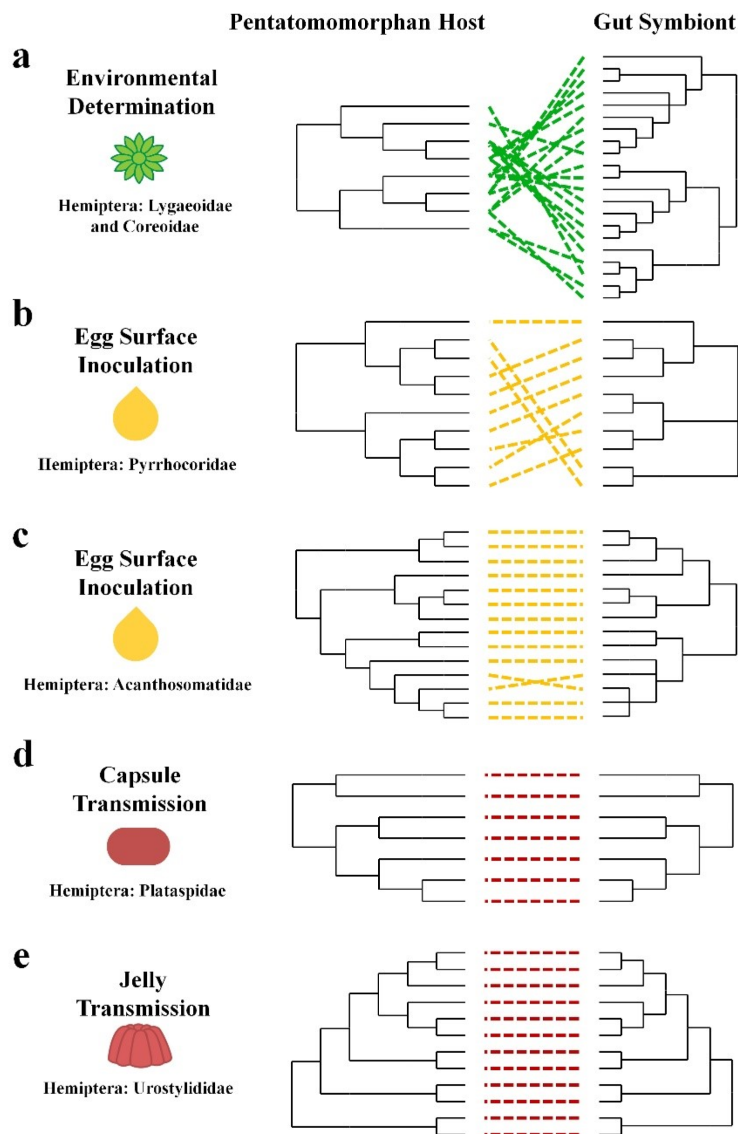

Figure 4. Comparison of evolutionary relationships between bugs and their gut symbionts as it relates to the symbionts' extracellular transmission routes. These relationships were established for (**a**) Lygaeoidea and Coreoidea and their environmentally-acquired *Burkholderia* symbionts [17], (**b**) Pyrrhocoridae [66] and (**c**) Acanthosomatidae [39] relying on egg smearing, as well as (**d**) Plataspidae [44] and (**e**) Urostylididae [45] utilizing symbiont capsules and jelly, respectively, for the transmission of their gut symbionts.

3.8. Conclusions and future perspectives

In addition to providing insights into evolutionary aspects of symbiosis, insect-bacterial mutualisms that rely on extracellular mechanisms for symbiont transmission present excellent opportunities to elucidate functional aspects of these partnerships. Given a transiently aposymbiotic phase during the early stages of insect development, alongside the ability of the microbe to survive outside of the host's body for part of its lifetime – two conditions universally shared across the aforementioned systems - it is in many cases experimentally feasible to physically separate both partners by disrupting the transmission cycle (*e.g.*[34,39,40,44,60]). Such experiments have been successfully employed to elucidate symbiont contributions towards host fitness, to assess host-symbiont specificity, and to detail the effects of symbiont replacement on host ecology (*e.g.* [67,68]).

Furthermore, the extracellular nature of the symbionts contributes to the likelihood that they can be cultured and genetically manipulated, which is not possible for most intracellular symbionts. *In vitro* cultivation and manipulation can facilitate introduction of genetically modified symbionts into their insect hosts. Thereby, the importance of

candidate symbiont genes for establishment or maintenance of a mutualistic association, as well as for the fitness benefits conferred to the host, can be directly assessed [55,56]. Additionally, this strategy may prove valuable to manage agricultural pest species or disease vectors by modification of their symbionts [22]. Combining symbiont manipulation with targeted knock-down of host genes potentially involved in mediating symbiosis will undoubtedly provide unprecedented opportunities to study host-symbiont molecular interactions and investigate the genomic and physiological underpinnings of these associations.

Conflict of interest statement

The authors declare that they have no competing interests.

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3.10. Supplement

Supplementary Table 1. Diversity of extracellularly transmitted bacterial mutualists in insects. Only those associations are listed for which the molecular identification and/or characterization of the symbionts are available, and a description of the transmission route as well as at least a putative function is reported. Exemplary references are given for each symbiosis (not exhaustive).

Insect Host		Bacterial Symbiont		Transmission Route	Biological Function(Putative)[a]	References
Order	Family - Genus/Species	Phylum	Genus/Species			
Hemiptera	Plataspidae - Various species	γ-Proteobacteria	'*Candidatus* Ishikawaella capsulata'	Capsule transmission	Nutrition[a]	[1-4]
	Acanthosomatidae - Various species	γ -Proteobacteria	'*Candidatus* Rosenkranzia clausaccus'	Egg smearing: gland secretion	Nutrition[a]	[5]
	Parastrachiidae - *Parastrachia japonensis*	γ -Proteobacteria	'*Candidatus* Benitsuchiphilus tojoi'	Egg smearing: gland secretion	Nutrition: Uric acid recycling[a]	[6,7]
	Pentatomidae - *Plautia stali*	γ -Proteobacteria	*Erwinia* sp.	Egg smearing: feces	Nutrition[a]	[8,9]
	Pyrrhocoridae - Various species	Actinobacteria	*Coriobacterium glomerans* *Gordonibacter* sp.	Egg smearing: feces	Nutrition: B vitamin supplementation	[10-13]
	Lygaeoidea and Coreoidea superfamilies - Various species	β-Proteobacteria	*Burkholderia* sp.	Environmental determination	Nutrition[a], Insecticide resistance	[14-16]
	Reduviidae - *Rhodnius prolixus*	Actinobacteria	*Rhodococcus rhodnii*	Coprophagy	Nutrition: B vitamin supplementation	[17]
	Aleyrodidae - *Bemisia tabaci*	α-Proteobacteria	*Rickettsia* sp.	Environmental determination	Unknown	[18,19]
	Cicadellidae - *Scaphoideus titanus*[b]	α-Proteobacteria	*Asaia* sp.	Egg smearing : bacterial colonies on ovarian egg surface Environmental determination	Unknown	[20]
	Urostylididae	γ -Proteobacteria	'*Candidatus* Tachikawaea gelatinosa'	Jelly transmission	Unknown	[21]

Thysanoptera	Thripidae - *Frankliniella occidentalis*	γ -Proteobacteria	*Erwinia* sp.	Environmental determination	Unknown	[22,23]
Coleoptera	Chrysomelidae - *Macroplea mutica, Macroplea appendiculata*	γ -Proteobacteria	'*Candidatus* Macropleicola'	Egg smearing: bacterial mass droplets	Habitat colonization: secretion provision for cocoon building in wetlands	[24,25]
	Chrysomelidae - *Donacia marginata, Donacia semicuprea*	γ -Proteobacteria	Unspecified	Egg smearing: bacterial mass droplets	Habitat colonization: secretion provision for cocoon building in wetlands	[25]
	Staphylinidae - *Paederus sabaeus*	γ -Proteobacteria	*Pseudomonas* sp.	Egg smearing : unspecified	Defense: biosynthesis of defensive toxin	[26,27]
Diptera	Tephritidae - *Bactrocera oleae*	γ -Proteobacteria	'*Candidatus* Erwinia dacicola'	Egg smearing: gland secretion	Nutrition[a]: protein hydrolisis and amino acid provision	[28,29]
	Tephritidae - *Bactrocera dorsalis*	γ -Proteobacteria	*Klebsiella oxytoca*	Egg smearing: gland secretion	Reproductive behavior: adult attractant	[30]
	Tephritidae - *Ceratitis capitata*	γ -Proteobacteria	*Klebsiella oxytoca* *Pectobacterium cypripedii*	Egg smearing: biofilm on egg surface, potential fecal contamination	Nutrition: pectin degradation and nitrogen metabolism. Reproductive behavior: shortened mating latency in males	[31,32]
	Muscidae - *Musca domestica*	γ-Proteobacteria, Firmicutes	*Klebsiella oxytoca* *Bacillus cereus*	Egg smearing: unspecified	Defense: against competing fungi in substrate for larval nutrition. Nutrition: diet supplement for larvae Reproductive behavior: influence on oviposition decisions.	[33]

Diptera (Cont'd)	Culicidae - *Anopheles* spp	α-Proteobacteria	*Asaia* sp.	Egg smearing: bacterial colonies on ovarian egg surface Environmental determination	Nutrition, defense, mediation of gut homeostasis and microbial equilibrium[a]	[34-38]
	Culicidae - *Aedes aegypti*	α-Proteobacteria	*Asaia* sp.	Egg smearing: unspecified Environmental determination	Nutrition, defense, mediation of gut homeostasis and microbial equilibrium[a]	[36]
Hymenoptera	Crabronidae - *Philanthus* spp., *Trachypus* spp., *Philanthinus* spp.	Actinobacteria	'*Candidatus* Streptomyces philanthi'	Brood cell contamination: antennal gland secretion	Defense: Protection against detrimental fungi	[39-42]
	Formicidae - Various Attine species	Actinobacteria	*Pseudonocardia* sp.	Social acquisition	Defense: production of antifungal compound against cultivar pathogen (*Escovopsis* sp.)	[43-45]
	Apidae - *Apis* spp.	α-, β-, and γ-Proteobacteria, Firmicutes	*Gilliamella apicola*, *Snodgrassella alvi* *Lactobacillus* sp., *Bifidobacterium* sp. and unspecified Acetobacteraceae	Social acquisition	Pectin degradation[a]	[46-50]
	Apidae - *Bombus* spp.	γ, β-Proteobacteria	*Burkholderia* *Gilliamella apicola*, *Snodgrassella alvi*	Social acquisition: coprophagy	Defense: protection against parasites	[46,51]
Blattaria	Blattidae - *Shelfordella lateralis*	Firmicutes, Bacteroidetes, δ- Proteobacteria	*Clostridium* sp., *Succinispira* sp. *Enterococcus* sp., *Erysipelothrix* sp.,	Coprophagy	Nutrition[a]	[52]
	Polyphagidae - *Cryptocercus punctulatus*	Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes	*Treponema* sp., *Spirochaeta* sp., others unspecified	Coprophagy	Nutrition[a]	[53]

Isoptera	Rhinotermitidae - *Reticulitermes* spp. Kalotermitidae - *Cryptotermes* spp. *Neotermes castaneus* Termopsidae - *Zootermopsis angusticollis* (lower termites)	Spirochaetes, Firmicutes, Bacteroidetes, Proteobacteria	*Treponema* sp. *Clostridiales* spp. *Lactococcus* sp. *Enterococcus* sp. *Bacteroides* spp. *Desulfovibrio* sp.	Social acquisition	Nutrition: N_2 fixation, acetogenesis, nitrogen recycling, cellulolytic activity.	[54-56]
	Termitidae - *Microcerotermes* spp. *Nasutitermes* spp. (higher termites)	Spirochaetes, Firmicutes, Bacteroidetes, Fibrobacteres.	*Treponema* sp., *Clostridiales* sp. *Bacteroidales* sp.,	Social acquisition, Environmental determination	Nutrition: N_2 fixation, acetogenesis, nitrogen recycling, cellulolytic activity	[57-59]
Orthoptera	Acrididae - *Schistocerca gregaria*	γ -Proteobacteria	*Pantoea, Enterococcus, Serratia, Klebsiella, Acinetobacter*	Environmental determination	Unknown	[60,61]

[a] No conclusive evidence, but hypotheses or suggestive results are reported.

Supplementary Table 2. Genome size and GC content for intra- and extracellularly transmitted bacterial symbionts in insects (as illustrated in Figure 3).

Symbiont	Symbiont Localization	Symbiont Transmission Route	Genome Size (Mb)	GC content (in %)	Reference
'Candidatus Zinderia insecticola'	Intracellular	Intracellular	0.21	13.5	[62]
'Candidatus Sulcia muelleri GWSS'	Intracellular	Intracellular	0.25	22.4	[63]
'Candidatus Uzinura diaspidicola'	Intracellular	Intracellular	0.26	30.2	[64]
'Candidatus Moranella endobia'	Intracellular	Intracellular	0.54	43.5	[65]
'Candidatus Blattabacterium sp.'	Intracellular	Intracellular	0.59	27.5	[66]
Buchnera aphidicola	Intracellular	Intracellular	0.66	26.4	[67]
Baumannia cicadellinicola	Intracellular	Intracellular	0.69	33.2	[68]
'Candidatus Tachikawaea gelatinosa'	Extracellular	Extracellular (Jelly transmission)	0.70	37.5	[21]
'Candidatus Ishikawaella capsulata'	Extracellular	Extracellular (Capsule transmission)	0.75	38.5	[4]
'Candidatus Rosenkranzia clausaccus'	Extracellular	Extracellular (Egg smearing)	0.94	37.6	[5]
Gut symbiont of *Adomerus triguttulus*	Extracellular	Extracellular (Egg smearing)	1,22	53.6	[7]
Coriobacterium glomerans	Extracellular	Extracellular (Egg smearing)	2.11	60.4	[69]
Treponema azonutricium	Extracellular	Extracellular (Social acquisition)	3.91	50	[56]
Gilliamella apicola	Extracellular	Extracellular (Social acquisition)	2.26	35	[70]
Snodgrassella alvi	Extracellular	Extracellular (Social acquisition)	2.3	43	[70]
Burkholderia sp. strain RPE64	Extracellular	Extracellular (Environmental determination)	6.96	63.5	[71]

Supplementary Table 3. Non-exhaustive list of intracellularly transmitted symbionts in insects.

Symbiont	Host (Order: Family)	Reference
'Candidatus Zinderia insecticola'	Spittlebugs Hemiptera: Clastopteridae	[62]
'Candidatus Sulcia muelleri GWSS'	Spittlebugs Hemiptera: Clastopteridae	[63]
'Candidatus Uzinura diaspidicola'	Scale insects Hemiptera: Diaspididae	[64]
'Candidatus Moranella endobia'	Mealybugs Hemiptera: Pseudococcidae	[65]
Buchnera aphidicola	Aphids Hemiptera: Aphididae	[67]
Baumannia cicadellinicola	Sharpshooters Hemiptera: Homalodisca	[68]
'Candidatus Portiera aleyrodidarum'	Whiteflies Hemiptera: Sternorrhyncha	[72]
'Candidatus Hodgkinia cicadicola'	Cicadas Hemiptera: Cicadidae	[73]
Wolbachia Spp.	Bed bugs Hemiptera: Cimicidae	[74]
'Candidatus Cardinium hertigii'	Midges Diptera: Ceratopogonidae	[75]
'Candidatus Blochmannia floridanus'	Carpenter ants Hymenoptera: Formicidae	[76]
'Candidatus Blattabacterium cuenoti '	Termites Isoptera: Mastotermitidae	[77]
Nardonella spp	Weevils Coleoptera: Curculionidae	[78]
'Candidatus Blattabacterium sp.'	Cockroaches Blattodea: Dictyoptera	[66]

Supplementary Table 4. Evolutionary implications of symbiont transmission routes in insects

Insect Host (Order: Family)	Bacterial Symbiont	Symbiont Localization	Extracellular Transmission Route	Maternal Provisioning of Symbionts	Specialized Structures for Symbiont Cultivation and/or Transmission	Strict Host Symbiont Co-cladogenesis	Symbiont Genome Erosion	References
Urostylis spp. (Hemiptera: Urostylididae)	'*Candidatus* Tachikawaea gelatinosa'	Midgut crypts	Jelly transmission	+	+	+	+	45
Megacopta spp. (Hemiptera: Plataspidae)	'*Candidatus* Ishikawaella capsulata'	Midgut crypts	Symbiont capsule	+	+	+	+	44,63
Elasmostethus spp. (Hemiptera: Acanthosmatidae)	γ-Proteobacteria	Midgut crypts	Egg smearing	+	+	+	+	39
Philanthus spp. (Hymenoptera: Crabronidae)	*Streptomyces philanthi*	Antennal gland reservoirs	Brood cell smearing	+	+	+/-	+/-	69,70
Pyrrhocoris apterus (Hemiptera: Pyrrhocoridae)	*Coriobacterium glomerans*	Midgut lumen	Egg smearing	+	-	-	-	38,71
Plautia spp. (Hemiptera: Pentatomidae)	*Erwinia* spp.	Midgut crypts	Egg smearing	+	-	-	Unexamined	60,65
Apis spp. and *Bombus* spp. (Hymenoptera: Apidae)	*Snodgrassella alvi*, *Gilliamella apicola*	Midgut lumen	Social transmission	+	-	-	Unexamined	32,72
Riptortus pedestris (Hemiptera: Alydidae)	*Burkholderia* spp.	Midgut crypts	Environmental uptake	-	-	-	-	17,73

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CHAPTER 4

Antibiotic-producing beetle mutualists evolved from plant-pathogenic bacteria

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4.1. Summary

Symbiotic interactions with microorganisms are widespread among plants and animals and have profound implications for the development, reproduction, and genome evolution of hosts and symbionts[1]. It is remarkable that pathogenic and mutualistic microbes are often closely related and generally lack consistent distinguishing features on the genomic level, indicating regular transitions between pathogenicity and mutualism[2]. However, the mechanistic basis and ecological context of such transitions have remained largely elusive[3,4]. Here we show that antibiotic production mediates a dynamic transition from plant pathogenicity to insect defensive mutualism in symbiotic *Burkholderia gladioli* bacteria. In a group of widespread herbivorous beetles (Lagriinae), these symbionts protect the vulnerable egg stage against detrimental fungi by producing a blend of antimicrobial compounds; namely toxoflavin, caryoynencin, and two novel polyketides, lagriene and lagriamide. Despite vertical transmission and a high degree of specificity in the beetle-*Burkholderia* association, the symbionts can be horizontally exchanged via the host plant and retain the ability to initiate a systemic infection at the expense of the plant's fitness. Our findings shed light on the evolution and chemical ecology of a novel defensive mutualism and provide a paradigm for the transition between pathogenic and mutualistic lifestyles. Furthermore, symbiont-mediated antimicrobial defense of the immobile egg stage may help explain the frequent evolution of egg-surface contamination as a route for vertical symbiont transmission across a wide range of insect taxa. The discovery of two previously unknown secondary metabolites with antimicrobial potential highlights insect-associated bacteria as promising sources of novel bioactive compounds.

4.2. Main text

Symbiosis is ubiquitous in nature and constitutes a major source of evolutionary innovation that has played a fundamental role in the origin and diversification of eukaryotic life on earth[5]. Microbial symbionts influence virtually all aspects of eukaryote biology[1], and their impact on host fitness ranges from detrimental to beneficial, occasionally shifting along this continuum[6]. Although such shifts have important implications for the ecological and evolutionary dynamics of symbiosis, observations of recent or dynamic transitions between parasitic and mutualistic lifestyles are scarce, and reports on their occurrence rely largely on indirect evidence[3]. Interestingly, genomic and phylogenetic analyses across bacterial groups reveal a lack of general signatures that distinguish pathogenic and mutualistic microbes[2-4]. Yet, ecological evidence documenting lifestyle transitions across eukaryotic hosts is largely lacking. Here we report on a dynamic transition between pathogenicity and mutualism in *Burkholderia* bacteria associated with the widespread group of herbivorous Lagriinae beetles (Coleoptera: Tenebrionidae). We unveil that this defensive insect mutualist evolved from plant pathogenic bacteria, elucidate the chemical mediators of defense, and propose an ecological context in which the lifestyle transition occurred.

Lagriinae beetles harbor extracellular bacteria in a pair of accessory glands connected to the female reproductive system[7] (Fig. 1a), which are transmitted vertically via the egg surface[7] (Fig. 1b). Shortly before hatching, a few bacterial cells enter the egg and colonize invaginations of the cuticle located dorsally in the embryo, which later close to form three compartments in the larva[7] (Fig 1c). To identify the bacterial symbionts associated with the invasive South American soybean pest *Lagria villosa*[8] and to confirm their vertical transmission route, we characterized the bacterial community in the symbiont-bearing structures of field-collected adult females and eggs laid by these females, as well as in larvae of laboratory cultures using 454 sequencing of bacterial 16S rRNA amplicons and quantitative PCR. We identified *Burkholderia* as the most prevalent bacterial taxon in female accessory glands and eggs (65-86% and 30-71% of reads per individual gland or egg clutch, respectively) (Extended Data Fig. 1a and b), and we found the same bacteria in mean abundances of 1.39×10^{6}, 1.83×10^{7} and 1.59×10^{8} 16S rRNA gene copies per egg, larva, and adult female gland, respectively (Extended Data Figure 1c). Longer 16S rRNA reads (1.1-1.3 kb) obtained by Sanger sequencing revealed that the symbionts are most similar to *Burkholderia gladioli*, a well-known plant pathogen, and that at least three highly similar strains coinfect *L. villosa* beetles (Extended Data Figure 2 and Table 1). One of these could be successfully isolated and cultured *in vitro* (*B. gladioli* Lv-StA). Additionally, *Burkholderia*-specific fluorescence *in situ* hybridization (FISH) confirmed symbiont localization in the adult female reproductive glands, on the egg surface, and in the unusual dorsal organs of larvae (Fig. 1).

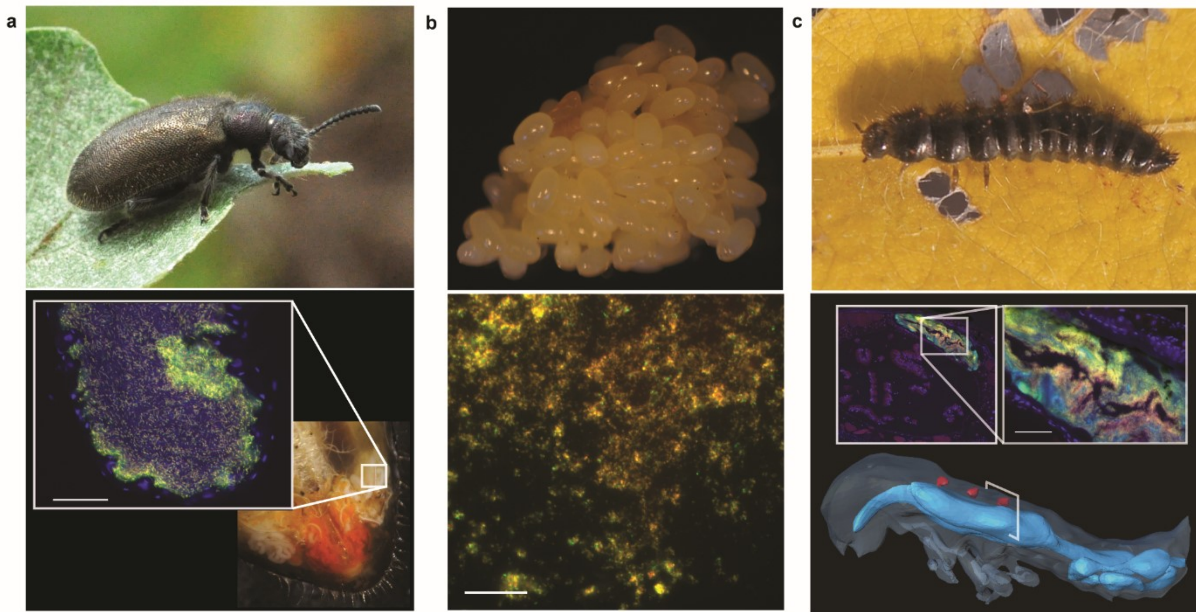

Figure 1. *Burkholderia gladioli* symbionts are transmitted vertically via egg-smearing in *Lagria villosa* beetles. (a) Adult females carry the symbionts within two pairs of accessory glands associated to the reproductive system as confirmed by FISH on cross-sections of a female reproductive system (inset). **(b)** Host eggs are covered with a secretion containing *Burkholderia* bacteria, as revealed by FISH on an egg wash. **(c)** The symbionts colonize invaginations of the cuticle that result in three dorsal compartments represented in red in a 3D-reconstruction of an *L. hirta* larva. The *Burkholderia* symbionts were localized by FISH in a cross section of an *L. villosa* larva (inset). FISH pictures show *Burkholderia*-specific staining in red (Burk16S_Cy3), general eubacterial staining in green (EUB338_Cy5), the overlap of these two in yellow, and host cell nuclei in blue (DAPI). Scale bars: 20 µm **(a)** and 50 µm **(b,c)**.

The specialized localization of the symbionts in the larval and adult stage, and the vertical transmission route suggested an important functional role of *Burkholderia* in the insect host. We therefore generated symbiont-free (aposymbiotic) beetles by egg-surface sterilization to evaluate potential differences to their untreated symbiotic counterparts. Notably, aposymbiotic eggs suffered more frequently from fungal infestation, pointing to a protective role of the symbionts. To test this hypothesis, we isolated spores of the most frequently encountered fungal antagonist of *L. villosa* eggs under laboratory conditions, *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*), which has been previously reported as an egg entomopathogen[9], and as a natural enemy of *L. villosa* adults and larvae[10]. Upon exposure to the fungal pathogen, surface-sterilized eggs experienced fungal growth significantly more often and at higher levels than control eggs (Fig. 2a and 2d, and Extended Data Fig. 3). Importantly, reinfection of surface-sterilized eggs with *Burkholderia* symbionts from egg washes or cultured *B. gladioli* Lv-StA significantly reduced fungal infestation, confirming that the absence of the symbionts rather than the surface-sterilization procedure itself was responsible for increased susceptibility to fungal growth (Fig. 2a and Extended Data Fig. 3).The symbionts' protective effect was further corroborated by the significantly higher probability of fungal growth on untreated eggs from aposymbiotic as compared to symbiotic mothers (Extended Data Fig. 4). In addition to *P. lilacinum*, the symbionts also inhibited the growth of the fast-growing soil fungus *Trichoderma harzianum* and the entomopathogen *Beauveria bassiana in vivo*, revealing a generalized antifungal protection by the symbionts (Fig. 2b and 2c). Although eggs suffering from *P. lilacinum* infection hatched at similar rates as those without fungus (Extended Data Fig. 5a), larvae hatching from infected eggs had significantly lower chances of surviving the first instars, demonstrating that fungal inhibition by the symbionts confers a benefit to the host (Fig. 2e and Extended Data Fig. 5b). Furthermore, the impact of fungal growth on survival varied among the different

treatments and was most pronounced for aposymbiotic individuals, suggesting costs of the symbiosis in the absence of fungal infection (Extended Data Fig. 5b).

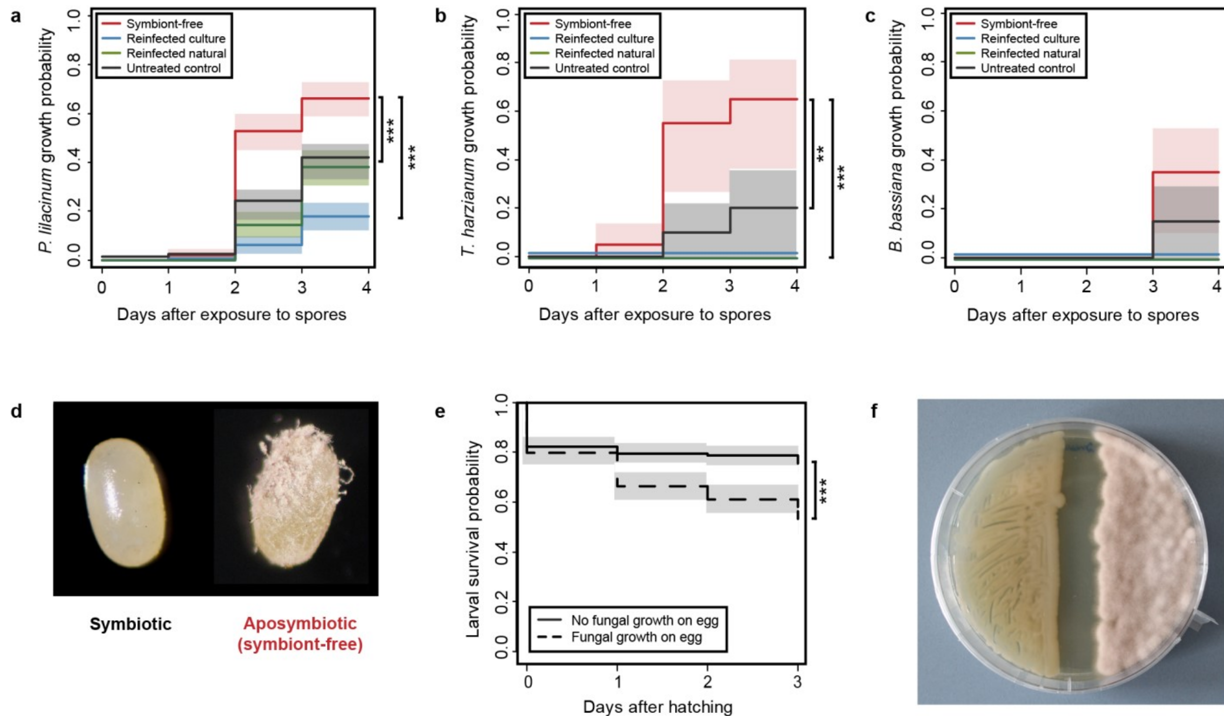

Figure 2. *B. gladioli* symbionts protect *L. villosa* eggs from fungal infestation. In the absence of the symbionts on *L. villosa* eggs, there is a significantly higher probability of the following three fungi to grow: (**a**) *Purpureocillium lilacinum* (Cox Mixed-Effects Model, $p<0.001$ compared to all controls), (**b**) *Trichoderma harzianum* (Mantel-Cox Log Rank test, $p<0.01$ compared to untreated control and $p<0.001$ compared to reinfected controls), and (**c**) *Beauveria bassiana* (Mantel-Cox Log Rank test, $p<0.01$ compared to reinfected controls). (**d**) Picture of a representative symbiotic and aposymbiotic egg after 4 days of exposure to *P. lilacinum* spores. (**e**) The growth of *P. lilacinum* on the egg has a negative effect on the survival of the larvae during the first days after hatching (Cox Mixed-Effects Model, $p<0.001$). (**f**) *In vitro* inhibition of *P. lilacinum* by *B. gladioli* Lv-StA. Asterisks indicate statistically significant differences: $p < 0.01$ (**) and $p < 0.001$ (***).

Given the antifungal activity of the cultured bacterial symbiont (*B. gladioli* Lv-StA) both *in vivo* (Fig. 2a-c) and *in vitro* (Fig. 2f), we used this strain to investigate the chemical nature of the symbiont-conferred protection based on whole-genome sequencing. Bioinformatic mining revealed several secondary metabolite biosynthesis gene clusters including those putatively coding for the previously described bioactive compounds toxoflavin[11,12] and caryoynencin[13], as well as an orphan gene cluster coding for a complex polyketide, homologous to the etnangien biosynthetic assembly line characterized in *Sorangium cellulosum*[14] (Fig. 3a and Supplementary Information). HPLC-MS-based metabolic profiling of *B. gladioli* Lv-StA culture extracts, which exhibit antibiotic activity (Extended Data Fig. 6a), confirmed the production of the azapteridine toxoflavin (1) and the polyyne caryoynencin (2), as well as a polyketide structurally related to etnangien, which we named lagriene (3) (Fig. 3b and Supplementary Information). Although these compounds were not detected in extracts from beetle eggs, likely due to low abundance and the high instability previously reported for caryoynencin[13,15], the antimicrobial activity of pure toxoflavin[11], caryoynencin[13] and lagriene (Supplementary Information) supports a potential role in defense. To gain additional insight into the antifungal effect *in vivo*, we extracted 156 *L. villosa* clutches (ca. 28,000 eggs) and analyzed the pooled extracts. HPLC-based micro-

fractionation in combination with an antifungal bioassay and subsequent bioassay-guided isolation (Extended Data Fig. 6b-c) led to the identification of another unprecedented metabolite with antifungal properties, named lagriamide (4) (Fig. 3b and Supplementary Information). The structure of lagriamide (4) was fully elucidated by MS and NMR (Fig. 3b and Supplementary Information).

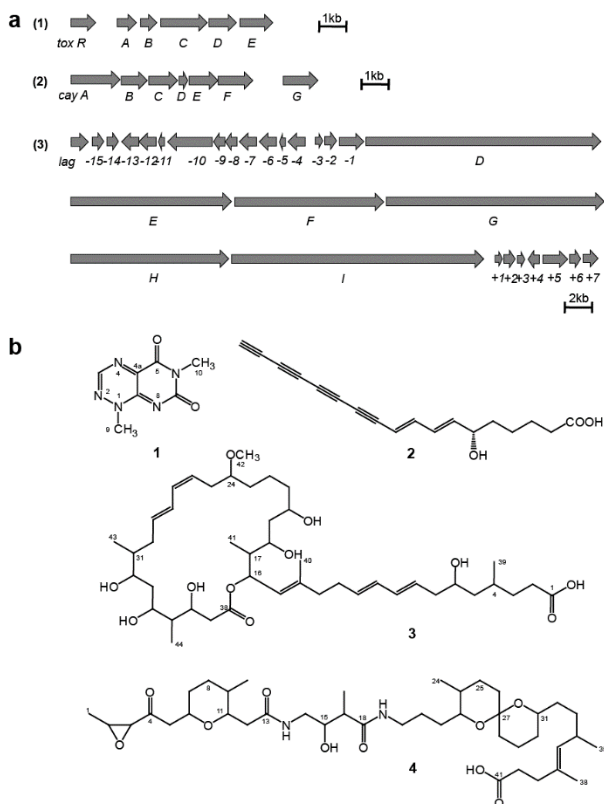

Figure 3. The *Burkholderia* symbionts of *L. villosa* produce an array of potent antimicrobial compounds. (a) Organization of biosynthetic gene clusters in *B. gladioli* Lv-StA corresponding to (**1**) toxoflavin (*tox*), (**2**) caryoynencin (*cay*) and (**3**) lagriene (*lag*). **(b)** Chemical structures of toxoflavin (**1**), caryoynencin (**2**), lagriene (**3**) and lagriamide (**4**).

HPLC-MS analyses and quantitative PCR revealed that the amount of lagriamide was strongly correlated with the abundance of *Burkholderia* on *L. villosa* eggs (Extended Data Fig. 6d). Since a putative lagriamide biosynthesis gene cluster is not present in the genome of the cultured isolate, we concluded that one of the two yet unculturable *B. gladioli* symbiont strains is responsible for lagriamide production on the eggs. In this context, it is particularly noteworthy that lagriamide is closely related to bistramides, a family of compounds isolated from the marine ascidian *Lissoclinum bistratum*[16]. The structures of bistramides suggest that they are produced by bacterial symbionts, too. Furthermore, the structural similarity of lagriamide and bistramides points to a common biosynthetic origin and possible horizontal transfer of the responsible gene cluster, as has been postulated for the symbiotically produced group of compounds comprising onnamide in sponges[17], pederin in staphylinid beetles[18], diaphorin in psyllids[19], and nosperin in lichens[20].

The finding that *B. gladioli* strains protect an insect was unexpected given that *B. gladioli* strains are well-known plant pathogens[21]. Hence, we set out to investigate (i) whether the beetle symbionts evolved from plant-pathogenic ancestors, and (ii) if they retained the ability to successfully infect host plants. Characterization of the bacterial symbionts in Lagriinae beetles from Europe (*Lagria hirta),* Brazil (*L. villosa*), Japan (*Lagria nigricollis*, *Lagria rufipennis* and *Lagria okinawana*), and Australia (*Ecnolagria* sp.) revealed the presence of *B. gladioli* in homologous accessory glands of females in all six species. Thus, considering also the morphological description of symbiont-bearing organs in numerous other Lagriinae species[8], the association with *B. gladioli* is likely ancient and widespread in this beetle subfamily. A 16S rRNA-

based phylogeny revealed that the symbionts are interspersed within the monophyletic clade of plant-pathogenic *B. gladioli,* strongly supporting the symbionts' plant pathogenic ancestry (Fig. 4d). The lack of symbiont monophyly indicates that – despite a considerable degree of symbiont specificity and a characterized route for vertical transmission – lagriid beetles at least occasionally exchange *B. gladioli* strains with their environment. Experimental exposure of soybean plants to female beetles and subsequent screening for *Burkholderia* indeed revealed the transfer of symbionts to the plant tissue (Fig. 4a). Furthermore, artificial infection of soybean with *B. gladioli* Lv-StA resulted in systemic infection (Extended Data Fig. 7) and reduced seed production compared to water-treated controls (Fig. 4b). Concordantly, cotyledon assays revealed that soybean mounts a defense response against *B. gladioli* Lv-StA (Fig. 4c, Extended Data Fig. 8)*,* showing that the plant recognizes the beetle symbiont as a pathogen. It is well conceivable that toxoflavin production by Lv-StA is involved in plant pathogenicity, as has been previously demonstrated for *B. gladioli* and *B. glumae*[11,12,22]. Thus, the beetle is capable of transmitting its symbiotic *B. gladioli* to soybean plants, where the bacteria can reproduce, spread systemically and ultimately impact plant fitness, confirming that the bacteria maintain the potential to interact pathogenically with a plant host, as well as mutualistically with the beetle.

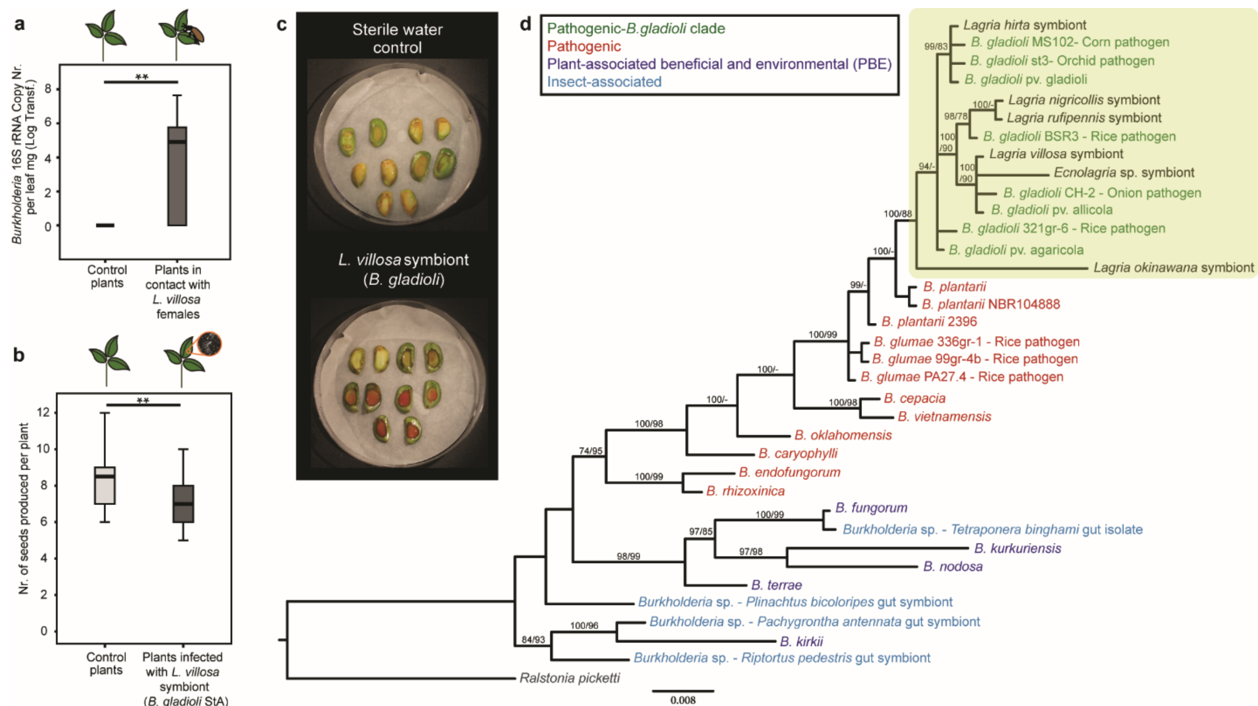

Figure 4. The symbionts of *L. villosa* evolved from plant-pathogenic *B. gladioli* and retain their ability to infect a plant host. (a) *L. villosa* females transmit *Burkholderia* to soybean plants (control plants N=9, *L. villosa*-exposed plants N=13; Mann Whitney *U* test, p<0.01). **(b)** Soybean plants infected with the symbiotic *Burkholderia* from *in vitro* cultures show reduced seed output (N=18 for each treatment; Mann Whitney *U* test, p<0.01). (**c)** Cotyledon assays reveal recognition of pathogenic elicitors by soybean (red coloration of wounded tissue) upon exposure to symbiotic *B. gladioli*. (**d)** Phylogenetic reconstruction based on Bayesian and approximately-maximum-likelihood algorithms of selected *Burkholderia* using partial 16S rRNA gene sequences (1,148 bp) showing the placement of lagriid-associated *Burkholderia* clustering with plant-pathogenic *B. gladioli*. Posterior probabilities (Bayesian inference) and local support values (FastTree) above 0.7 are reported at the nodes. References to sequences extracted from public databases and their categorization are listed in Extended Data Table 1.

The defensive symbiosis with *Burkholderia* constitutes a potential key innovation in Lagriinae beetles for the protection of the vulnerable and immobile egg stage exposed to the soil environment. Considering that egg-surface contamination is a widespread route for symbiont transmission in insects[23], this mechanism may have originally evolved for protection

or at least have been reinforced by the additional protective benefit. Symbiont-mediated egg defense is known for some marine crustaceans[24], and immobile larval stages are symbiotically protected against pathogenic fungi in beewolves[25] and leaf-rolling weevils[26], indicating that the protection of immature animals through mutualistic microbes may be a common phenomenon. In this context, the prolific production of a broad spectrum of secondary metabolites[27] and the ability to engage in pathogenic or mutualistic interactions with a wide range of eukaryotic hosts[28] may predispose members of the genus *Burkholderia* for defensive symbioses. The *in vitro* and genomics-guided metabolic profiling of the cultured *Lagria* symbiont as well as the chemical analysis of the not yet culturable symbionts directly *in vivo* on beetle eggs revealed that the associated *B. gladioli* strains provide a diverse antimicrobial armory. From a translational point of view, it should be highlighted that two of these symbiont-derived antibiotics, lagriene and lagriamide, are new. In addition, we showed that secondary metabolite production is important for beetle protection and likely also for plant pathogenicity[12,22], providing a plausible explanation for the evolutionary switch from pathogenicity to a dual lifestyle. There are many examples of insect-vectored plant pathogens[29,30], and some of these indirectly benefit their insect vector by altering plant physiology or suppressing plant defenses[29,30]. However, the Lagriinae symbiosis is exceptional in that the symbionts are not only consistently associated with their insect host, but also provide a direct benefit to the insect that is independent of its plant pathogenic effects. Our findings show that expanding from a plant pathogenic to an insect mutualistic lifestyle can be evolutionarily successful and describe an ecological setting in which it occurred. This not only contributes to our knowledge on lifestyle transitions in microorganisms, but also broadens our understanding on the evolution of defensive host-microbe associations and their dynamic nature. In addition, our findings highlight arthropod-associated microbes as promising sources for novel bioactive compounds.

4.3. Methods

Insect collection and rearing.

L. villosa individuals were collected in the localities of Itajú, São Carlos and Corumbataí within the state of São Paulo, Brazil between January and February 2015 (ICMBio authorization Nr. 45742-1, CNPq process nº 01300.004320/2014-21). Adults were fed with soybean leaves and kept at 23-26 °C with a natural light regime. Autoclaved water was supplied in centrifuge tubes with cotton, and moist cotton was provided for egg laying. Specimens from all other *Lagriinae* species were provided by collaborators in Germany (*L. hirta*), Japan (*L. nigricollis*, *L. rufipennis* and *L. okinawana*) and Australia (*Ecnolagria* sp.).

Fluorescence in situ hybridization (FISH).

Fluorescence *in situ* hybridization was carried out on sections of an *L. villosa* larva and female reproductive system, respectively, and on a suspension containing bacteria recovered from the egg surface. The Cy3-labeled *Burkholderia*-specific probe Burk16S (5'-TGCGGTTAGACTAGCCACT-3') (modified from primer BKH1434Rw[31]) and the Cy5-labeled general eubacterial probe EUB338 (5'-GCTGCCTCCCGTAGGAGT-3')[32] were used for hybridization, and DAPI (4′,6-diamidino-2-phenylindole) for host DNA counterstaining. Embedding, sectioning and FISH were performed as described previously[33], using a hybridization temperature of 55 °C.

3D-reconstruction.

An *L. hirta* larva was fixated in Bouin solution at 4 °C, dehydrated in a graded ethanol series and in isopropanol, and embedded using the Epoxy Embedding Medium kit (Sigma-Aldrich, Germany) following the manufacturer's instructions. Hardened epoxy blocks were cut in a rotation microtom (Mikrom HM355S, Thermo-Scientific,

Germany) to sections 2 μm thick and stained with toluidine blue-pyrimidine solution, humidified with xylol and covered with Entellan (Merck, Germany). Section images were acquired in an Axioimager Z1 Microscope (Carl Zeiss, Germany), and the reconstruction was carried out using Amira 5.4.1. software.

In vitro cultivation of symbiotic B. gladioli for in vivo bioassays and genome sequencing. Live *L. villosa* female adults were placed at –20 °C for 20 min and subsequently surface sterilized by rinsing in 70% ethanol. The paired glandular structures associated to the ovipositor were dissected in sterile PBS, and one of these was stored at –80 °C for nucleic acid extraction. The second one was homogenized in 100 μL of sterile PBS and diluted to a factor of 10^{-3}, 10^{-4} and 10^{-5}. 100 μL of each dilution were plated on Nutrient Agar, R2A Agar (Carl Roth GmbH, Germany), and Actinomycete Isolation Agar (Sigma Aldrich, Germany) and incubated at 30 °C. After 3 days, colonies with distinct morphologies were selected, and part of their biomass was transferred into a lysis solution (67 mM Tris–HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 5 mM beta-mercaptoethanol, 6.7 mM $MgCl_2$, 6.7μM EDTA (pH 8.0), 1.7 μL SDS) and kept at 90 °C for 5 min. This suspension containing free DNA was used for a diagnostic PCR with primers specific to the 16S rRNA gene of *Burkholderia* BKH1434Rw (3'- TGCGGTTAGRCTASCYACT-5')[31] and Burk3fwd (3'- CGGCGAAAGCCGGAT -5') (modified from[34]). Pure cultures of colonies corresponding to *B. gladioli* Lv-StA were kept as glycerol stocks until further use. Genomic DNA isolation was performed with the QIAGEN Genomic-tip 100/G kit (Qiagen, Germany) following the manufacturer's instructions. Genome sequencing was carried out using Single Molecule, Real-Time (SMRT) technology provided by Eurofins Genomics, Germany. To identify candidate biosynthesis gene clusters, antiSmash 2.0[35,50] and the Artemis genome browser and annotation tools [36] were used.

Cultivation and extraction of B. gladioli Lv-StA for metabolic profiling. Bacteria were grown in MGY liquid medium consisting of yeast extract (1.25 g L^{-1}) and M9 salts (50x, part A: 350 g L^{-1} K_2HPO_4; 100 g L^{-1} KH_2PO_4; part B: 29.4 g L^{-1} tri-Na-citrate-dihydrate; 50 g L^{-1} $(NH_4)_2SO_4$; 5 g L^{-1} $MgSO_4$) and glycerol (10 g L^{-1}) (toxoflavin/lagriene production) or in PDB (Difco™) (caryoynencin production) at 30 °C and 110 rpm for 5 days, respectively. The cultures were extracted with ethyl acetate, dried with sodium sulfate and concentrated under reduced pressure. For LC-MS measurements the extracts were dissolved in 500 μL methanol. For lagriene production, 50 L of MGY medium were inoculated with a one-day-old bacterial pre-culture (1.5 L in MGY) and incubated at 30 °C for 26 h followed by incubation at 28 °C for 76 h. The extraction was performed as described above.

Fungal inhibition on eggs and survival assays.

A layer of vermiculite substrate was added to 96-well plates and moistened with sterile water. Filter paper discs were then added individually to each well, excluding outermost rows and columns to avoid heterogeneous humidity conditions. 50 fungal (*P. lilacinum, T. harzianum or B. bassiana*) spores suspended in water were inoculated into each well.

For the assay with *P. lilacinum*, we used a total of 720 *L. villosa* eggs from six different clutches (120 eggs per clutch) laid by field-collected females. Eggs from each clutch were divided into four groups of equal size (30x) and randomly assigned to four different treatments. For the assays with *T. harzianum* and *B. bassiana,* 80 eggs from a same clutch were used (20x per treatment), respectively. All eggs were placed individually and distributed randomly in relation to treatment in the 96-well plates containing the fungal spores. The first group remained untreated as a control. The three remaining groups were washed in PBS and then surface sterilized by submerging them for 5 min in 90% ethanol, followed by 30 s in 12% NaClO, and a final rinse with sterile water. From the three surface sterilized groups, one (reinfected culture) was reinfected with a PBS suspension (2.5 μL per egg) of symbiotic *B. gladioli* Lv-StA (isolated

from *L. villosa*) previously grown in King B medium and adjusted to a concentration of $2x10^6$ cells μL^{-1} (to achieve a cell number comparable to naturally infected *L. villosa* eggs). The second (reinfected natural) was reinfected with the PBS suspension (2.5 µL per egg) recovered from the egg-washing step previous to sterilization, which contained *B. gladioli* and possibly other microbes naturally present on the eggs. 2.5 µL of PBS were added to each egg of the final group (Apo). Plates were stored in closed boxes at 25 °C and monitored daily for visible growth of fungal mycelia on the egg surface. Corresponding treatments were not labeled during monitoring (blind assessment). Hatching rate and survival during the first larval instar and early days of the second instar were also assessed in the assay using *P. lilacinum*. For this assay, statistical analyses were carried out in R 2.14.1. using the coxme package[37]. Cox Mixed Effects Models with a random intercept per clutch were used to analyze the effect of treatment on *P. lilacinum* growth on eggs, as well as the effect of treatment and fungal growth at the egg stage on the survival of early instar larvae. To analyze *T. harzianum* and *B. bassiana* the effect of treatment on fungal growth on the eggs was assessed using Mantel-Cox Log Rank tests in SPSS 17.0. Growth probability of all fungi and larval survival probability (*P. lilacinum* assay) were plotted based on Kaplan-Meier models using the rms package[38].

Nucleic acid extraction, amplification and sequencing.

The accessory glands dissected from adult Lagriinae beetles as described above, whole larvae and eggs (previously submitted to chemical extraction in methanol) were used for nucleic acid isolation. Tissue samples were homogenized in liquid nitrogen and subjected to DNA extraction using the MasterPure™ complete DNA and RNA isolation Kit (Epicentre). Prior to protein precipitation, samples were incubated at 37 °C with 4 µL lysozyme (100 mg mL^{-1}). The rest of the procedure was carried out following the manufacturer's instructions. Isolated nucleic acids were resuspended in Low TE buffer and stored at –20 °C. The 16S rRNA gene fragment was amplified using general eubacterial primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (3'- ACGGCTACCTTGTTACGACTT-5')[39] and *Burkholderia*-specific primers BKH1434Rw (3'- TGCGGTTAGRCTASCYACT-5')[31] and Burk16S_1F (3'- GTTGGCCGATGGCTGATT -5'). The PCR conditions were 3 min at 94 °C, followed by 32 cycles (eubacterial primers) or 42 cycles (*Burkholderia* primers) of 40 s at 94 °C, 60 s at 65 °C (eubacterial primers) or 62 °C (*Burkholderia* primers) and 60 s at 72 °C, and a final extension step of 4 min at 72 °C. Purified PCR products were then sequenced bidirectionally on an ABI 3730xl capillary DNA sequencer (Applied Biosystems, Foster City, CA, USA). For symbiont quantification in *L. villosa* eggs, larvae and female accessory glands, primers Burk16S_1F (3'-GTTGGCCGATGGCTGATT-5') and Burk16S_1R (3'-AAGTGCTTTACAACCCGAAGG-5'), which amplify a 172 bp region of *Burkholderia* 16S rRNA, were used for quantitative PCR in a RotorgeneQ cycler (Qiagen, Hilden, Germany) following the protocol described for the Rotor-Gene SYBR Green PCR Kit. PCR conditions were as follows: 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s and 65 °C for 30 s. A melting curve was subsequently performed with a temperature ramp from 60 °C to 99 °C within 4.25 min.

Phylogenetic analyses.

Burkholderia sequences obtained from accessory glands from females of the six different Lagriinae species were curated manually in Geneious 6.0.5 (http://www.geneious.com[40]) and aligned using the SINA alignment software[41]. The phylogenetic reconstruction including a representative or the single available symbiont sequence for each investigated Lagriinae species, and *Burkholderia* references, was based on an approximately-maximum-likelihood algorithm in FastTree 2.1.8[42], using a generalized time reversible model, and on Bayesian inference in MrBayes 3.1.2[43] using a HKY substitution model. The Bayesian analysis was run for 100,000 generations, sampling every 100 generations, and a

'burn-in' of 100 was applied. The phylogenetic reconstruction including the *L. villosa* symbiont strains and corresponding references was based on an approximately-maximum-likelihood algorithm in FastTree 2.1.8[42].

Microbial community analysis.

DNA samples from 16 *L. villosa* egg clutches and single accessory glands from five adult females were used individually for bacterial community characterization. 454 pyrosequencing and sequence processing and analyses were carried out as described previously[44], with minor modifications. Briefly, sequences were obtained from MR DNA (Shallowater, TX, USA) by bacterial tag-encoded FLX amplicon pyroseqencing (bTEFAP) using 16S rRNA primers Gray28F (5'-GAGTTTGATCNTGGCTCA-3') and Gray519R (5'-GTNTTACNGCGGCKGCTG-3')[45] and subsequently analyzed in Qiime[46]. After quality filtering, between 10,168 and 26,597 high-quality reads per sample were available for analysis. Sequences were clustered into operational taxonomic units (OTUs) using 97% similarity cutoffs, and one representative sequence per OTU was extracted for taxonomy assignment using the uclust consensus taxonomy assigner. For graphical representation, OTUs corresponding to the same genus were combined, and the percentage of reads in each genus relative to the total reads per sample was plotted.

Insect-mediated transmission of *Burkholderia* to soybean plants.

To test for transmission of *B. gladioli* from the insect host to soybean plants (*Glycine max*), we confined 18 individual *L. villosa* adults to single leaves and later used quantitative PCR to assess the presence and abundance of live *Burkholderia* in the leaf tissue. In a first group of 9 plants, we attached magnetic cages containing a single beetle to 2 independent leaves per plant. As a control, a second group of 9 plants had an empty magnetic cage attached to one leaf. After 3 days, all beetles were removed, and plants were maintained at 21-23 °C with a 16 h light regime for 12 more days until leaves were removed and stored at –80 °C for nucleic acid extraction.

Stored leaves were weighed individually, ground in liquid nitrogen and homogenized. For each sample, a fraction of known weight was separated for RNA extraction and processed using the MasterPure™ complete DNA and RNA isolation Kit (Epicentre) following the manufacturer's instructions. Reverse transcription was carried out using the Quantitect Reverse Transcription Kit (Qiagen) with *Burkholderia* - specific primers Burk16S_1F (3'-GTTGGCCGATGGCTGATT-5') and Burk16S_1R (3'-AAGTGCTTTACAACCCGAAGG-5'). Obtained cDNA was used for quantitative PCR as described above ("Nucleic acid extraction, amplification and sequencing" section). Beetles recovered from the experiment were dissected to confirm sex, revealing that 5 out of the original 18 individuals were males. Since adult males lack symbiotic *B. gladioli*, only the 13 replicates involving female beetles were included in the analysis. To test for statistically significant differences in *B. gladioli* titers between *L. villosa*-exposed and control plant leaves, a Mann-Whitney *U* test was carried out in SPSS 17.0.

Plant fitness effect upon *B. gladioli* infection.

A total of 36 soybean plants (*Glycine max)* were grown for 28 days before treatment. A single leaflet of the first trifoliate leaf on each plant was wounded in a circular area (0.5 cm diameter) using a robotic device that mimics herbivory damage (MecWorm[47]). Symbiotic *B. gladioli* Lv-StA previously isolated from *L.villosa* as described above were cultured overnight in King B liquid medium at 30 °C and constant shaking (200 rpm) and resuspended in sterile water at a concentration of 10^5 cells μL^{-1}. Half of the plants (N = 18) were inoculated with 10 µL of the bacterial suspension on the wounded area, and the second half were treated with the same volume of sterile water as a control. Plants were kept at room temperature with a 16 h light regime for 38 days after inoculation. Total seed number was determined

for all plants, and tissue samples were recovered from three regions on each plant: (i) the wounded area, (ii) a different area on the same leaflet, and (iii) a leaflet of a younger leaf (not wounded). RNA was extracted from the recovered tissues, and quantitative PCR specific for the 16S rRNA gene of *Burkholderia* was carried out on the corresponding cDNA on a 167 bp fragment using primers Burk16S_StAG_F (5'-CTGAGGGCTAATATCCTTCGGGG-'3) and Burk 3.1_R (5'-TRCCATACTCTAGCTTGC-3') as described for the horizontal transmission experiment. Statistical analyses regarding *Burkholderia* abundance and seed output were carried out in SPSS 17.0.

Cotyledon assay.

Symbiotic *B. gladioli* Lv-StA from *L. villosa,* and *E. coli* K-12 (Agilent Technologies, USA) were cultured overnight in King B liquid medium at 30 °C and continuous shaking (200 rpm). A fraction of the cultured *B. gladioli* cells were killed in 70% ethanol for 5 min. All cultures were centrifuged and resuspended in sterile water. The cotyledon bioassay procedure was based on a previously described protocol[48] with minor modifications. Briefly, 150 cotyledons from 5-day old *G. max* seedlings were washed in distilled water, placed in 10% NaClO and submerged in distilled water. Groups of ten cotyledons, using three replicates per treatment were cut and placed on moist filter paper. 50 µL of bacterial suspension containing 10^6 cells, sterile water (negative control), or ß-glucan (200 µg mg^{-1}) (positive control elicitor from the cell wall of the phytopathogen *Phytophtora sojae*) were applied on the wounded area of each cotyledon. After a 24 h incubation period, only cotyledons that retained the liquid (eight cotyledons per treatment) were individually washed in millipore water, and the content of mixed glyceolin isomers was determined by measuring absorbance at 285 nm. A Kruskall Wallis test with Dunn post-hoc test was carried out on the absorbance data in SPSS 17.0.

Antimicrobial bioassays.

The bioactivity of lagriene and lagriamide was studied by agar diffusion tests. Fifty microliters of a solution of the respective compound (1 mg mL^{-1} in methanol as a stock solution and respective dilutions) were filled in agar holes of 9 mm diameter (PDA, seeded with a spore suspension). After incubation at 30 °C for 24 h the inhibition zone was measured. Antibacterial activity was tested as described before[49].

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Sequence availability in public databases

The 16S rDNA nucleotide sequences of *Burkholderia* symbionts have been deposited in the GenBank genetic sequence database under the accession numbers KT888026 - KT888030 and KU358660 - KU358661. Raw 454 sequencing data corresponding to bacterial 16S rDNA amplicons from *L. villosa* female accessory glands and egg clutches have been deposited in the Sequence Read Archive of NCBI within BioProject PRJNA306502, under the accession numbers SAMN04364624 - SAMN04364628 and SAMN04510296 - SAMN04510311, respectively.

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4.5. Extended Data

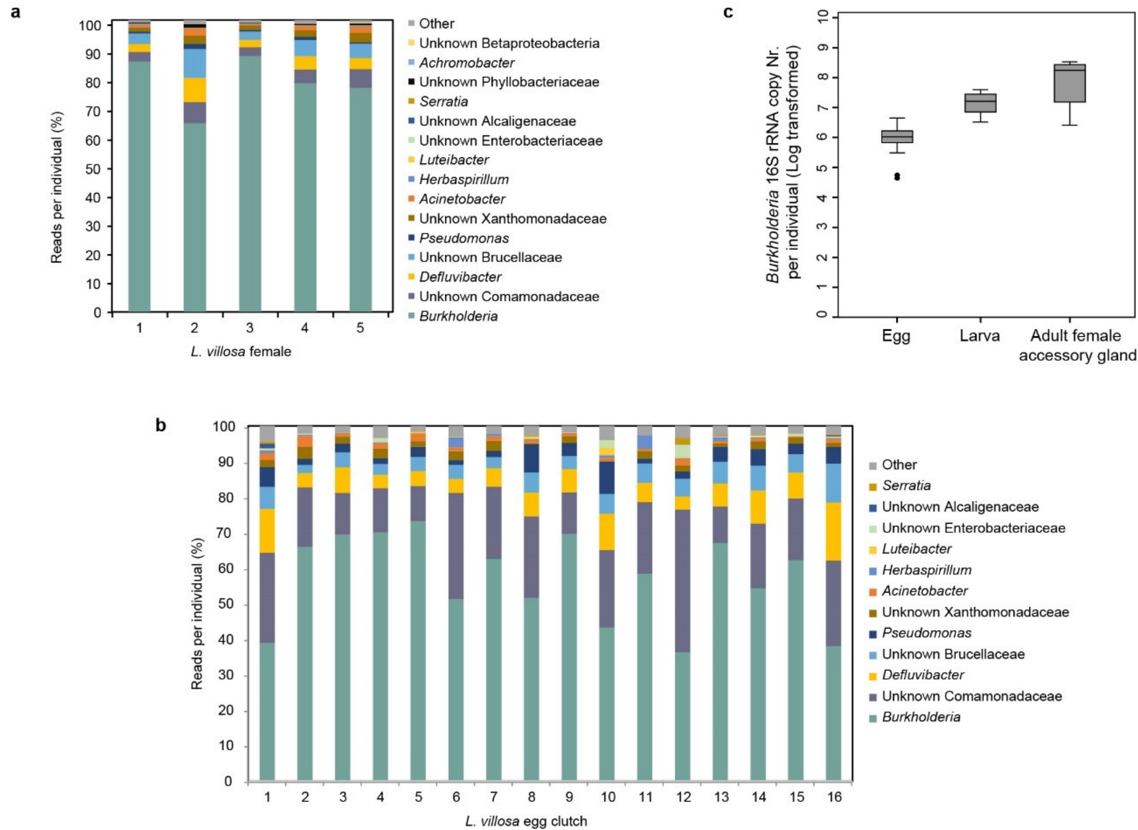


Figure 1. *Burkholderia* is consistently the most abundant taxon in *Lagria villosa* eggs, larvae and the accessory glands associated with the reproductive tract of adult females. Microbial composition as revealed by 454 pyrosequencing of partial 16S rRNA gene sequences carried out on (**a**) the accessory glands of six field-collected *L. villosa* females and (**b**) 16 egg clutches laid by field-collected *L.villosa* females, based on 97% similarity OTU clustering as described in the methods section (Microbial community analysis); (**c**) Quantification of *Burkholderia* symbionts by qPCR using a 172 bp region of the 16S rRNA gene as described in the methods section (Horizontal Transmission experiment) in 15 egg clutches (abundance per individual egg is represented), six larvae between 32 and 43 days old, and eight accessory glands from adult females (abundance for a single gland per individual is represented).

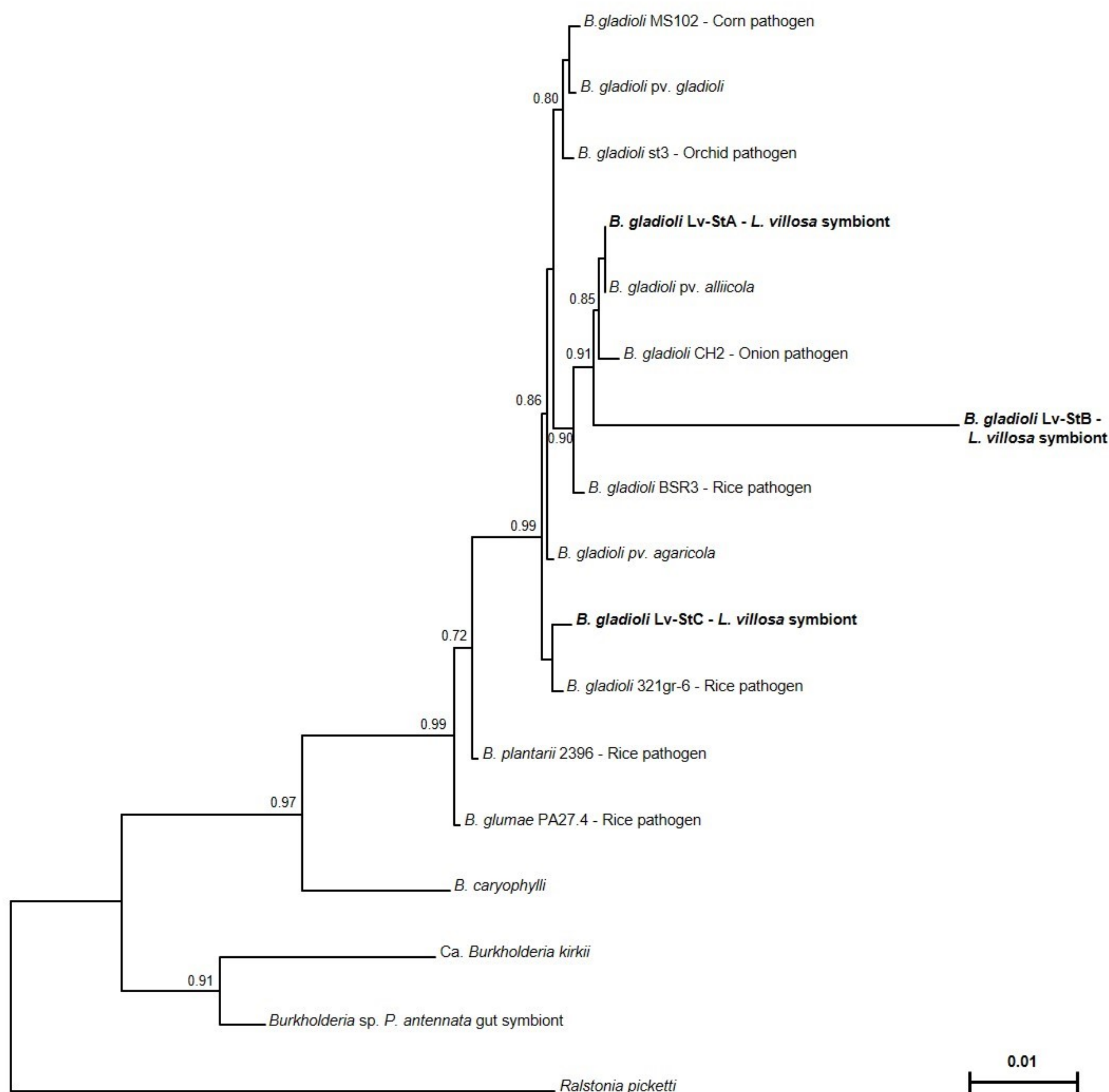


Figure 2. *L. villosa* beetles carry at least three symbiotic *Burkholderia gladioli* strains. Phylogenetic reconstruction based on an approximately-maximum-likelihood algorithm of selected *Burkholderia* using partial 16S rRNA gene sequences (1,120 bp), showing the placement of the *L. villosa* - associated strains relative to other *Burkholderia*. Local support values above 0.7 are reported at the nodes. References to sequences extracted from public databases are listed in Extended Data Table 1.

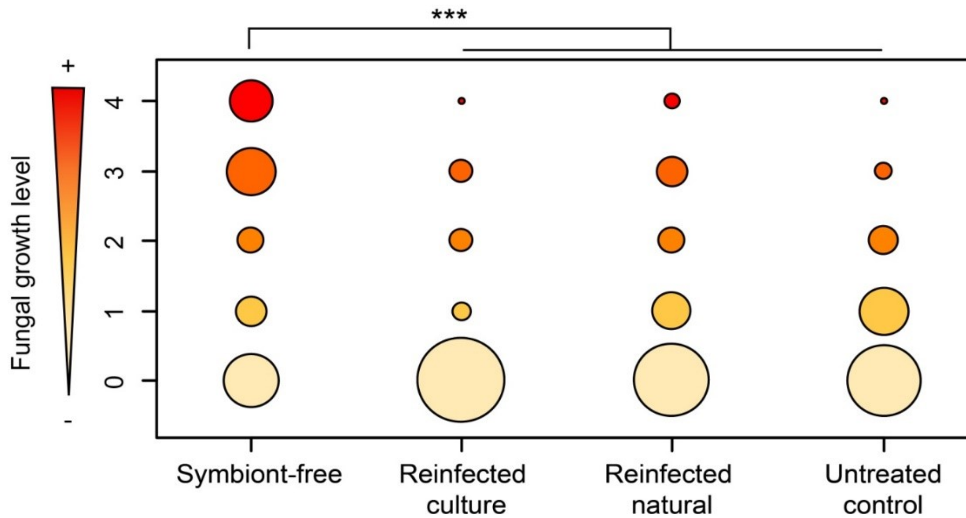


Figure 3. When present, *P. lilacinum* reaches higher biomass on *L. villosa* eggs in the absence of the *Burkholderia* symbionts. Fungal growth was estimated qualitatively during blind monitoring of 720 eggs (180 eggs per treatment from 6 independent clutches) as described in the methods section (*Fungal inhibition on eggs and survival assays*), assigning the level of growth to one of the following categories (0 = no visible growth, 1 = minor growth directly on surface and barely noticeable, 2 = multiple mycelia in contact with surface, 3 = considerable growth on surface, 4 = surface completely covered by mycelia). For statistical analysis, a generalized linear mixed model with a Poisson distribution and clutch as random factor was used (***p < 0.001).

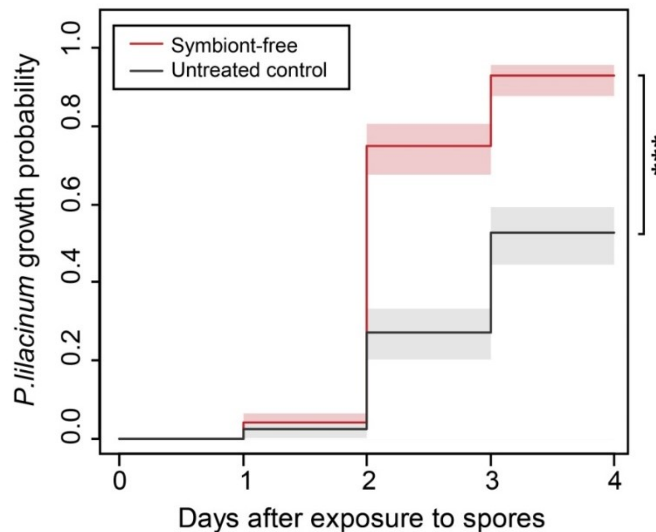


Figure 4. Eggs laid by symbiont-free *L. villosa* females are less protected against *P. lilacinum* fungal growth in comparison to their symbiotic counterparts. Symbiont-free females were obtained by rearing from surface-sterilized eggs, while symbiotic females were taken from the normal beetle culture. For each treatment, six clutches and 30 eggs per clutch were tested as described in the methods section excluding reinfection procedures (*Fungal inhibition on eggs and survival assays*). For statistical analysis, a Cox mixed effects model including clutch as a random factor was used (***p < 0.001).

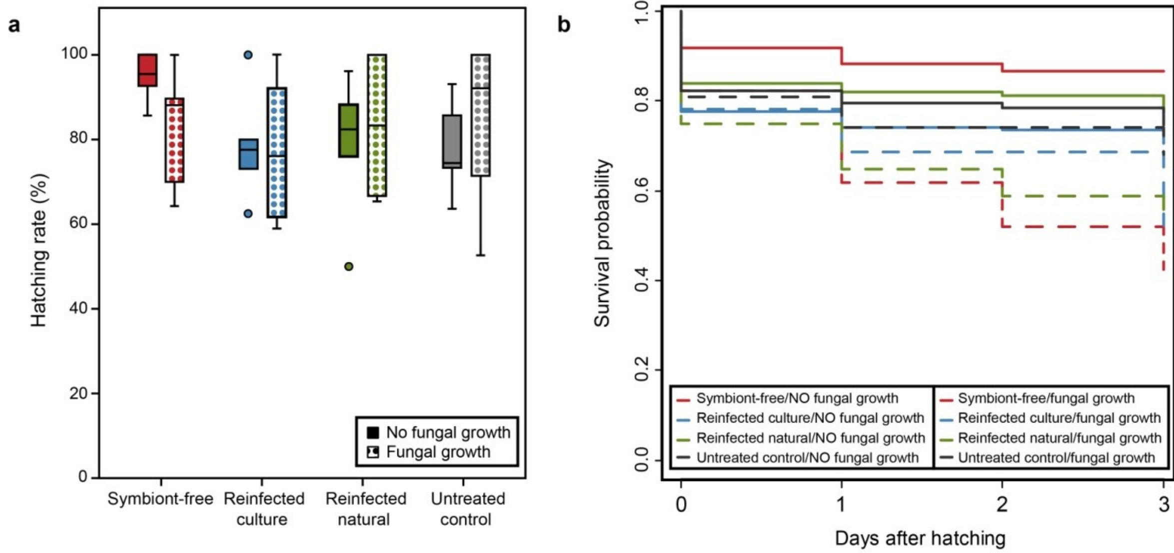


Figure 5. The detrimental effect of *P. lilacinum* growth on *L. villosa* eggs does not significantly reflect on hatching rate, but it causes increased larval mortality in the first days after hatching. (**a**) There was no statistically significant effect of either treatment or fungal growth level on hatching rate of the six egg clutches (Generalized linear mixed model with a Poisson distribution, $p > 0.05$). (**b**) Fungal growth on the eggs has a negative effect on the survival of the larvae during the first days after hatching and affects individuals from the treatments differently, with aposymbionts showing the most pronounced effect (Cox Mixed-Effects Model; Fungus, $p < 0.001$; Treatment:Fungus, $p < 0.05$).

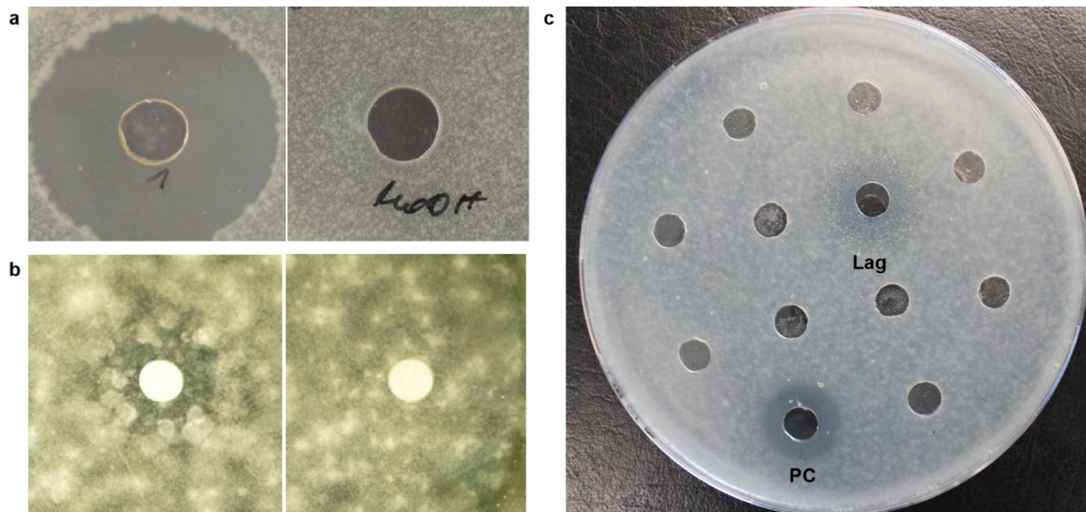


Figure 6. Compounds produced by *L. villosa* symbionts exhibit antimicrobial activity. (**a**) Antibiotic activity of *B. gladioli* Lv-StA (crude extract) against *B. subtilis* (left) and the corresponding MeOH control (right); (**b**) Antifungal activity of a crude extract from *L. villosa* eggs against *A. niger* (left) and the corresponding control (MeOH) (right) in PDA; (**c**) Bioassay of fractions from *L. villosa* egg extracts showing antifungal activity against *A. niger* by the lagriamide-containing fraction (Lag) and Nystatin (50 µg/mL) as positive control (PC); (**d**) The amount of lagriamide on the egg surface shows a highly significant correlation with the abundance of *Burkholderia* on the eggs (N = 51 clutches, Spearman's $\rho = 0.846$, $p < 0.001$).

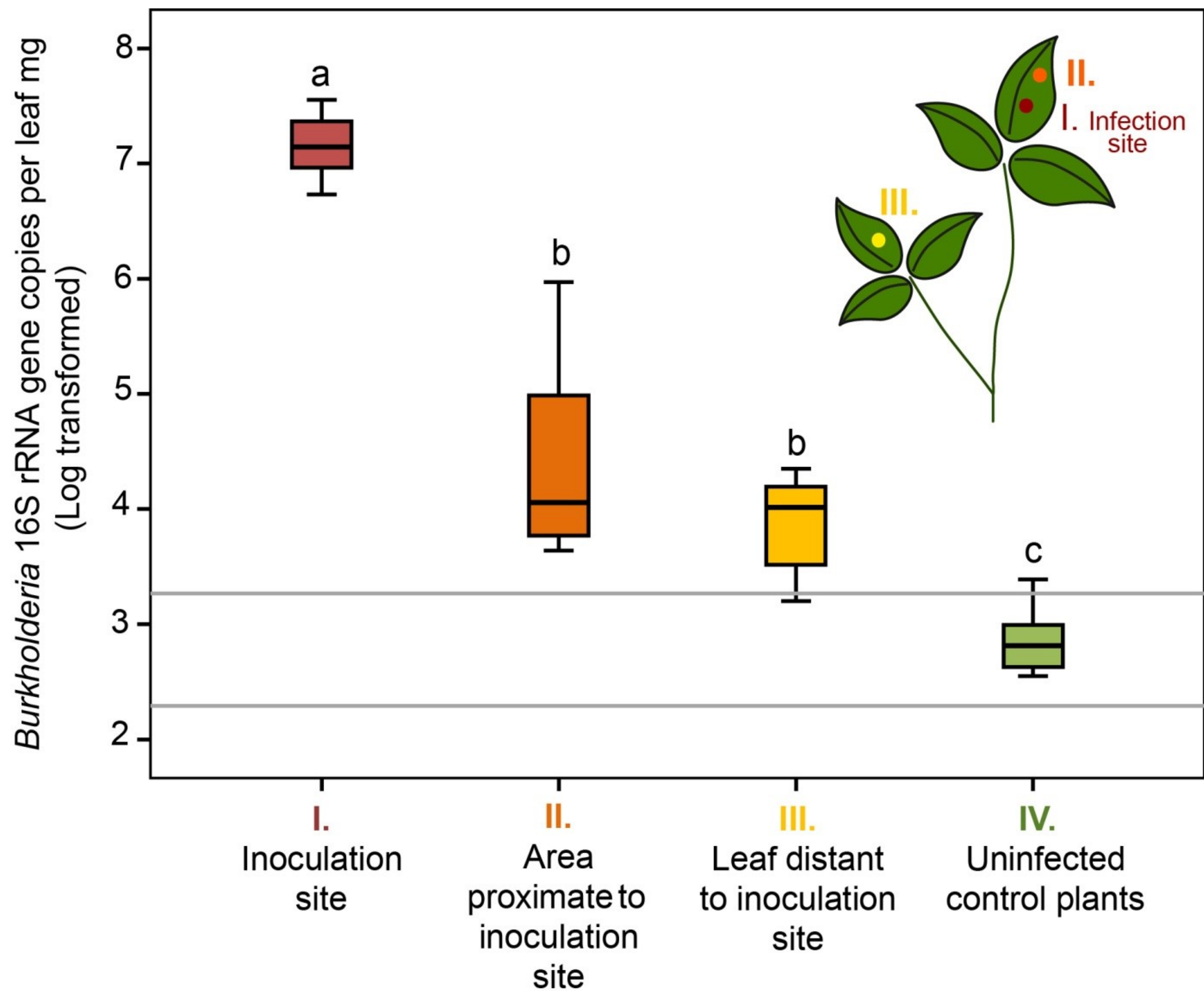


Figure 7. *Burkholderia* symbionts of *L. villosa* can establish a systemic infection in soybean plants. 38 days after infection, *B. gladioli* Lv-StA were found in higher concentration in plant tissues adjacent to the infection site. Significant titers of bacteria were also detected in areas proximate to the inoculation site, as well as in distant leaves (ANOVA, $p < 0.001$; Tukey test), demonstrating dispersal within the plant, probably via the vascular tissues. Bacteria were quantified using qPCR of a 167 bp region of the 16S rRNA gene as described in the methods section (*Plant fitness effect upon B. gladioli infection*). Gray lines correspond to the maximum and minimum values obtained for negative controls in the same qPCR run. Different letters above boxes represent significant differences according to an ANOVA with Tukey post-hoc tests.

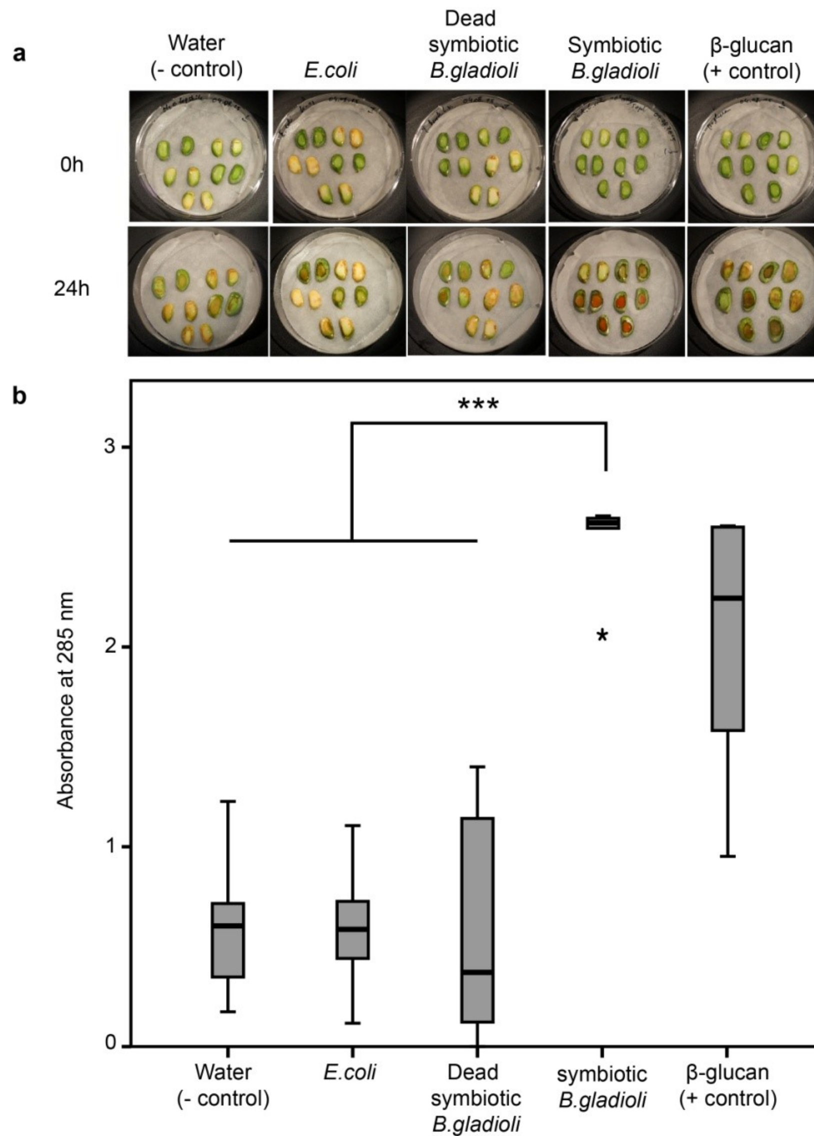


Figure 8. *Burkholderia* symbionts of *L. villosa* elicit a defense response in soybean cotyledons. Cotyledon assay determining glyceollin production by soybean upon inoculation with *B. gladioli* symbionts isolated from *L. villosa*, in comparison with negative (water, *E. coli* and dead *B. gladioli*) and positive controls (β-glucan). (**a**) Red coloration after 24h is indicative of glyceollin production as observed in *B. gladioli* and β-glucan treatments; (**b**) UV-spectrophotometric measurements at 285 nm support significant differences in glyceollin amounts in the different treatments (Kruskall Wallis test with Dunn post-hoc test, ***$p < 0.001$).

Table 1. 16S rRNA gene sequences from *Burkholderia* symbionts of Lagriinae, and selected references used for phylogenetic reconstruction (Figure 2b and Extended Data Figure 2).

Sequence Id. (Labels Fig.2b)	Category*	Strain	Accession Nr.
Lagria hirta Symbiont	Lagriinae symbiont (this study)	Lh_StG	KT888026
Lagria nigricollis Symbiont	Lagriinae symbiont (this study)	Ln	KT888028
Lagria okinawana Symbiont	Lagriinae symbiont (this study)	Lo	KT888029
Lagria rufipennis Symbiont	Lagriinae symbiont (this study)	Lr	KT888030
Lagria villosa Symbiont	Lagriinae symbiont (this study)	Lv_StA	KT888027
Lagria villosa Symbiont	Lagriinae symbiont (this study)	Lv_StB	KU358661
Lagria villosa Symbiont	Lagriinae symbiont (this study)	Lv_StC	KU358660
Ecnolagria sp. Symbiont	Lagriinae symbiont (this study)	Ec	KT888031
Burkholderia gladioli BSR3 - Rice pathogen	Plant pathogen - *B.gladioli* clade	BSR3	NR_102847
Burkholderia gladioli MS102 - Corn pathogen	Plant pathogen - *B.gladioli* clade	MS 102	EU053154
Burkholderia gladioli CH-2 - Onion pathogen	Plant pathogen - *B.gladioli* clade	CH-2	AY500138
Burkholderia gladioli st3 - Orchid pathogen	Plant pathogen- *B.gladioli* clade	strain 3	DQ090078
Burkholderia gladioli - Rice pathogen	Plant pathogen - *B.gladioli* clade	321gr-6	DQ355169
Burkholderia gladioli pv. *agaricicola*	Fungal pathogen - *B.gladioli* clade	CFBP 3580	GU936678
Burkholderia gladioli pv. *alliicola*	Plant pathogen - *B.gladioli* clade	CFBP 2422	GU936679
Burkholderia gladioli pv. *gladioli*	Plant pathogen - *B.gladioli* clade	CFBP 2427	GU936677
Burkholderia plantarii	Plant pathogen	-	U96933
Burkholderia plantarii - 2396	Plant pathogen	2396	AB183679
Burkholderia plantarii - NBRC104888	Plant pathogen	NBRC104888	AB682222.1
Burkholderia glumae 336gr-1 - Rice pathogen	Plant pathogen	336gr-1	DQ355164.1
Burkholderia glumae - 99gr-4b - Rice pathogen	Plant pathogen	99gr-4b	DQ355167
Burkholderia glumae - PA27.4 - Rice pathogen	Plant pathogen	PA27.4	EF193641.1
Burkholderia caryophylli	Plant pathogen	ATCC 25418	AB021423
Burkholderia endofungorum	Fungal endosymbiont- plant pathogen	HKI 456T	AM420302
Burkholderia rhizoxinica	Fungal endosymbiont- plant pathogen	HKI 454	AJ938142
Burkholderia cepacia	Plant and animal pathogen - BCC	ATCC 25416	U96927
Burkholderia vietnamiensis	Opportunistic animal pathogen - BCC	LMG 10929	AF097534
Burkholderia oklahomensis	Animal pathogen - Pseudomallei group	C6786	DQ108388
Burkholderia fungorum	PBE	LM16225	AF215705
Burkholderia kururiensis	PBE	-	AB024310
Burkholderia nodosa	PBE	Br3437	AY773189
Burkholderia terrae	PBE	KMY02	AB201285
'*Ca.* Burkholderia kirkii'	PBE	835462	AF475068
Burkholderia sp. [*P.antennata* symbiont]	Insect-associated	PAN136	AB558189
Burkholderia sp. [*P.bicoloripes* symbiont]	Insect-associated	PBI_clone1	AB558203.1
Burkholderia sp. [*R. pedestris* symbiont]	Insect-associated	RPE64	AB558208
Burkholderia sp. [*Tetraponera binghami* gut isolate]	Insect-associated	-	AF459796
Ralstonia pickettii	Outgroup	12J	NC010678

* Except for Lagriinae symbionts and insect-associated strains, the categorization is based on studies by Suárez-Moreno et al.[51] and Verstraete et al.[52]

4.6. Supplementary Information

Supplementary Methods

General analytical procedures. Analytical HPLC was performed on a Shimadzu LC-10Avp series HPLC system consisting of an autosampler, high-pressure pumps, column oven and PDA. HPLC conditions: C18 column (Eurospher 100-5, 250 x 4.6 mm) and gradient elution (MeCN/0.1 % (*v/v*) TFA 0.5/99.5 in 30 min to MeCN/0.1 % (v/v) TFA 100/0, MeCN 100 % for 10 min), flow rate 1 mL min^{-1}. Preparative HPLC was performed on a Shimadzu LC-8a series HPLC system with PDA. LC-MS measurements were performed using an Exactive Orbitrap High Performance Benchtop LC-MS with an electrospray ion source and an Accela HPLC system (Thermo Fisher Scientific, Bremen). HPLC conditions: C18 column (Betasil C18 3 µm 150 x 2.1 mm) and gradient elution (MeCN/0.1 % (*v/v*) HCOOH (H_2O) 5/95 for 1 min, going up to 98/2 in 15 min, then 98/2 for another 3 min; flow rate 0.2 mL min^{-1}; injection volume: 3 µL). For MS/MS measurements, a Q Exactive Orbitrap mass spectrometer with an electrospray ion source (Thermo Fisher Scientific, Bremen) was used. NMR spectra were recorded on a Bruker AVANCE III 600 MHz instrument equipped with a Bruker cryo platform. Spectra were normalized to the residual solvent signals. IR spectra were recorded on a JASCO FT/IR-4100typeA.

Isolation of lagriene (3) and toxoflavin (1) and identification of caryoynencin (2). The crude extract was defatted with hexane and fractionated by size exclusion chromatography with Sephadex LH20 using MeOH as eluent. Final purification of compound **3** was achieved by preparative HPLC using a Phenomenex Synergi 4 µm Fusion-RP80A column (250 x 21.2 mm) with a flow rate of 10 mL min^{-1} and a gradient method (MeCN/0.01 trifluoroacetic acid (H_2O, v/v) 40/60 for 5 min, going up to 75/25 in 25 min, then increasing to 100% MeCN in 5 min). Total yield: 5 mg. For compound **1**, the following HPLC gradient was applied: MeCN/0.01 trifluoroacetic acid (H_2O, v/v) 1/99 for 5 min, going up to 40/60 in 25 min. Caryoynencin (**2**) was identified by LC-HRESI-MS and comparison to an authentic reference.

Bioassay-guided fractionation and isolation of lagriamide (4). To determine the bioactive compound on *L. villosa* eggs, 50 µL of crude eggs extract were fractionated via analytical HPLC. Fractions with a volume of 1 mL were collected, concentrated using a Speedvac and redissolved in 50 µL MeOH. The bioactivity of each fraction was determined using an agar diffusion assay with *Aspergillus niger* as an indicator organism (Extended Data Fig. 6). The active compound was isolated from the combined extracts (156 *L. villosa* egg clutches, containing an estimated 28,000 eggs in total) by *semi*-preparative HPLC using a Nucleodur C18HTec column (250 x 10 mm, 5µm) with a flow rate of 5 mL min^{-1} and a gradient method (MeCN/H_2O 25/75 for 2 min, going up to 100/0 in 20 min). Total yield: 600 µg.

Quantification of lagriamide on *L. villosa* egg clutches. Crude extracts of individual *L. villosa* egg clutches laid by field collected mothers were extracted in methanol, and analyzed by LC-MS. Lagriamide formation was semi-quantitatively measured by integration of the peak areas of the extracted mass traces. In order to assess if these amounts correlated with the abundance of *Burkholderia* per egg clutch, we used quantitative PCR data on the *Burkholderia* 16S rRNA gene copy numbers from the corresponding egg clutches. Quantitative PCR was carried out as described in the methods section (*Nucleic acid extraction, amplification and sequencing*).

Structure elucidation of lagriene (3) and lagriamide (4). For compound **3**, a molecular formula of $C_{44}H_{74}O_{11}$ was deduced from HRESI-MS measurements. ^{13}C and DEPT135 spectra revealed the presence of three quaternary, 22 methine and 13 methylene and 6 methyl carbon atoms. The proton and carbon NMR data indicated the structural relatedness to etnangien[53]. Analysis of the H,H-COSY and the HMBC couplings identified the backbone of **3** (Fig. S1). A coupling constant of $J_{H,H}$ =11 Hz for the protons H-26/H-27 disclosed the *Z* configuration of the respective double

bond, whereas all other double bonds were found to be in *E* configuration ($J_{H,H}$=15Hz). HMBC coupling of H-16 and C-38 indicated the position of cyclization. For compound **4**, a molecular mass of *m/z* 749.4949 amu $(M+H)^+$ and a molecular formula of $C_{41}H_{69}N_2O_{10}$ (calcd. 749.4947) was determined by HRESI-MS. The number of carbon atoms was corroborated by ^{13}C NMR analysis and the multiplicity was assigned by DEPT135 measurements. Analysis of the H,H-COSY spectra revealed the spin system H14-H17 and the spin system of the pyran ring (Fig. S1). A chemical shift of δ 94.7 ppm for C-27 pointed to the presence of a spirocyclic ring system which was confirmed by characteristic HMBC couplings (Fig. S1). Chemical shifts of δ 53.7 ppm and δ 59.7 ppm for C-2 and C-3, respectively, and a coupling constant of $J_{H,H}$= 2 Hz of the corresponding protons disclosed the epoxide moiety. HMBC couplings of C-4 and H-2/H-3 and H-5/H-6 indicated the connectivity of the partial structures.

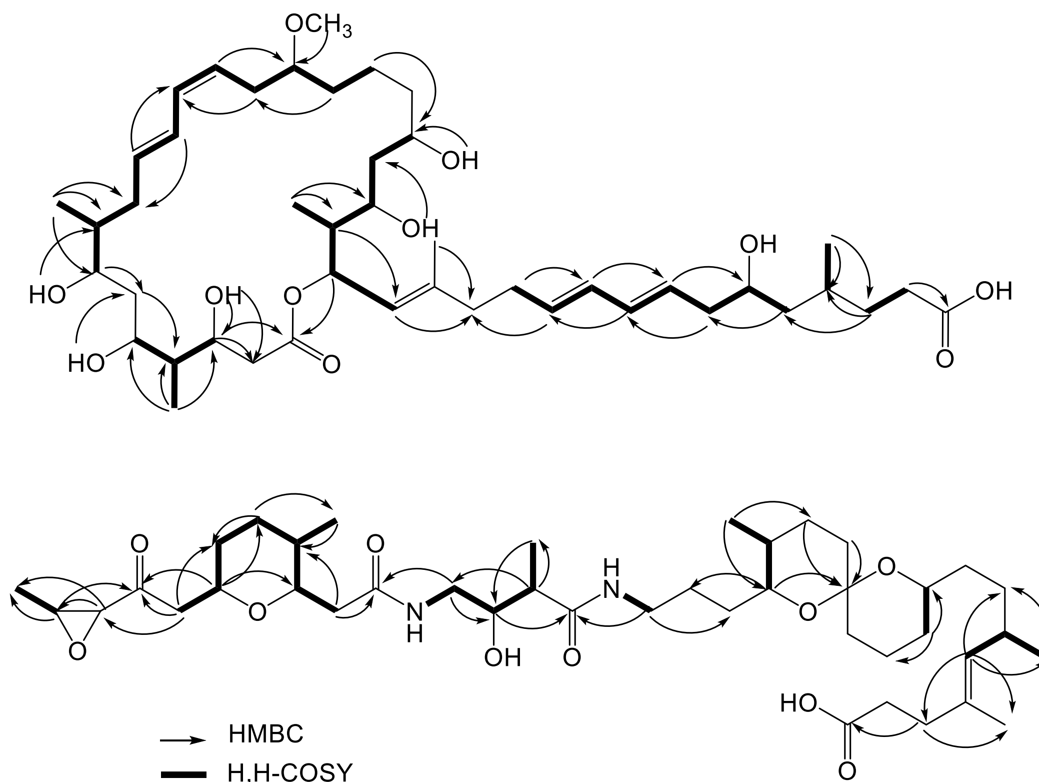


Figure S1: Key 2D NMR couplings of **3** and **4**

Supplementary data

Physicochemical data and antimicrobial activity of the symbiont-produced compounds

Toxoflavin (1)

ESI(+) *m/z* 194 $(M+H)^+$, HRESI(+)-MS *m/z* 194.0674 (calcd. for $C_7H_8N_5O_2$ 194.0673)
UV (PDA): λ_{max}= 258, 397 nm
NMR: d_6-DMSO, ^1H NMR 600 MHz, ^{13}C NMR 150 MHz

Carbon	^{13}C	^1H (mult., *J* in Hz)
3	144.7	8.96 (s)
4a	146.5	-
5	154.1	-
7	159.0	-
8a	150.9	-
9	42.4	3.94 (s)
10	28.2	3.24 (s)

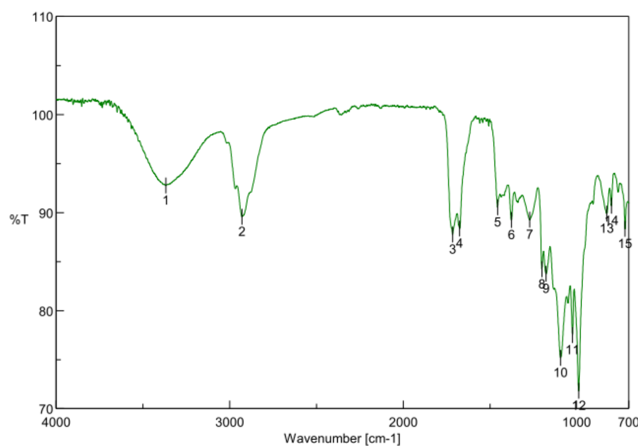
Caryoynencin (2)

ESI(-) *m/z* 279 $(M-H)^-$, HRESI(-)-MS *m/z* 279.1031 (calcd. for $C_{18}H_{17}O_3$ 279.1027)
UV (PDA): λ_{max}= 294, 239, 280, 268, 358 nm

Lagriene (3)

ESI(-) *m/z* 777 $(M-H)^-$, HRESI(-)-MS *m/z* 777.5172 (calcd. for $C_{44}H_{73}O_{11}$ 777.5158)
UV (PDA): λ_{max}= 231 nm

IR-spectrum



NMR: d_6-DMSO, ^{1}H NMR 600 MHz, ^{13}C NMR 150 MHz

Carbon	^{13}C	^{1}H (mult., *J* in Hz)
1	174.1	-
2	31.4	2.16 (m)
3	32.4	1.47 (m) 1.32 (m)
4	28.2	1.60 (m)
5	43.8	1.26 (m) 1.03 (m)
6	67.5	3.49 (m)
7	41.5	2.08 (m)
8	129.3	5.55 (m)
9	126.6	6.29 (m)
10	125.1	5.26 (m)
11	131.2	5.48 (m)
12	30.2	2.27 (m) 2.11 (m)
13	38.9	2.06 (m) 2.02 (t, *7.2*)
14	139.1	-
15	122.9	5.00 (d, *9.48*)
16	72.1	5.26 (m)
17	41.8	1.78 (m)
18	66.5	3.77 (s)
19	74.5	3.47 (m)
20	39.3	1.45 (m) 1.32 (m)
21	66.8	3.62 (m)
22	20.6	1.35 (m) 1.21 (m)
23	31.9	1.36 (m)
24	79.6	3.21 (m)
25	30.1	2.38 (m) 2.27 (m)
26	125.1	5.26 (m)
27	130.1	6.00 (m)
28	126.6	6.29 (m)
29	133.6	5.72 (m)
30	36.5	2.18 (m)
31	37.7	1.48 (m)
32	71.7	3.53 (m)
33	39.3	1.45 (m) 1.32 (m)
34	69.2	3.84 (m)
35	43.6	1.33 (m)
36	68.6	3,81 (m)
37	39.6	1.45 (m) 1.32 (m)
38	170.4	-
39	18.8	0.80 (m)
40	16.6	1.68 (s)
41	10.5	0.71 (d, *7.02*)
42	55.6	3.22 (s)
43	13.1	0.79 (m)
44	9.7	0.76 (d, *6.97*)

Antimicrobial activity (1 mg mL^{-1}): inhibition zones of 30 mm against *Mycobacterium vaccae*, 19 mm against vancomycin-resistant *Enterococcus faecalis*, and 18 mm against methicillin-resistant *Staphylococcus aureus* were measured (procedure described in Methods section, *Antimicrobial bioassays*).

Lagriamide (4)

ESI(+) *m/z* 749 $(M+H)^+$, HRESI(+)-MS *m/z* 749.4949 (calcd. for $C_{41}H_{69}N_2O_{10}$ 749.4947)

UV (PDA): λ_{max}=UVend

NMR: d_6-DMSO, ^1H NMR 600 MHz, ^{13}C NMR 150 MHz

Carbon	^{13}C	^1H (mult., *J* in Hz)
1	17.2	1.29 (d, *5.1*)
2	53.7	3.10 (m)
3	59.7	3.34 (d, *2.0*)
4	205.4	-
5	44.6	2.47 (d, *6.4*)
6	74.1	3.70 (m)
7	25.4	1.33 (m)
8	30.2	1.71 (m) 1.59 (m)
9	29.3	1.60 (m)
10	11.6	0.85 (d, *7.0*)
11	76.6	3.77 (m)
12	39.8	2.20 (dd, *14.5, 8.3*) 2.06 (dd, *14.4, 5.4*)
13	170.2	-
14	42.9	3.21 (m) 2.92 (dt, *13.0*, *5.8*)
15	71.4	3.50 (m)
16	43.5	2.25 (m)
17	14.4	0.95 (d, *7.0*)
18	174.1	-
19	38.6	3.05 (m)
20	25.6	1.69 (m) 1.35 (m)
21	30.1	1.53 (m) 1.20 (m)
22	73.8	3.06 (m)
23	34.4	1.20 (m)
24	17.9	0.76 (d, *6.5*)
25	27.6	1.41 (m)
26	35.5	1.50 (m) 1.34 (m)
27	94.6	-
28	34.9	1.44 (m)
29	18.8	1.49 (m)
30	31.0	1.47 (m) 1.05 (m)
31	68.0	3.40 (m)
32	33.4	1.41 (m)* 1,27 (m)
33	33.3	1.27 (m)*
34	31.4	2.30 (m)
35	21.2	0.86 (d, *6.6*)
36	131.0	4.87 (d, *9.1*)
37	132.4	-
38	16.0	1.55 (d, *1.2*)
39	34.4	2.15 (m)
40	33.0	2.24 (m)
41	174.3	-

* overlapping signals, might be interchangeable

Antimicrobial activity (100 µg mL^{-1}): an inhibition zone of 20 mm against *A. niger* was measured (procedure described in Methods section, *Antimicrobial bioassays*).

HPLC profiles of crude extracts indicating the production of compounds (1-4) *in vitro* (*B. gladioli* Lv-StA liquid cultures) or *in vivo* (on *L. villosa* egg clutches)

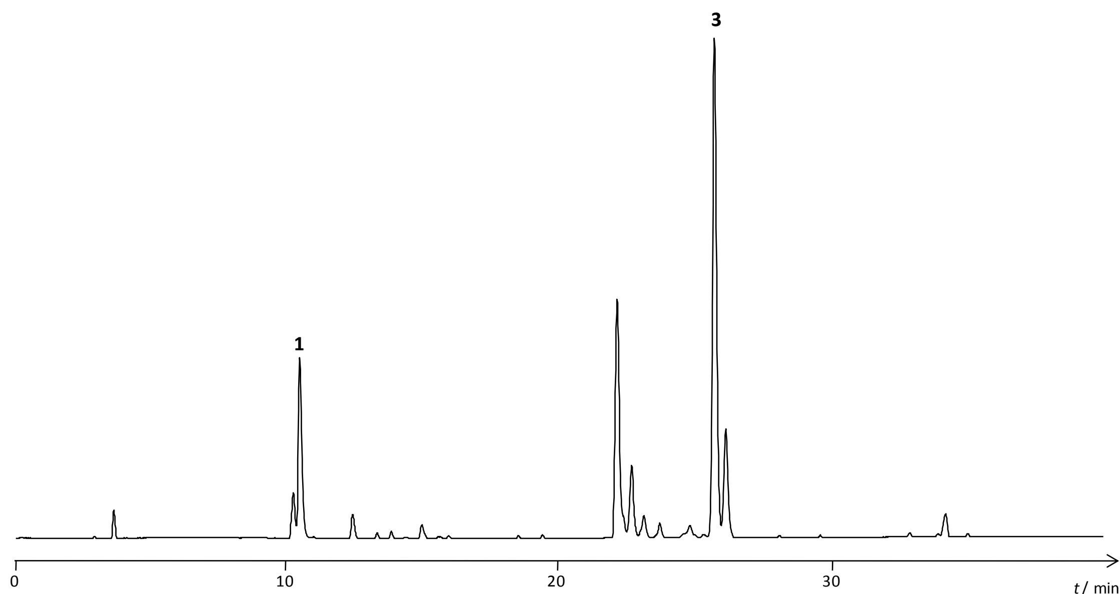


Figure S2. HPLC profile (PDA total scan) of the crude extract of *B. gladioli* Lv-StA cultured on MGY medium. Numbers indicate production of toxoflavin (**1**) and lagriene (**3**).

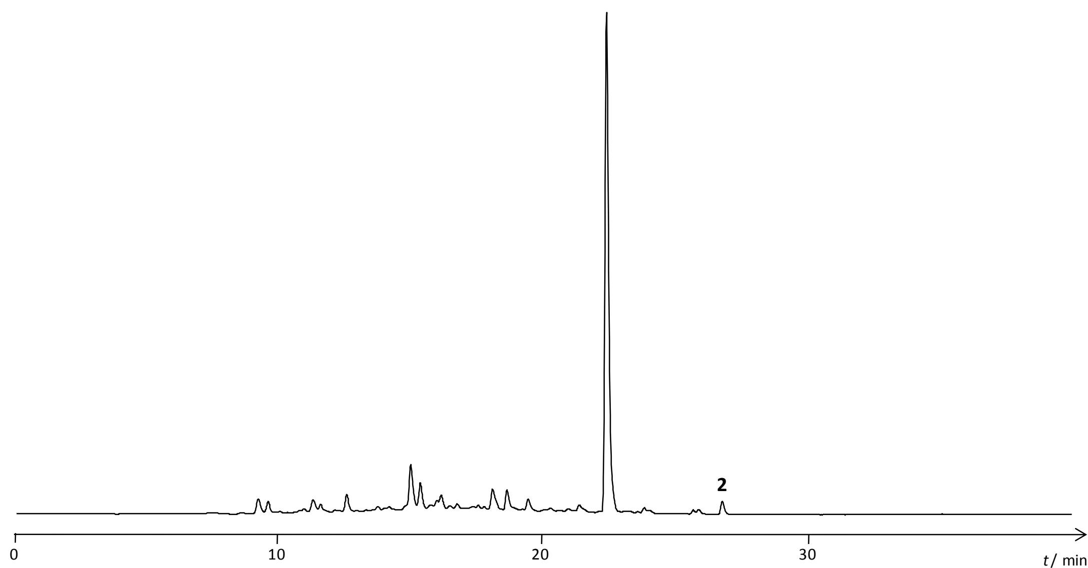


Figure S3. HPLC profile (294 nm) of the crude extract of *B. gladioli* Lv-StA cultured on PDB medium. Number indicates production of caryoynencin (**2**).

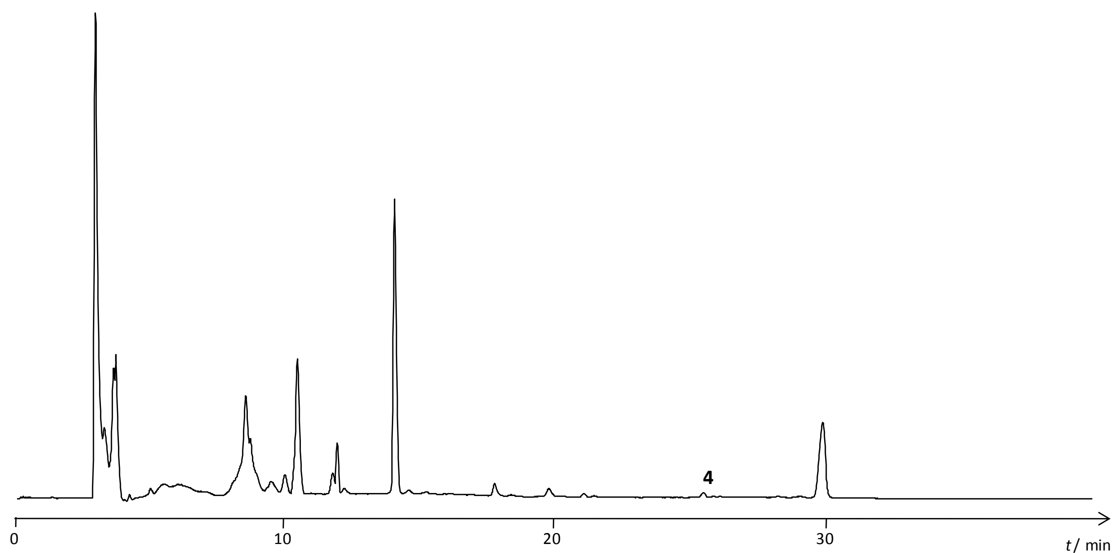


Figure S4. HPLC profile (PDA total scan) of the crude extract of *L. villosa* egg clutches. Number indicates production of lagriamide (**4**).

NMR spectra for structure elucidation of lagriene (3) and lagriamide (4).

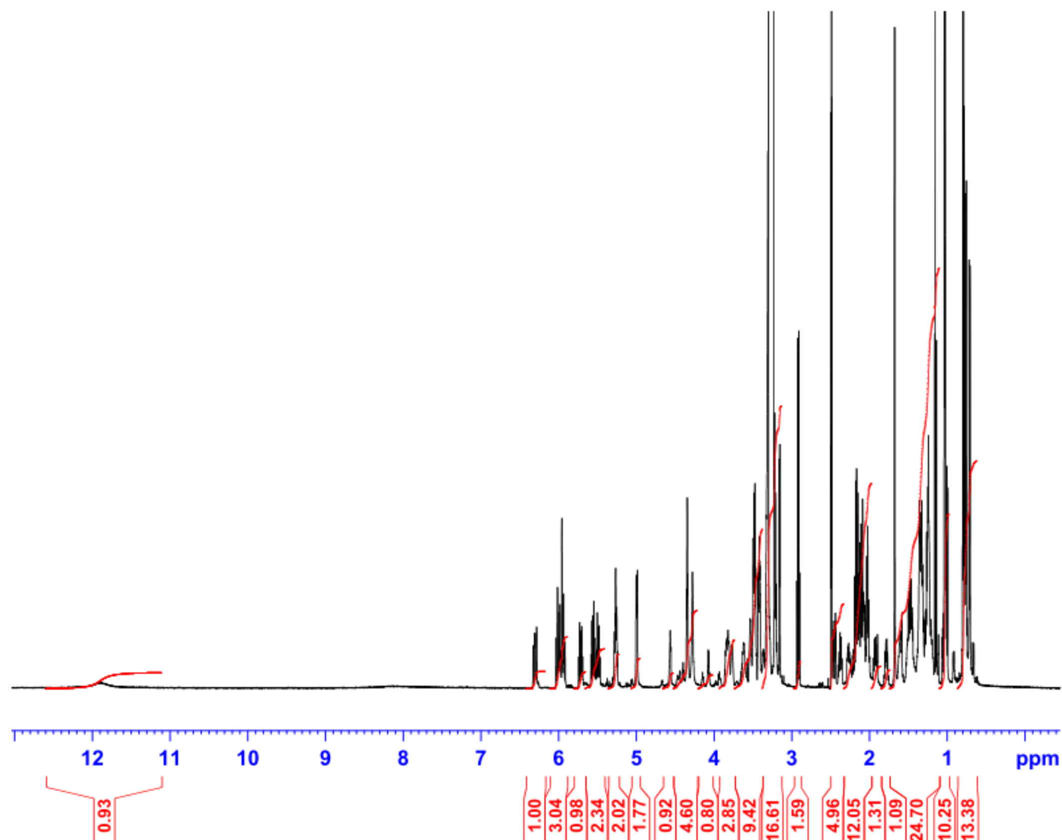


Figure S5. ^{1}H NMR spectrum of lagriene (**3**).

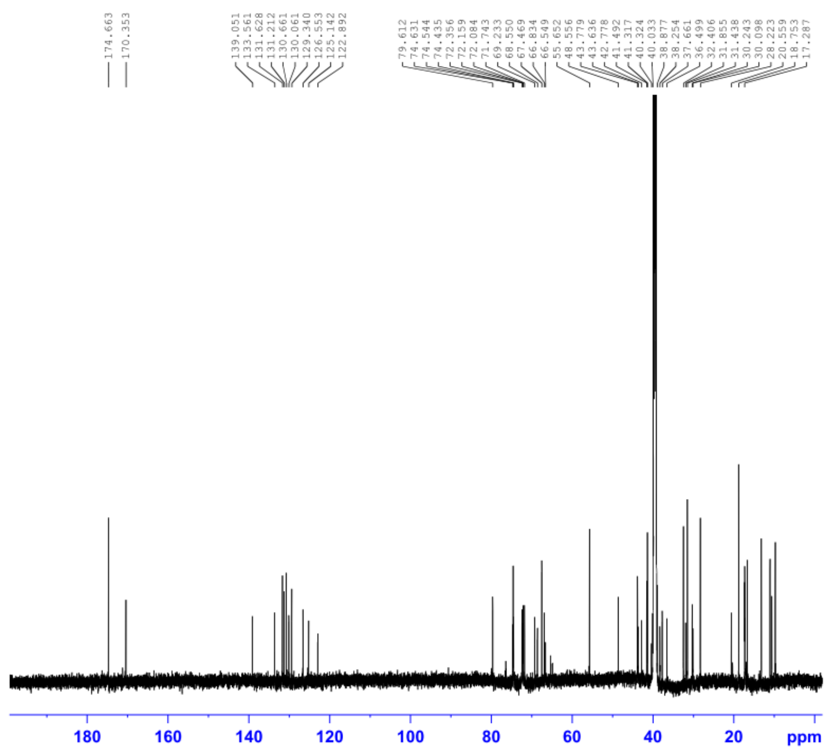


Figure S6. ^{13}C NMR spectrum of lagriene (**3**).

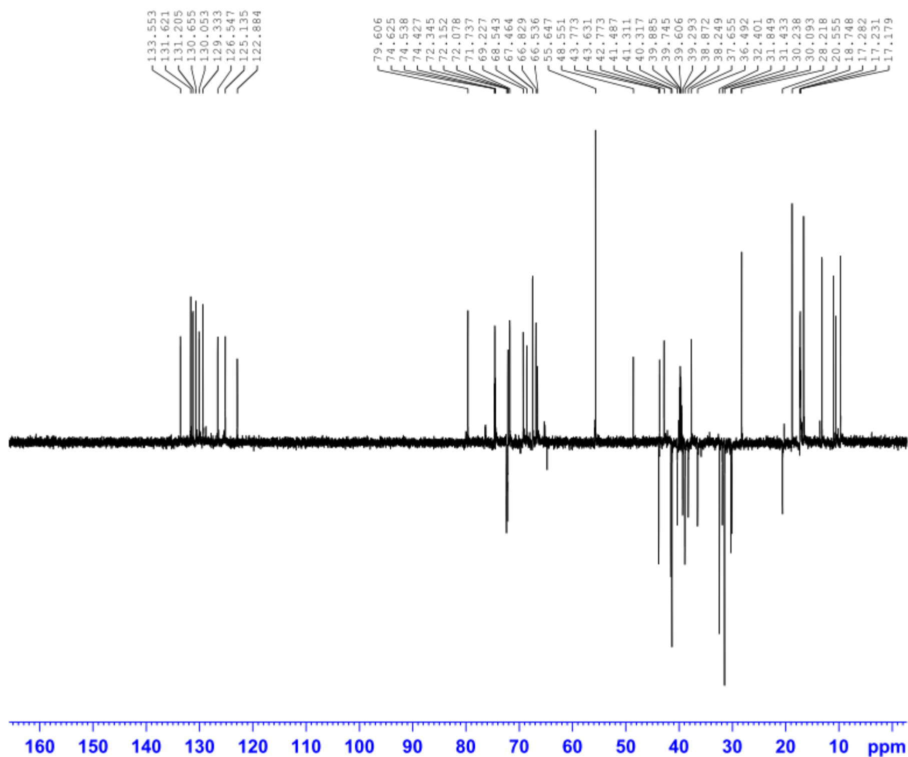


Figure S7. DEPT135 NMR spectrum of lagriene (**3**).

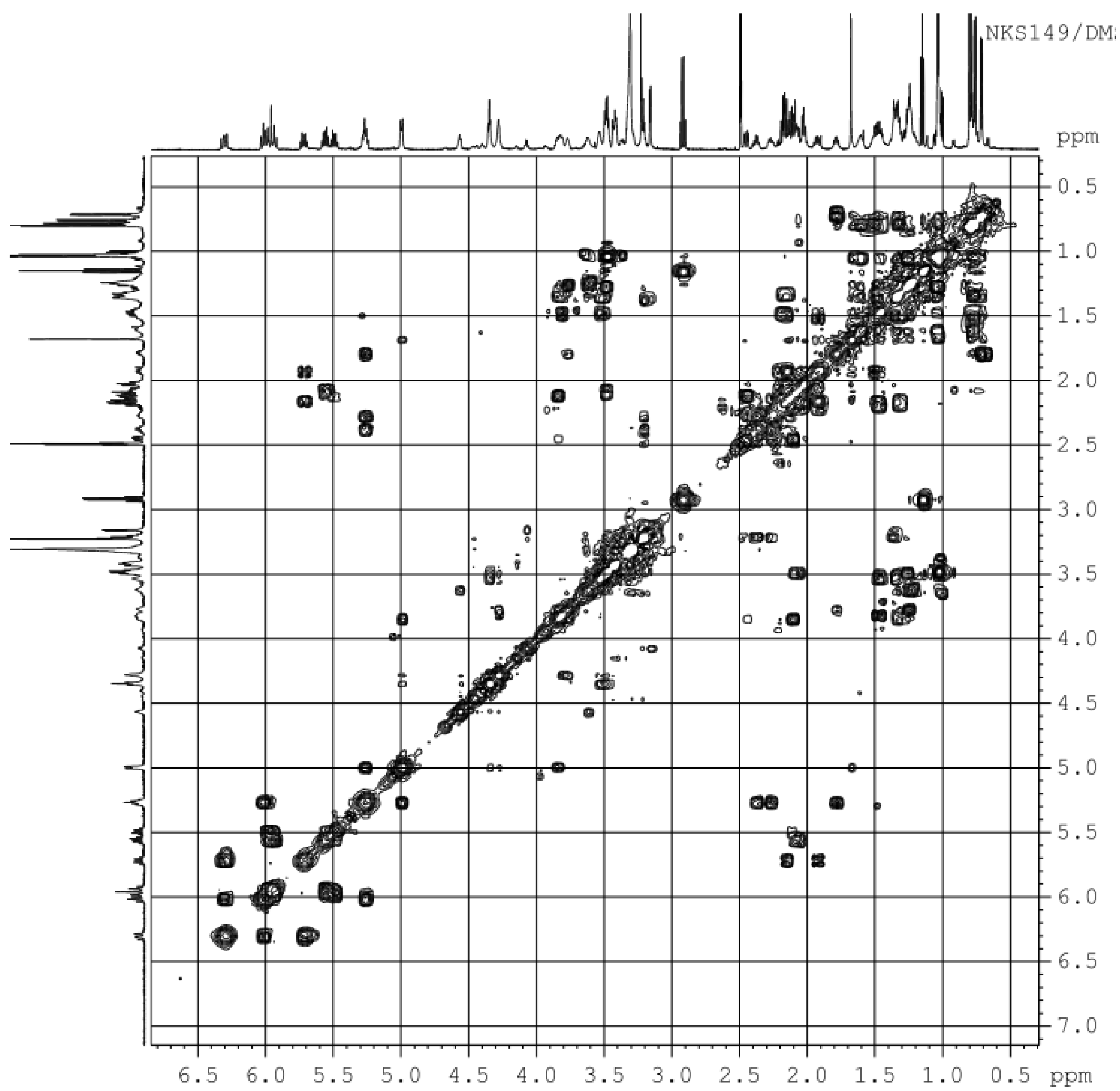


Figure S8. H,H-COSY spectrum of lagriene (**3**).

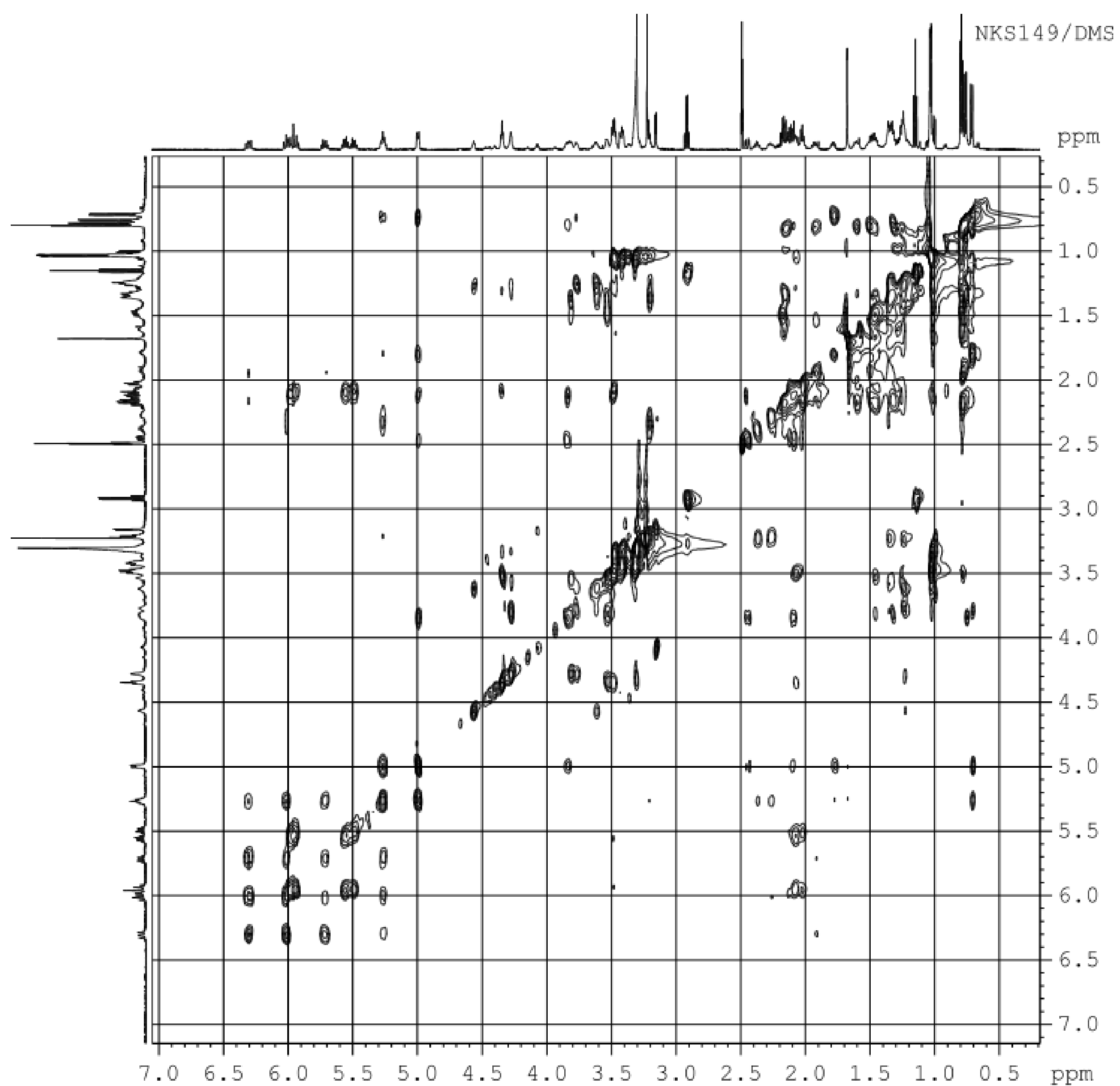


Figure S9. TOCSY spectrum of lagriene (**3**).

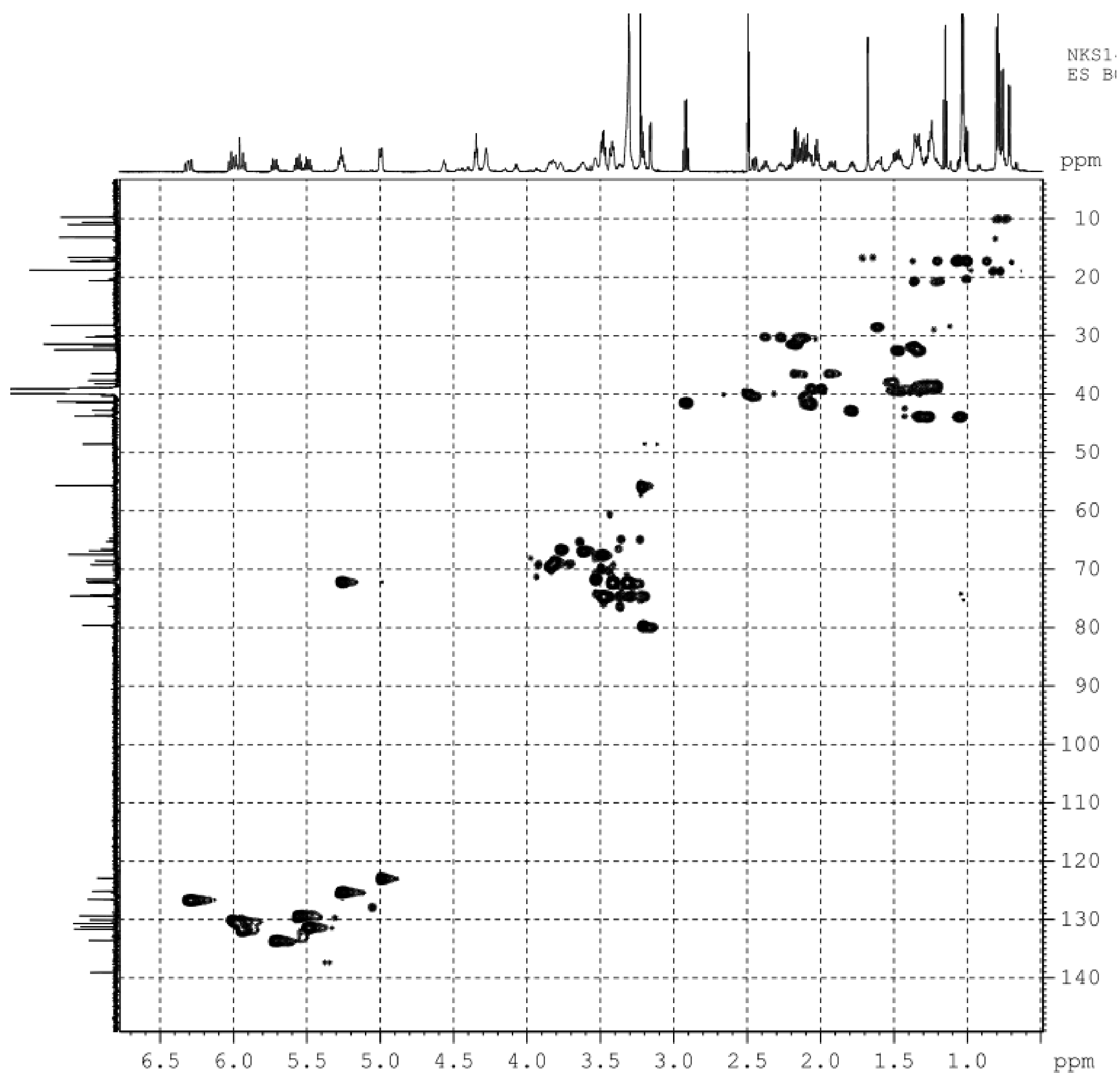


Figure S10. HSQC spectrum of lagriene (**3**).

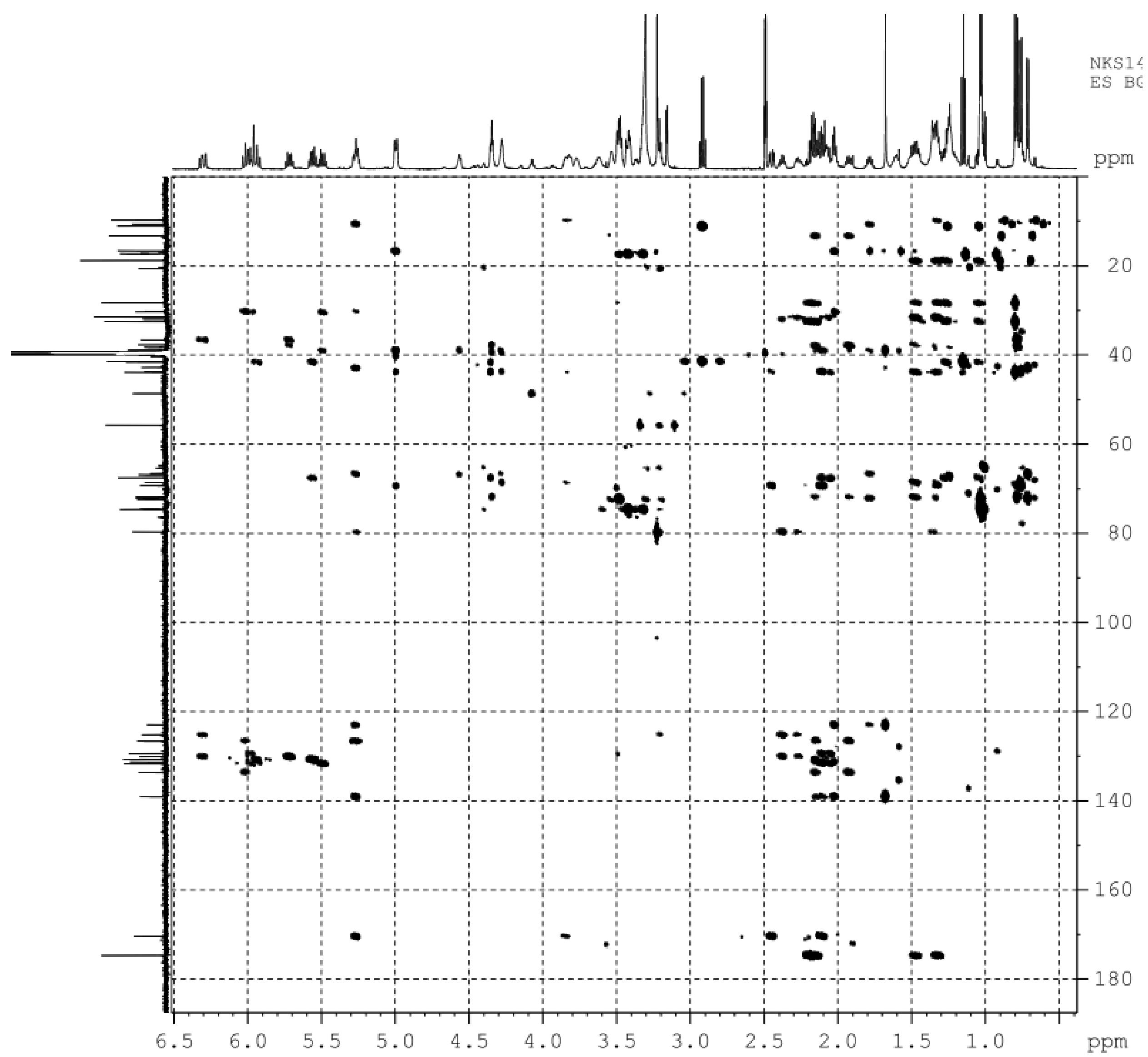


Figure S11. HMBC spectrum of lagriene (**3**).

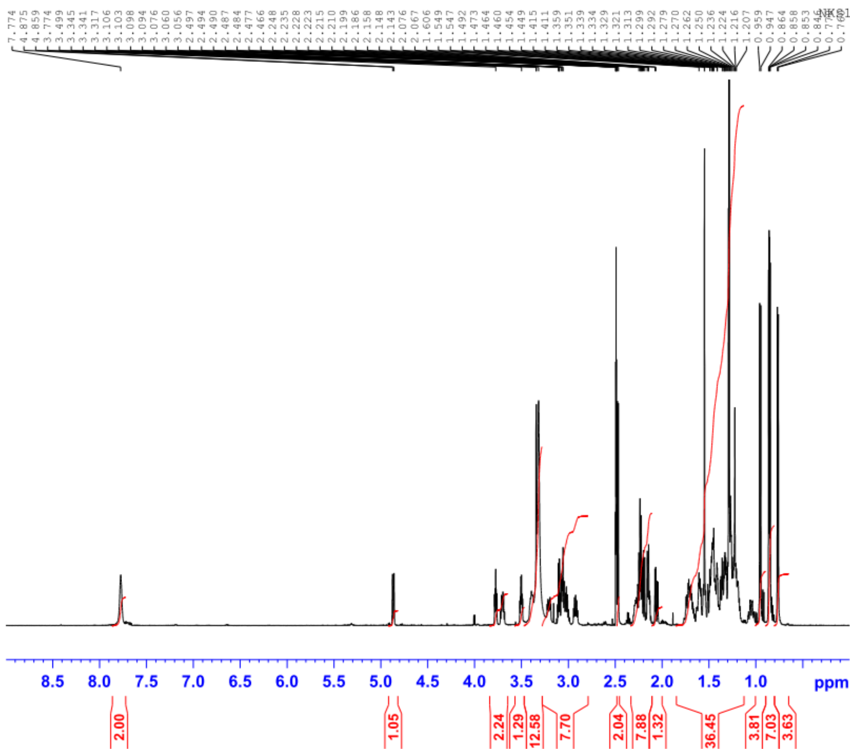


Figure S12. ^{1}H NMR spectrum of lagriamide (**4**).

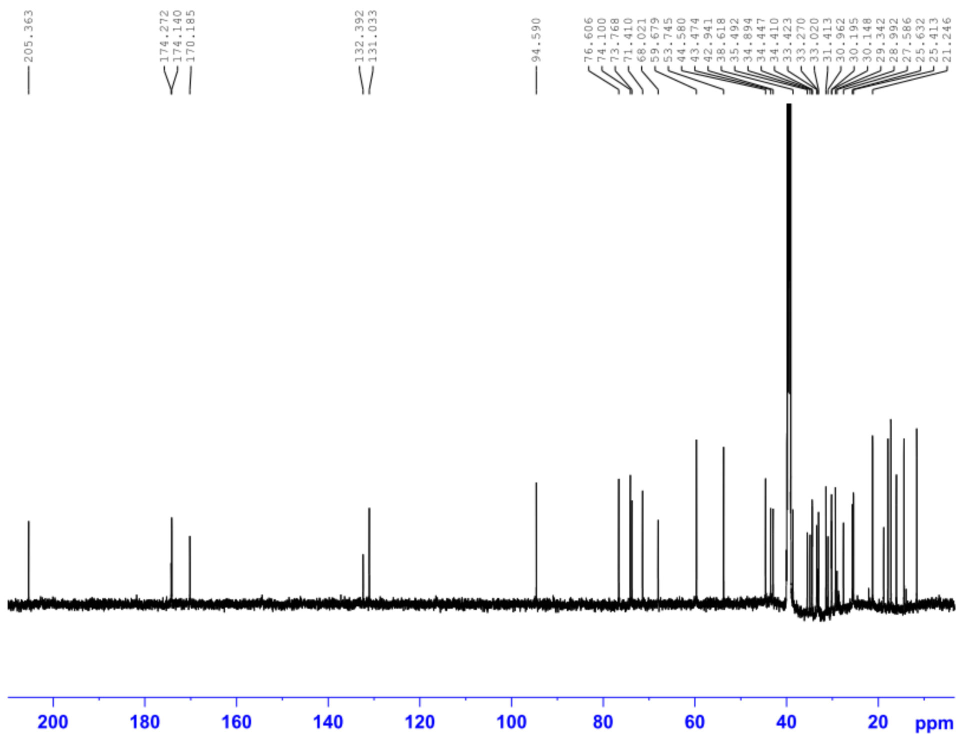


Figure S13. ^{13}C NMR spectrum of lagriamide (**4**).

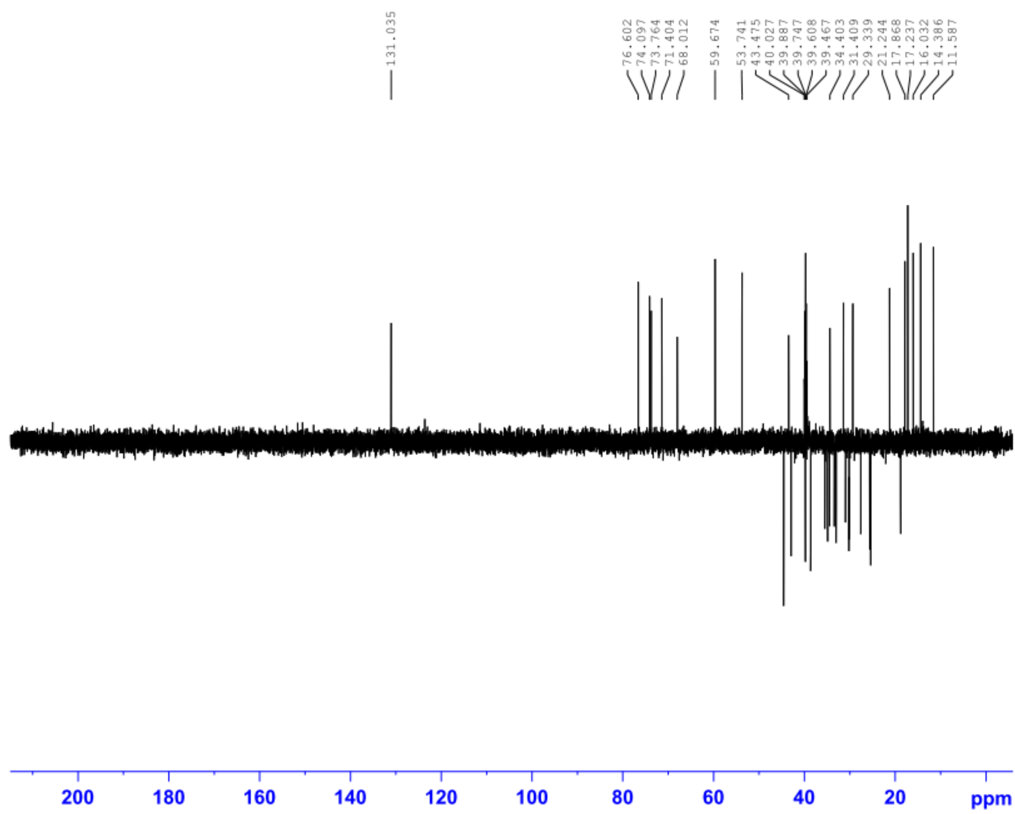


Figure S14. DEPT135 NMR spectrum of lagriamide (**4**).

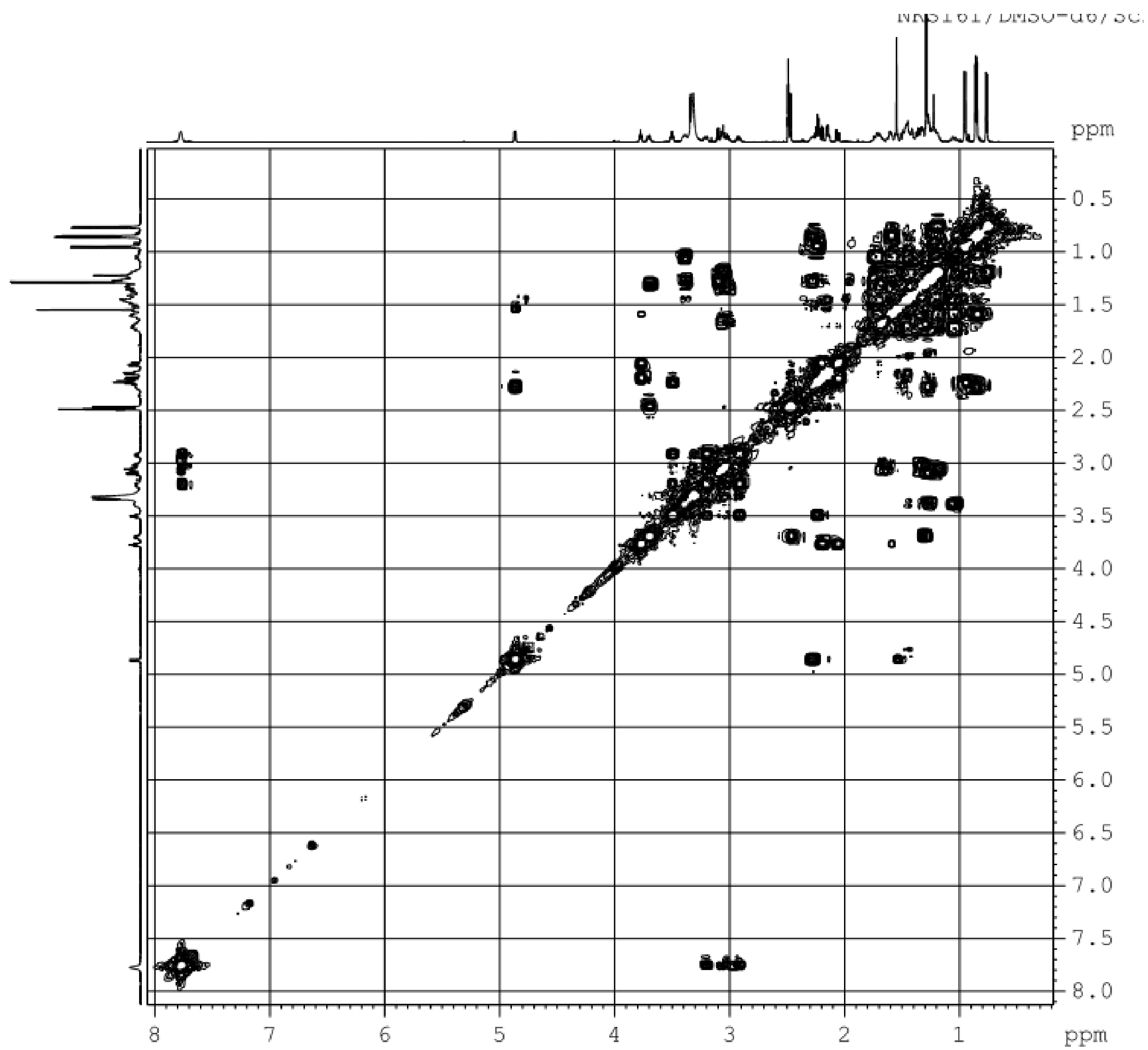


Figure S15. H,H-COSY spectrum of lagriamide (**4**).

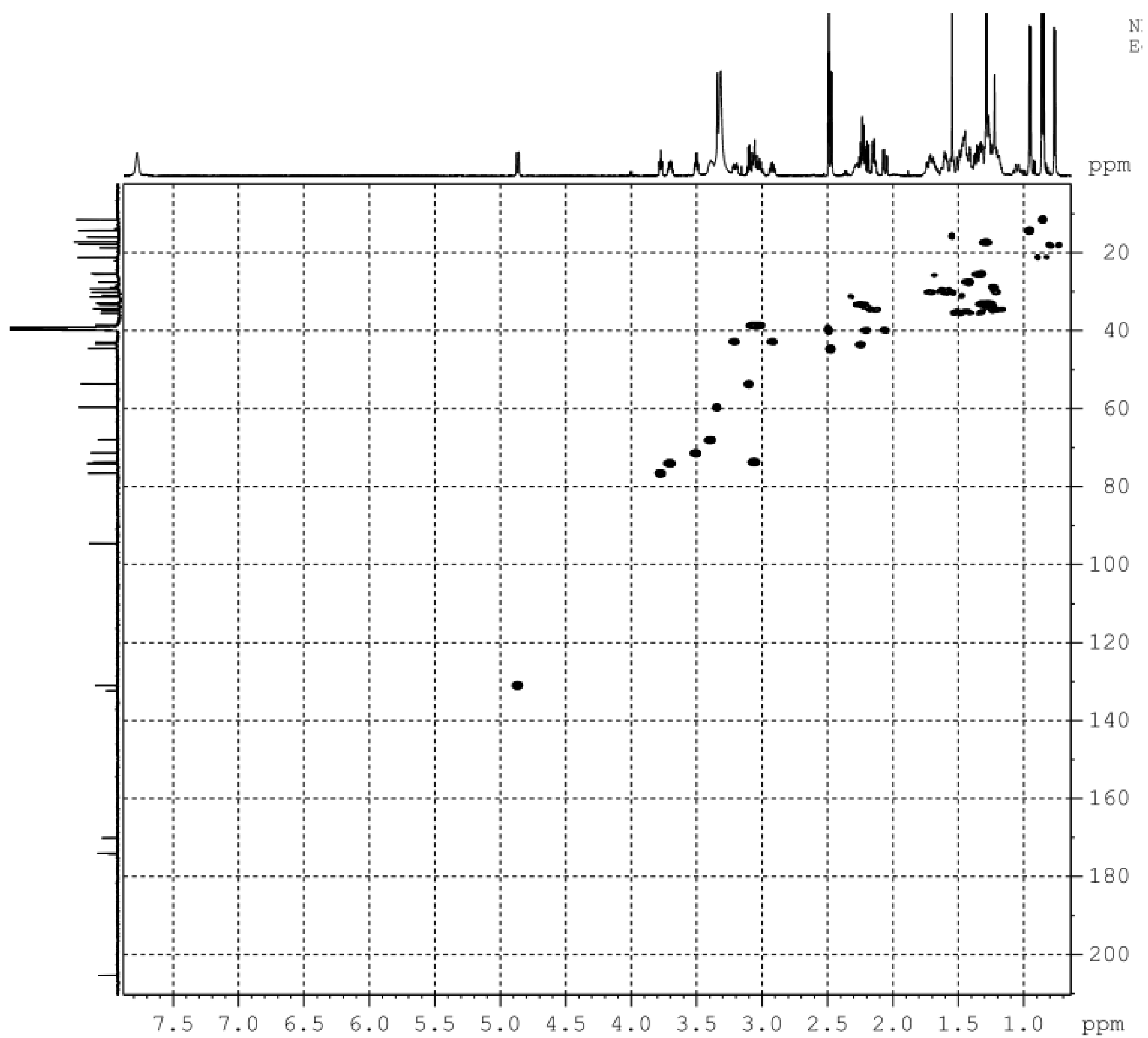


Figure S16. HSQC spectrum of lagriamide (**4**).

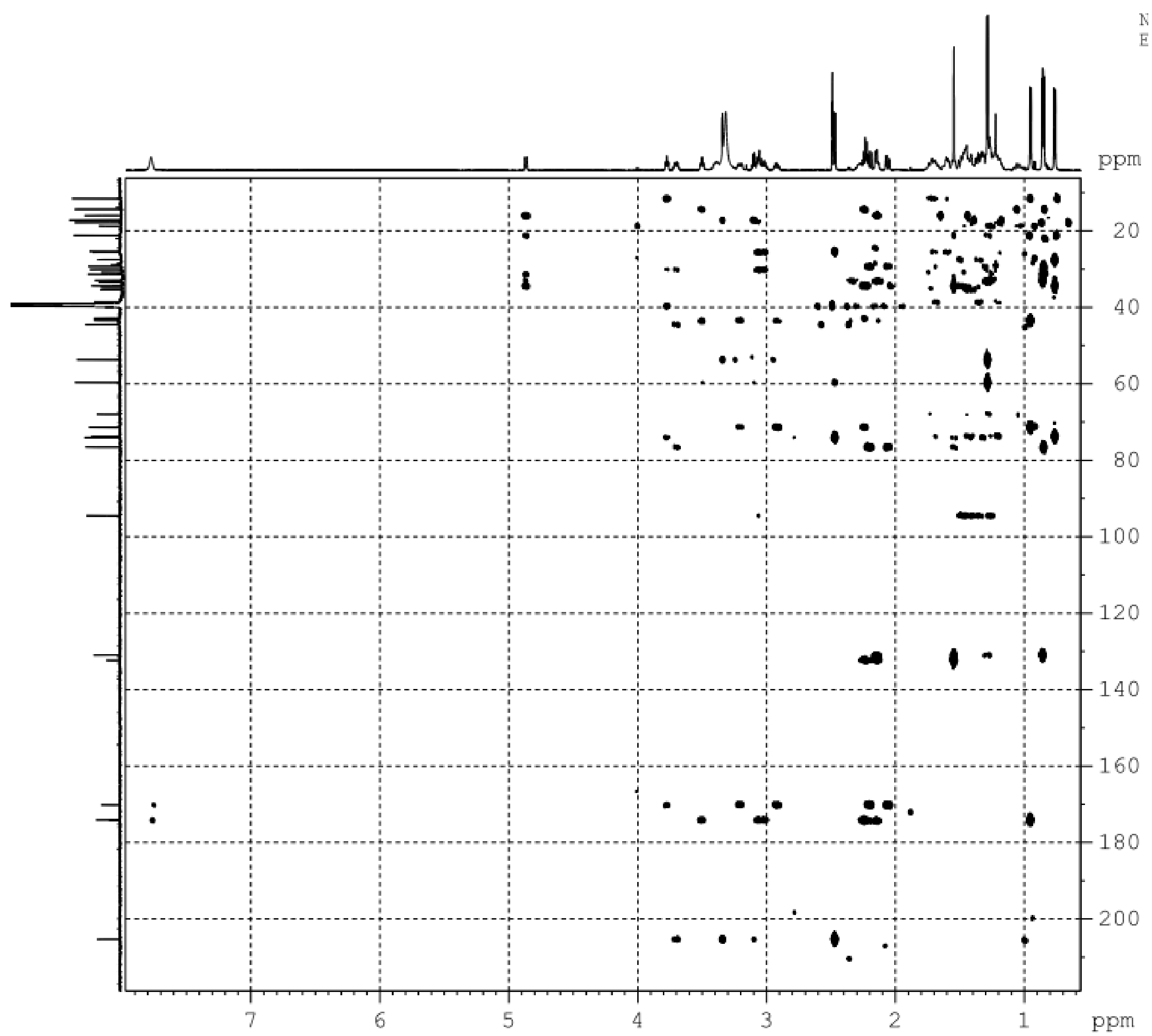


Figure S17. HMBC spectrum of lagriamide (**4**).

Biosynthetic gene clusters coding for toxoflavin (1), caryoynencin (2) and lagriene (3)

Table S1. Toxoflavin (*tox*) biosynthetic genes and predicted functions of proteins

Gene	Length, bp	Putative protein	Homologous protein	Accession number	Identity/ Similarity
toxA	408	*S*-Adenosylmethionine-dependent methyltransferase	ToxA (*Burkholderia glumae*)	BAA92862.2	98%/98%
toxB	507	GTP cyclohydrolase II	ToxB (*Burkholderia glumae*)	BAB88913.1	98%/98%
toxC	1701	WD-repeat protein	ToxC (*Burkholderia glumae*)	BAB88914.2	97%/98%
toxD	903	TRP-2	ToxD (*Burkholderia glumae*)	BAB88915.1	89%/91%
toxE	1143	Deaminase	ToxE (*Burkholderia glumae*)	BAB88916.2	92%/94%
toxR	600	LysR family transcriptional regulator	ToxR (*Burkholderia glumae*)	BAC77727.1	88%/90%

Table S2. Caryoynencin (*cay*) biosynthetic genes and predicted functions of proteins

Gene	Length, bp	Putative protein	Homologous protein	Accession number	Identity/ Similarity
cayA	1536	Fatty acyl-AMP ligase	CayA (*Burkholderia caryophylli*)	AIG53814.1	76%/85%
cayB	897	Fatty acid desaturase	CayB (*Burkholderia caryophylli*)	AIG53817.1	85%/89%
cayC	951	Fatty acid desaturase	CayC (*Burkholderia caryophylli*)	AIG53820.1	85%/91%
cayD	300	Phosphopantetheine attachment site	CayD (*Burkholderia caryophylli*)	AIG53823.1	81%/93%
cayE	1083	Fatty acid desaturase	CayE (*Burkholderia caryophylli*)	AIG53826.1	88%/94%
cayF	945	Alpha/Beta hydrolase	CayF (*Burkholderia caryophylli*)	AIG53829.1	74%/83%
cayG	1167	Cytochrome P450 monooxygenase	CayG (*Burkholderia caryophylli*)	AIG53832.1	79%/90%

Table S3. Lagriene (*lag*) biosynthetic genes and predicted functions of proteins

Gene	Length, bp	Description	Domains
2388/*lagD*	16284	Polyketide synthase	KS-ACP-ACP ER KS-KR-ACP KS-DH-KR-ACP KS-DH-KR
2389/*lagE*	11178	Polyketide synthase	ACP KS-KR-ACP KS-ACP-ACP KS-KR
2390/*lagF*	9927	Polyketide synthase	MT-ACP KS-KR-ACP KS-KR-ACP KS

2391/*lagG*	14991	Polyketide synthase	DH-KR-ACP KS-KR-ACP KS-MT-ACP KS-KR-ACP KS
2392/*lagH*	10800	Polyketide synthase	DH-ACP KS-KR-ACP KS-DH-KR-MT-ACP
2393/*lagI*	17523	Polyketide synthase	ER KS-KR-ACP KS-KR-MT-ACP KS-KR-ACP KS-KR TE

Table S4. Proteins encoded upstream and downstream of the *lagD-lagI*

Gene	**Length, bp**	**Putative protein**	**Homologous protein**	**Accession number**	**Identity/ Similarity**
2372/*orf-16*	1353	MATE efflux protein	sce3194 (*Sorangium cellulosum* So ce56)	CAN93353.1	68%/82%
2373/*orf-15*	975	Malonyl CoA-acyl carrier protein transacylase	sce3195 (*Sorangium cellulosum* So ce56)	CAN93354.1	47%/63%
2374/*orf-14*	855	4'-Phosphopantetheinyl transferase	sce5058 (*Sorangium cellulosum* So ce56)	CAN95221.1	41%/56%
2375/*orf-13*	1173	Malonyl CoA-acyl carrier protein transacylase	Malonyl CoA-acyl carrier protein transacylase (*Burkholderia pseudomallei*)	3G87_A	51%/62%
2376/*orf-12*	1401	Amidase	sce3176 (*Sorangium cellulosum* So ce56)	CAN93335.1	56%/69%
2377/*orf-11*	360	Alpha/beta-hydrolase	sce3177 (*Sorangium cellulosum* So ce56)	CAN93336.1	58%/76%
2378/*orf-10*	3234	Hypothetical protein	SorM (*Sorangium cellulosum* So ce12)	ADN68497.1	38%/51%
2379/*orf-9*	747	Enoyl-CoA hydratase	sce3179 (*Sorangium cellulosum* So ce56)	CAN93338.1	75%/83%
2380/*orf-8*	789	Enoyl-CoA hydratase	sce3180 (*Sorangium cellulosum* So ce56)	CAN93339.1	63%/78%
2381/*orf-7*	1260	3-Hydroxy-3-methylglutaryl CoA synthase	sce3181/EtnO (*Sorangium cellulosum* So ce56)	CAN93340.1	76%/86%
2382/*orf-6*	1227	Beta-ketoacyl synthase	sce3182/EtnP	CAN93341.1	64%/76%

			(*Sorangium cellulosum* So ce56)		
2383/*orf-5*	246	Acyl carrier protein	sce3183 (*Sorangium cellulosum* So ce56)	CAN93342.1	68%/81%
2384/*orf-4*	1395	Malonyl CoA-acyl carrier protein transacylase	sce3184 (*Sorangium cellulosum* So ce56)	CAN93343.1	68%/82%
2385/*orf-3*	264	Acyl carrier protein	sce3185 (*Sorangium cellulosum* So ce56)	CAN93344.1	60%/81%
2386/*orf-2*	609	Malonyl CoA-acyl carrier protein transacylase	sce3186/EtnB (*Sorangium cellulosum* So ce56)	CAN93345.1	55%/70%
2387/*orf-1*	1968	Asparagine synthase	sce3187/EtnC (*Sorangium cellulosum* So ce56)	CAN93346.1	66%/79%
2394/*orf+1*	486	Hypothetical protein	-	-	-
2395/*orf+2*	924	Hypothetical protein	-	-	-
2396/*orf+3*	609	Glutathione-*S*-transferase	Glutathione-*S*-transferase (*Yersinia pestis*)	4G9H_A	47%/65%
2397/*orf+4*	954	LysR family transcriptional regulator	LysR family transcriptional regulator (*Neisseria meningitidis*)	3HHG_A	34%/54%
2398/*orf+5*	2124	TonB-dependent siderophore receptor	TonB-dependent siderophore receptor (*Pseudomonas fluorescens*)	3QLB_A	24%/37%
2399/*orf+6*	858	Molybdate ABC transporter	Molybdate-binding protein (*Xanthomonas axonopodis* pv. *citri*)	3GZG_A	26%/39%
2400/*orf+7*	1092	Oxidoreductase	Luciferase-like monooxygenase (*Bacillus cereus*)	3RAO_A	36%/54%

CHAPTER 5

Symbiont dynamics and strain diversity in the defensive mutualism between *Lagria* beetles and *Burkholderia*

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[1]*Insect Symbiosis Research Group, Max Planck Institute for Chemical Ecology, Jena, Germany.*
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5.1. Summary

Defensive mutualisms are usually facultative in nature, and their evolutionary dynamics are often shaped by changes in the composition of local antagonist communities or arms races with coevolving antagonists. Under these conditions, selection may favor hosts that flexibly acquire novel symbionts producing secondary metabolites with bioactivity against the current antagonist community. Here we study the dynamics, prevalence, and strain diversity of *Burkholderia gladioli* bacteria in beetles of the genus *Lagria,* a recently described protective symbiosis involving vertical transmission via egg smearing and a broad-spectrum antifungal defense for the developing eggs. In *Lagria hirta*, we investigate the fate of the bacteria during the host life cycle. Despite a transmission route relying solely on the females, the bacteria are present in both sexes during the larval stage, suggesting a potentially multifaceted defensive role. In adults of *L. hirta* and *L. villosa*, culture-dependent and -independent techniques revealed that individual beetles harbor diverse *Burkholderia* strains from at least two different phylogenetic clades, yet all closely related to free-living *B. gladioli*. Interestingly, rearing the beetles in the laboratory strongly impacted symbiont strain profiles in both beetle species. Our findings highlight the dynamic nature of the *B. gladioli-Lagria* symbiosis and presents this as a valuable system for studying multiple strain coinfections, as well as the evolutionary and ecological factors regulating defensive symbiosis.

5.2. Introduction

A growing body of literature emphasizes the ubiquity of microbial symbionts in nature, as well as their impact on ecological and evolutionary processes of eukaryotes (1-3). In particular, the associations of a range of different microbial mutualists with insects have been fundamental to our understanding of symbiosis (4,5). While most research efforts have traditionally been directed towards obligate nutritional partnerships with intracellularly localized symbionts, numerous facultative associations are being discovered that are equally important for host biology (6) and provide a broader range of ecological functions to their hosts, including nutritional supplementation, degradation of otherwise indigestible dietary polymers, detoxification, heat tolerance, adaptive coloration, and defense against various antagonists (5).

In contrast to the highly intimate intracellular symbioses exhibiting low symbiont strain diversity or even monoclonal symbionts, extracellular and/or facultative symbiotic associations can be of a more dynamic nature, sometimes involving multiple symbiotic strains within individual hosts (7-11). The maintenance of multiple coinfecting strains can have considerable impact on symbiotic partnerships, especially since metabolic differences may exist even between closely related strains, thereby affecting functional roles within the host (11). The theoretical implications of mixed infections by symbionts have been discussed before, emphasizing on the possibility for increased competition between strains causing higher costs for the host (12,13). Also, maintaining multiple strains during transmission might be

problematic. There are, however, potential benefits of harboring diverse strains, like functional complementation or an increased chance of having or taking up the optimal symbiont if external conditions are variable.

The presence of coinfecting symbiont genotypes has often been related to their transmission route in the host. Whereas strict vertical transmission (transfer from parent to offspring) is usually coupled with obligate symbiosis and a high degree of partner fidelity, the opportunity for horizontal transmission (acquisition from the environment or unrelated hosts) increases the chance for multiple infections (14) and necessitates partner choice mechanisms to ensure cooperation (15). Indeed, multiple symbiont genotypes within an individual host are a common feature in systems in which horizontal transmission is the rule (9,10,16-22). Although horizontal transmission generally facilitates strain diversity, symbiont monoclonality has occasionally been observed in systems relying on environmental acquisition (23), and multiple coinfections can occur in systems relying on vertical transmission (7). This demonstrates that associating with a single or multiple symbiont strains is not solely determined by transmission mode, but by a combination of factors that likely depend on the particular ecological and evolutionary characteristics of the system. Thus, an integrated approach, including aspects like symbiont dynamics during the host life cycle and functional significance of the symbionts is relevant for understanding the causes and consequences of mixed infections in symbiosis.

Despite its ecological relevance, symbiont strain diversity is easily overlooked when studied using classical microbiological techniques and canonical sequence analysis pipelines (24), and a comprehensive view on the prevalence and consequences of multiple infections in symbiotic associations is lacking (10,25). Thanks to advances in sequencing technologies and molecular data analysis, tools to investigate microbial diversity at a higher resolution have become readily available (11,24,26).

A symbiotic association with bacteria of the genus *Burkholderia* for defense against pathogenic fungi was recently discovered in *Lagria villosa* beetles (Tenebrionidae: Lagriinae). Several lagriid beetle species host extracellular bacterial symbionts that are transmitted vertically (27, Chapter 4) and horizontally (Chapter 4). Females carry the symbiotic bacteria in a pair of accessory glands associated to the reproductive system and transmit these to the offspring in a secretion that is smeared on the egg surface during oviposition in the soil or leaf litter (27). From there, the symbionts migrate into specialized and unusual structures located dorsally in the larvae (27). The bacterial symbionts in *L. hirta* and *L. villosa*, as well as in four other Lagriinae species, have been identified as *Burkholderia gladioli* (Chapter 4), a taxon known for its plant pathogenic traits (28,29). Notably, experiments in *L. villosa* beetles demonstrate that the symbionts inhibit the growth of antagonistic fungi on the eggs of the insect host, indicating that the *Lagria*-associated *Burkholderia* have evolved from plant pathogenic ancestors into insect defensive mutualists (Chapter 4).

Here we investigated the symbiont dynamics during the life cycle of *L. hirta* and evaluated the prevalence and diversity of *Burkholderia* strains across different host populations. Our analyses reveal the presence of multiple symbiotic strains per individual female independent of geographical origin. Furthermore, we demonstrate significant and consistent shifts in the bacterial community and the symbiont strain profiles upon laboratory rearing in two species of *Lagria* beetles, which might be a common confounding effect when studying symbiotic associations in the laboratory.

5.3. Results and discussion

Symbiont localization and dynamics during host life cycle

Using a combination of molecular methods, we localized extracellular symbiotic *Burkholderia* bacteria in different life stages of *L. hirta* beetles. The observed transmission route and localization of *Burkholderia* symbionts resemble those in

the congeneric species *L. villosa* (Chapter 4), and support previous morphological descriptions and a proposed vertical transmission mechanism (27). Fluorescence *in situ* hybridization (FISH) revealed the presence of *Burkholderia* cells on the surface of eggs laid by field-collected *L. hirta* females (Fig. 1a and 1d), as well as inside three dorsal compartments of the larva (Fig. 1b and 1e), but not inside the larval gut. Symbiont titers within the dorsal structures increased in early larval instars and reached a plateau after 20-35 days of larval development, stabilizing around 10^6 *Burkholderia* 16S rRNA copies per host individual (Fig. 2a).

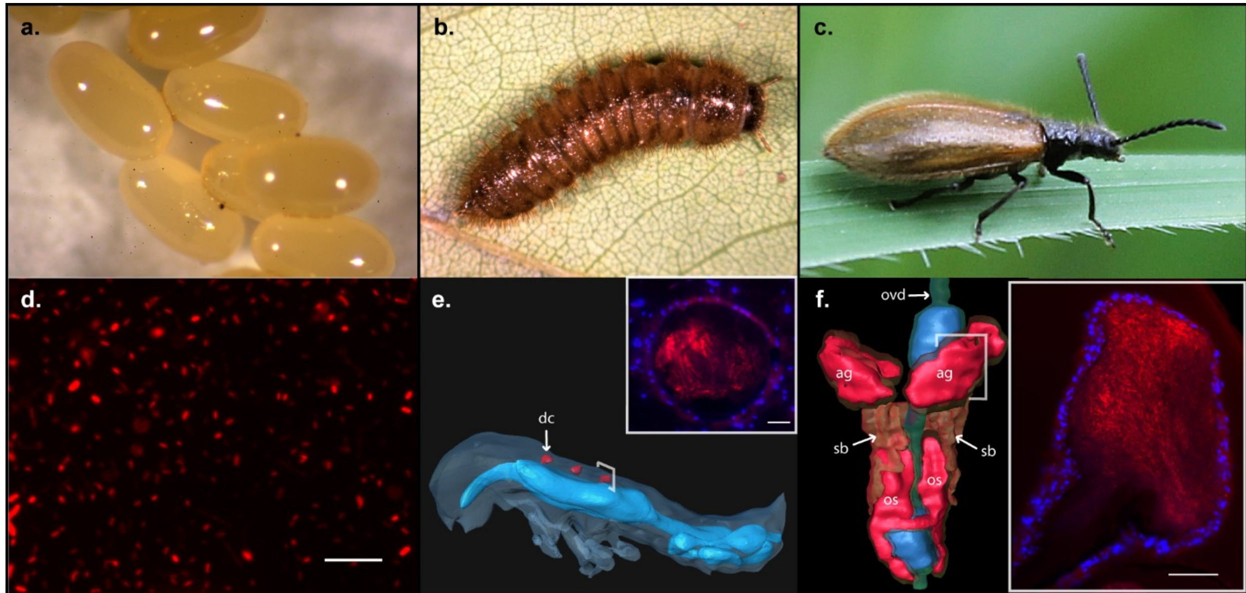

Figure 1. Localization of *Burkholderia* symbionts across *L. hirta* life stages. Host eggs (**a**) are covered with *Burkholderia* bacteria, as revealed by FISH on a 0.1% SDS solution in which eggs were previously washed (**d**). In the larvae (**b,e**), three dorsal compartments (dc) harbor the *Burkholderia* symbionts located by FISH in a cross section of a larva (inset in **e**). In female adults (**c**) the symbionts are found within a pair of accessory glands (ag) associated to the reproductive system as confirmed by FISH on cross sections of these glands (**f**). The symbionts are also present in elongated ovipositor-associated structures (os) and in the area proximate to these organs (sb, "surrounding bacteria"), also around the oviduct (ovd) (see also Fig. S2). 3D-reconstructions represent symbiont-bearing structures in red and surrounding organs in blue as a reference (**e-f**). Image (**e**) is adapted from Chapter 4. FISH pictures show *Burkholderia*-specific staining in red (Burk16S_Cy3 in **d** and **f**; Burk16S_Cy5 in **e**) and host nuclei cells in blue (DAPI). Scale bars: 10µm (**d**), 20µm (inset in **e**) and 50µm (**f**).

While in the adult stage of *L. hirta* the bacterial symbionts are only present in the females (27), sex-specific differences regarding the presence of the symbionts had not been investigated in larvae. Sex differentiation of larvae is not feasible in this species based on morphology. Therefore, we based our approach on the assumption that if only female larvae carry the symbionts, the proportion of *Burkholderia*-infected larvae as assessed by quantitative PCR should match the sex ratio observed in the sexually dimorphic adults. These proportions showed, however, statistically significant differences (Fisher's exact test, $p < 0.01$, Fig. 2b), while the proportion of *Burkholderia*-infected larvae corresponded to that of natural infection rates in adult females as assessed by the same method (quantitative PCR) (Fig. 2b). Although indirectly, this suggests that all larvae carry the symbionts and males lose them, most likely during metamorphosis. FISH experiments carried out on an *L. hirta* male during the first two days of pupation (total duration is six days) revealed the presence of *Burkholderia* in the newly-forming gut (Fig. S1a and S1b), possibly released previously from the dorsal organs. It is plausible that the bacterial cells are then removed with the meconium upon adult emergence, since microbial community analyses on the gut of field-collected adult males revealed no *Burkholderia* (Table S2). Notably, the maintenance of the symbionts during the larval stage by both sexes suggests that the bacteria confer an advantage in this life stage in addition to the egg, possibly also defensive. Although rare, the protection of two different life stages has been described in other symbiotic associations. In hoopoe birds, *Enterococcus* symbionts are known to confer antimicrobial protection to both eggs (30) and adults (31). Likewise, the bacterial symbionts of some bryozoans defend both the adult reproductive tissues as well as the larvae through the production of potent anti-predator toxins (32).

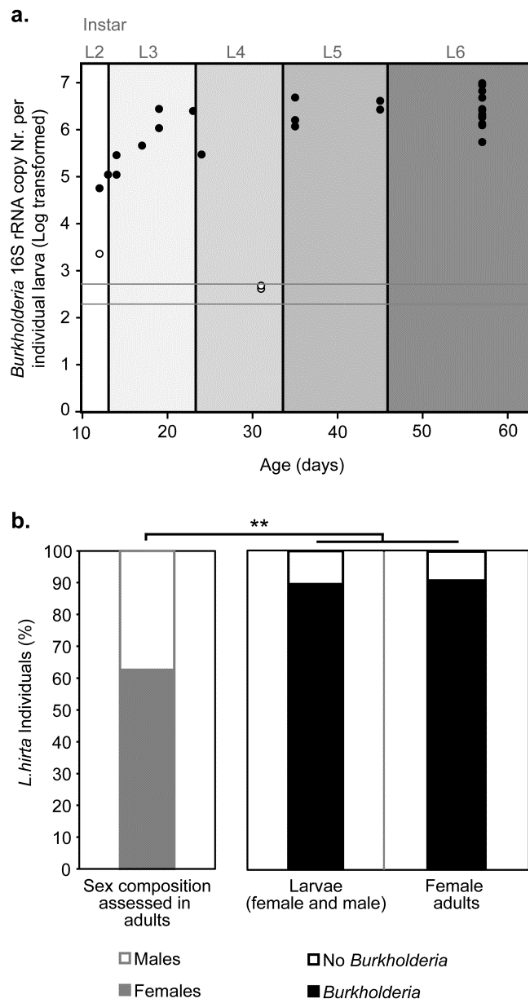

Figure 2. Symbiont dynamics during the life cycle of *L. hirta*. **(a)** Increase in *Burkholderia* symbiont titers during larval development, assessed by qPCR on the *Burkholderia* 16S rRNA gene in 29 larvae ranging from 12 to 57 days old. Horizontal gray lines represent the minimum and maximum negative control values, and shaded regions define expected instars according to Zhou et al. 2000. Empty dots represent individuals considered negative for *Burkholderia* in the infection rate calculation by quantitative PCR (Table S1). **(b)** *Burkholderia* are present in both female and male larvae, based on the significantly higher proportion of infected larvae compared to natural sex ratios recorded in adults ($N_{larvae} = 29$, $N_{adults} = 744$, Fischer's exact test, $p < 0.01$) and the exactly matching proportion to natural infection rates in adult females ($N_{adult\text{-}females} = 22$). Infection rates for larvae and adult females are based on quantitative PCR data (Table S1).

In *L. hirta* adult females (Fig. 1c), *Burkholderia* are localized within two sac-like accessory glands associated with the reproductive system and in the space directly surrounding the ovipositor (Fig. 1f and S2). Notably, the corresponding glands in *L. villosa* are tubular in shape and thus morphologically distinct to those of *L. hirta* (27). In the latter host species, *Burkholderia* were also detected inside a pair of elongated structures within the ovipositor, confirming early observations by Stammer (1929) (Fig. 1f and S2). These ovipositor-associated structures open caudally in the female body, next to the oviduct and the anus. Due to potentially higher exposition to the environment and feces, it is possible that additional non-*Burkholderia* bacteria are also present in the ovipositor-associated structures (Fig. S2). Interestingly, among the numerous Lagriinae species that have paired accessory glands proposed to harbor symbionts, not all have analogous ovipositor-associated structures (27). This suggests that these structures are not ancestral and might be only complementary to the accessory glands for maintaining and/or transmitting the symbionts.

The mechanism of symbiont transfer from the larval dorsal compartments to the accessory glands in the female reproductive system remains elusive. No *Burkholderia* have been observed in the gut of female pupa using FISH (Fig. S1c), and in adult female guts only traces of *Burkholderia* are found (Table S2), which could be due to contamination from the reproductive system. Thus, release of the symbionts from the dorsal organs into the gut and subsequent active migration through the digestive tract and into the accessory gland appears unlikely. As proposed previously, however, it is possible that during molting the dorsal compartments are exposed to the molting fluid and the motile *Burkholderia* migrate towards the developing accessory glands, which develop from invaginations of the intersegmental skin towards the caudal region of the body (33).

Prevalence of *Burkholderia*

To assess the prevalence of *Burkholderia* in *L. hirta* beetles from four different populations, we used culture independent (PCR/cloning/sequencing and quantitative PCR) as well as culture-based screening to determine the proportion of adult females carrying *Burkholderia*. According to the culture-dependent approach, 81.8 % of the individuals carried symbiotic *B. gladioli*, while both culture-independent methods revealed infection rates of 90.9 % (Table S1). Regardless of the difference in infection rates revealed by these approaches (likely explained by false negatives in culture-based screening), *B. gladioli* is highly prevalent in *L. hirta* populations. However, the absence of the symbionts in some of the individuals indicates a facultative rather than obligate relationship for the host. This is coherent with previous findings on the protective role of the *B. gladioli* symbionts in the congeneric beetle species *L. villosa* (Chapter 4), since the context-dependence of defensive symbioses is thought to account for their usually facultative nature. Maintaining a protective symbiont is expected to be influenced by the probability of encountering a relevant antagonist, as the costs might otherwise outweigh the benefits of symbiosis (34, Chapter 2). In fact, infection rates in numerous microbe-animal defensive symbioses are in line with this expectation (36-43). In Lagriinae, it is worth noting that earlier observations suggest that horizontal acquisition of *B. gladioli* also occurs (Chapter 4), which might ameliorate the risk of permanent symbiont loss.

As to the presence of the symbionts in individual hosts, high-throughput amplicon sequencing confirmed *Burkholderia* as the dominant taxon in the accessory glands of female beetles (75-90%) (Table S3 and Fig. 4a). Additionally, qPCR yielded an estimate for the average absolute abundance of $1.3x10^7$ *Burkholderia* 16S rRNA gene copies per individual female accessory gland (Fig. S3). Considering the presence of two glands with equivalent characteristics in each female, and using the 16S rRNA gene copy number per cell from the cultivated *B. gladioli* symbiotic strain as a reference (i.e., six), approximately $4.4x10^6$ cells are harbored in the accessory glands of a single female. Additionally, the presence of *Burkholderia* in the ovipositor-associated structures and around the ovipositor in *L. hirta* should result in an even higher total number of symbiont cells per female.

Symbiont strain diversity

Phylogenetic analyses of almost full-length 16S rRNA sequences obtained from cloned PCR amplicons as well as from cultured *Burkholderia* isolates revealed symbionts from two different clades to be associated with *L. hirta* (referred to henceforth as clades I and II). The two clades form a monophyletic group together with other *B. gladioli* strains. While members of both clades were detected using culture-independent methods, only isolates from clade II were recovered by culture-dependent methods (Fig. 3), including *B. gladioli* Lh-StG, which is referred to in other chapters. *B. gladioli* from both clades were represented in the four beetle populations that were investigated, even in those with low sampling numbers (Table S1), suggesting no geographical pattern associated to their occurrence (Fig. 3). However, more extensive sampling is required to determine precise infection rates for each strain in the different populations. Interestingly, *B. gladioli* from clade I and II can also co-occur in a single host (Fig. 3, see samples FreiburgF5, Krakow228, and Jena183). Additional deep sequencing of 16S rRNA amplicons (~300bp) followed by subspecies-level bioinformatic analyses (oligotyping and minimum entropy decomposition) (24,26) on single accessory glands of six field-collected females supported the existence of multiple *Burkholderia* oligotypes (hereafter referred to as strains) per insect host, from which three were found in all sampled females (*Burkholderia* - 3, 2 and 17), and one of these (*Burkholderia* - 3) was the most abundant across all six individuals (53- 91% of *Burkholderia* reads per female gland) (Fig. 4a). The partial 16S rDNA sequences of these and other less consistent but abundant strains (*Burkholderia* – 7, 8 and 9) were between 99.7 and 100% similar to the corresponding fragment in sequences assigned to clades I and II in the phylogenetic reconstruction (Fig. 3 and 4a). An analogous analysis in *L. villosa* revealed similar patterns of symbiotic *Burkholderia* strain diversity, with three consistent strains across field-collected individuals (Fig. S4a), suggesting that multiple infections with *Burkholderia* are a common feature in the interaction between these bacteria and Lagriinae beetles. The most abundant of these strains in *L. villosa* (*Burkholderia* - 1) does not correspond to any sequence within clades I or II from the *L. hirta* symbionts. However, the single culturable strain from *L. villosa* (refered to as *B. gladioli*-LvStA in all other chapters) falls within clade II and matches one from *L. hirta* (*Burkholderia* – 2) in the region used for oligotyping analysis. Also, the third consistent strain (*Burkholderia* – 3) falls within clade I (Fig. S4). It is worth noting that given the length of the fragment used for oligotyping, this does not necessarily imply complete identity between the corresponding symbionts of *L. hirta* and *L. villosa*.

Our results show that multiple symbiotic *Burkholderia* strains are associated with *L. hirta* and *L. villosa* beetles despite the vertical transmission route that involves specific morphological adaptations in the host. This regular coexistence of multiple similar symbiotic strains raises interesting questions in terms of the evolutionary stability of such an interaction, as has been discussed for other cases of mixed infections (12,13): is there competition between coinfecting strains, and if so, what keeps one of the strains from outcompeting the others? Does competition result in costs for the host and how can this remain stable over evolutionary timescales? Multiple infections in a single host can indeed influence symbiont-conferred functions (44,45) or result in symbiont virulence and physiological costs for the host (46). In addition to potential competition, polyclonality can cause relaxed selection on symbiotic functional roles of individual genotypes (47), like nutrient provision or defensive compound production. From an alternative perspective, carrying a diversity of symbiotic strains can be beneficial in certain circumstances, such as in arms-race scenarios that would favor a dynamic association. Hypothetically, this could hold true for herbivore symbionts that detoxify plant secondary metabolites (48,49), entomopathogenic nematode-associated strains that suppress the insect host's immune system (50,51), or defensive symbioses in general (Chapter 2).

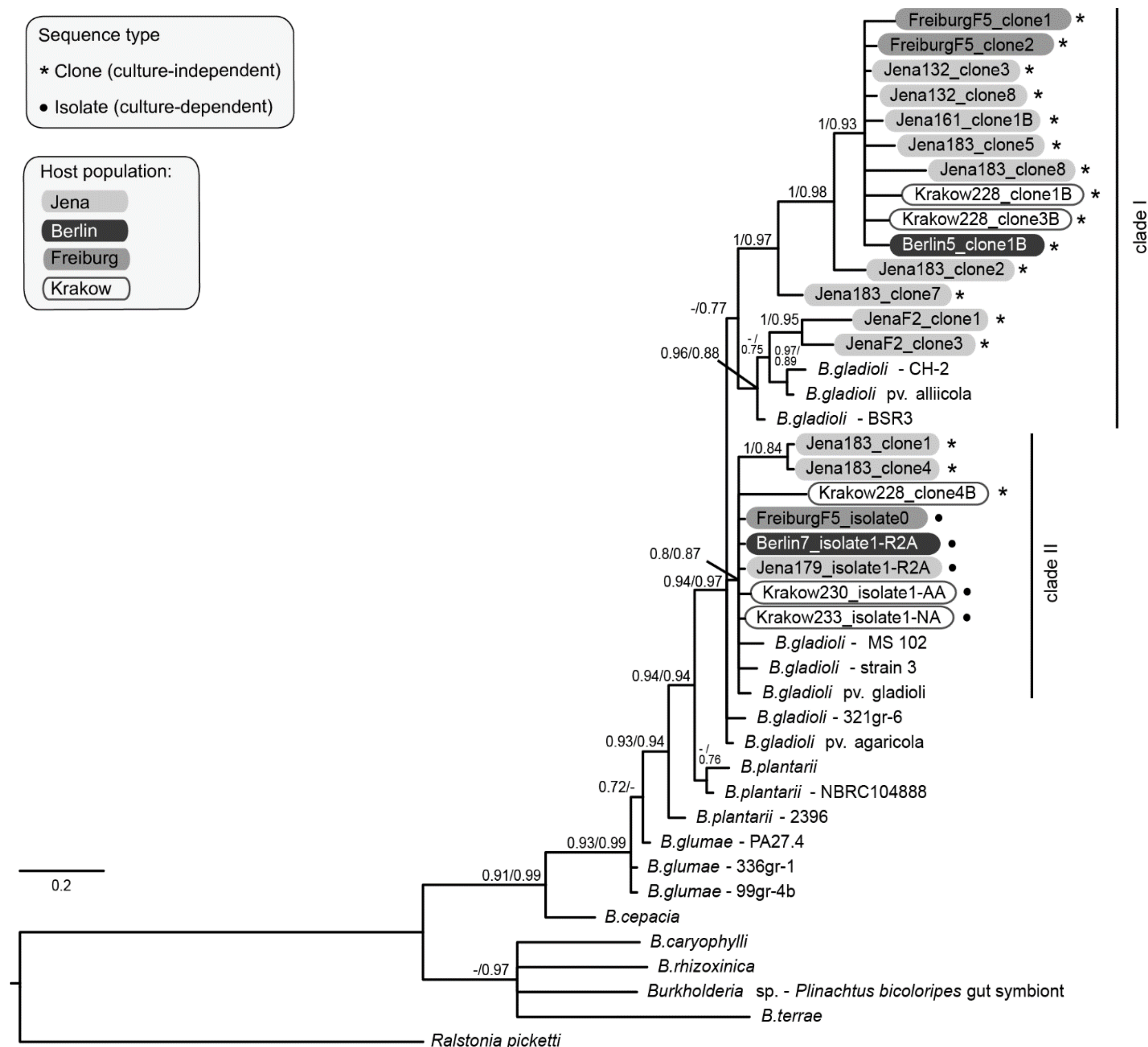

Figure 3. Phylogenetic placement of *Burkholderia* symbiont strains from different *L. hirta* populations. The phylogeny is based on almost complete 16S rRNA gene sequences (1300 bp) and tree reconstruction using Bayesian and approximately-maximum-likelihood algorithms (FastTree). Posterior probabilities and local support values above 0.7 are reported at the nodes. Sequence accession numbers are listed in Table S4. Host populations are represented in different background shadings, and characterization methods (culture dependent or independent) are represented by symbols.

Considering that the symbiosis with *Burkholderia* occurs in several beetles of this subfamily and is thus most likely ancient (Chapter 4), and that multiple strains were found in both Lagriinae species studied in depth (*L. hirta* and *L. villosa*), a benefit of harboring this strain diversity is plausible. Additionally, the inhibition of antagonistic fungi by *Burkholderia* symbionts has been demonstrated in *L. villosa* eggs (Chapter 4), so it is possible that the combined action of multiple strains results in the production of a larger diversity of secondary metabolites and thereby in the inhibition of a broader spectrum of antagonistic microbes of *Lagria* beetles. In *L. villosa*, different compounds seem to be produced by different strains. In an isolated symbiotic *B. gladioli* strain, we could identify the production of the bioactive substances toxoflavin and lagriene, and the additional presence of the biosynthetic gene cluster for the production of the antibiotic caryoynencin. The bioactive compound lagriamide, on the other hand, is presumably produced by another *B. gladioli* symbiotic strain in the microbial community. Concordantly, a diversity in bioactive compounds has been reported for

several other defensive symbioses, either due to the presence of multiple producing strains (44,52) or the production of several different compounds by a single symbiont (53,54). However, both a defensive role in *L. hirta* and the benefit of multiple coinfecting strains in this system require additional experimental evidence.

Interestingly, the occurrence of coinfecting strains appears to be common for symbiotic *Burkholderia*, as mixed infections in a single host individual have been found across several symbiotic systems involving this bacterial genus, namely in stinkbugs (9,10), bordered plant bugs (55), and in root nodules of legumes (18). *Burkholderia* are common bacteria in a variety of environments and exhibit extraordinary ecological versatility (29). Thus, for the host it might be favorable to maintain a flexible relationship as this allows for selecting the best available partner(s) (56). Additionally, genomic features of *Burkholderia* like the high incidence of genomic islands, plasmid-like genes, bacteriophages and insertion sequences indicate that members of this genus are susceptible to frequent genetic exchange (57). This relates to recurrent opportunities for the symbionts to reacquire genetic traits lost during host adaptation, thus avoiding host specialization. Replenishing genetic diversity through horizontal gene transfer with coinfecting symbionts and/or with environmental microorganisms during phases of host-independent existence is likely highly relevant in this context. Concordantly, the majority of *Burkholderia* strains described so far have large multireplicon genomes encoding diverse metabolic functions, rapid evolutionary rates and remarkable phenotypic diversity (57), which is in line with dynamic ecological interactions.

Influence of laboratory conditions on bacterial community and symbiont strain composition

Indications of occasional symbiont loss by *L. hirta* in the laboratory motivated us to investigate the influence of this artificial environment on the bacterial profiles in the accessory glands, as well as on the *Burkholderia* strain composition. While field collected females showed a consistent bacterial community dominated by *Burkholderia*, individuals that had been reared in the lab for at least one generation had more variable profiles (Fig. 4c-d and Fig. 5). In three out of six females (Nr. 7, 9, and 11) the prevalent bacterial genus was *Streptomyces*, rather than *Burkholderia*, which was only represented in less than 0.27% of the reads in the glands of these individuals (Table S3). Given the well-known potential of members of the genus *Streptomyces* for production of bioactive substances (58), this observation encourages further investigation on whether and how other antibiotic-producing bacteria could replace the native defensive mutualists. Additionally, comparing *Burkholderia* strain profiles between natural and lab rearing conditions revealed that the naturally dominant strain (contained in clade I) was either completely lost or at least strongly reduced in individuals from the lab. Instead, *Burkholderia* was represented mostly by a second strain (contained in clade II) in this group of females (Fig. 4a and b). However, at least in some of the individuals (Nr. 7, 9, and 11) the relative abundance of this strain might be explained by the drop in clade I instead of an enrichment of bacterial cells in clade II, given the very low representation of *Burkholderia* in the total eubacterial reads (Fig. 4b and Table S3). Furthermore, a comparable data set from *L. villosa* showed a similar impact of laboratory conditions on the consistency and relative proportions of symbiotic strains (Fig. S4). The pronounced differences in *Burkholderia* strain composition between lab-reared and field-collected individuals in both *Lagria* species were statistically significant (analysis of similarities "ANOSIM", $p < 0.01$), and the corresponding pattern was visualized based on non-metric multidimensional scaling (NMDS) (Fig. 5).

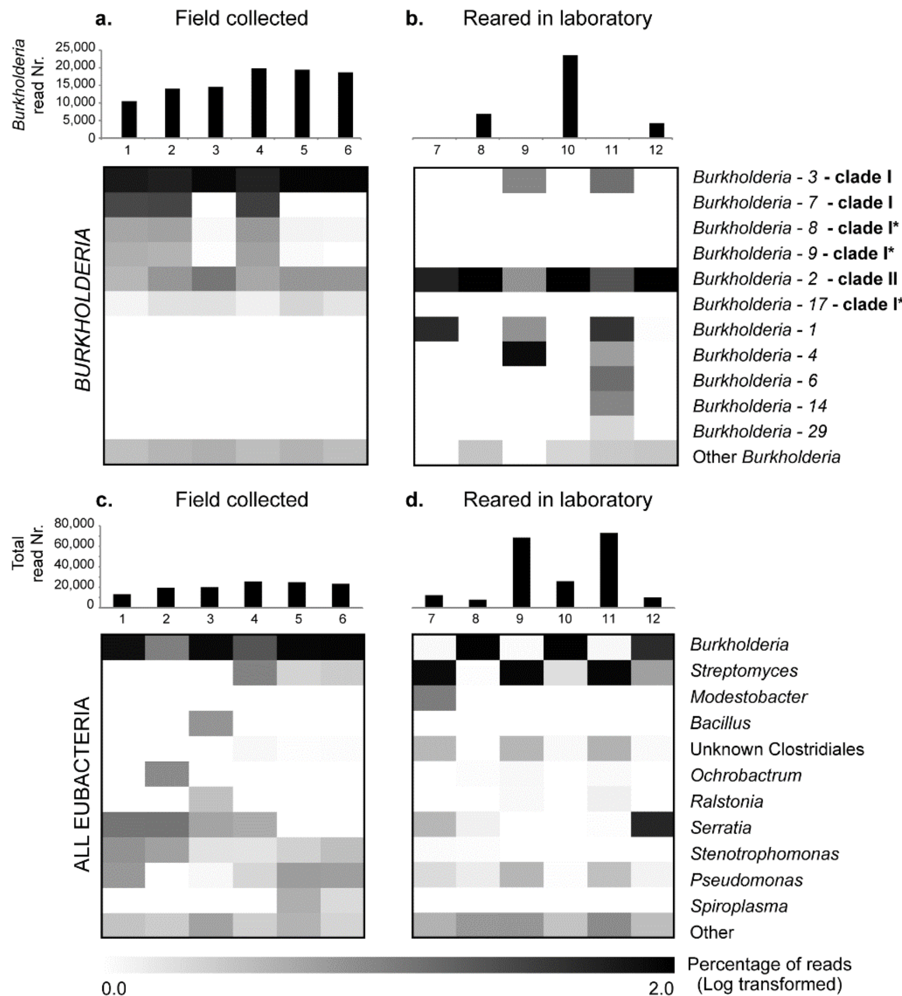

Figure 4. Bacterial community composition in the *L. hirta* female accessory glands under field and laboratory conditions as assessed by 454 pyrosequencing. (**a-b**) Total *Burkholderia* sequence numbers (bar graph – upper panel) and relative proportions of *Burkholderia* strains (log transformed) in female accessory glands based on an oligotyping analysis of individuals (**a**) collected in the field and (**b**) reared in the laboratory. Strains in close (99.7%, indicated by asterisks) or complete (100%) agreement with the corresponding fragment in any of the sequences from clades I or II (Fig. 3) are indicated. All others are at least 97% similar to one of the sequences used for the phylogeny. (**c-d**) Total eubacterial sequence numbers (bar graph – upper panel) and relative proportions (log transformed) of different bacterial genera per female accessory gland from individuals (**c**) collected in the field and (**d**) reared in the laboratory.

The near to complete loss of strains from clade I in *L. hirta* individuals that have been reared in laboratory conditions (Fig. 4a and 4b) strongly suggests that these are acquired primarily from the natural environment. Horizontal transmission in Lagriinae beetles has in fact been supported by previous phylogenetic analyses (Chapter 4), although currently there is no information on the rates of vertical and horizontal transmission in natural populations. In the light of this apparently recurrent opportunity for environmental acquisition, there should be efficient recognition and control mechanisms stabilizing the association specifically with closely related *B. gladioli* strains.

As in the *Lagria-Burkholderia* association, a few symbioses with described routes for vertical transmission also show cases of multiple strains within single host individuals, such as *Prochloron* in didemnid ascidians (44,59), *Wolbachia* in *Asobara* wasps (46) and other insects (60), or *Burkholderia* in the oriental chinch bug *Cavelerius saccharivorus* (61). In the oriental

chinch bug, horizontally acquired strains seem to account for a significant fraction of the observed diversity, while at least one of the strains can be vertically transmitted (61). A similar scenario could occur in *Lagria* beetles. Interestingly, in *L. hirta* the strain that is most likely to remain associated to females when displaced from their natural environment is also the only strain amenable to cultivation so far (Fig. 3a and b). Thus, despite lacking signs of strong host dependence, it shows a more stable association with this beetle species. This stability could be either due to higher success at horizontally infecting the beetles under lab conditions or explained by the maintenance of vertical transmission. It is certainly possible that all or some of the symbiotic strains are usually vertically transferred in natural conditions but are vulnerable to specific conditions in the laboratory that hinder transmission either directly (e.g. temperature, humidity) or via the host's regulation, in the form of reduced available nutrients or other physiological changes associated to this artificial environment (e.g. immune response). Under these circumstances, the results suggest that clade II is more resilient and thus more likely to be maintained in the laboratory, at least in the case of *L.hirta*. It will therefore be interesting to investigate in the future which strains can be vertically transmitted under field and lab conditions, and what specific factors explain the altered *Burkholderia* profiles in the laboratory.

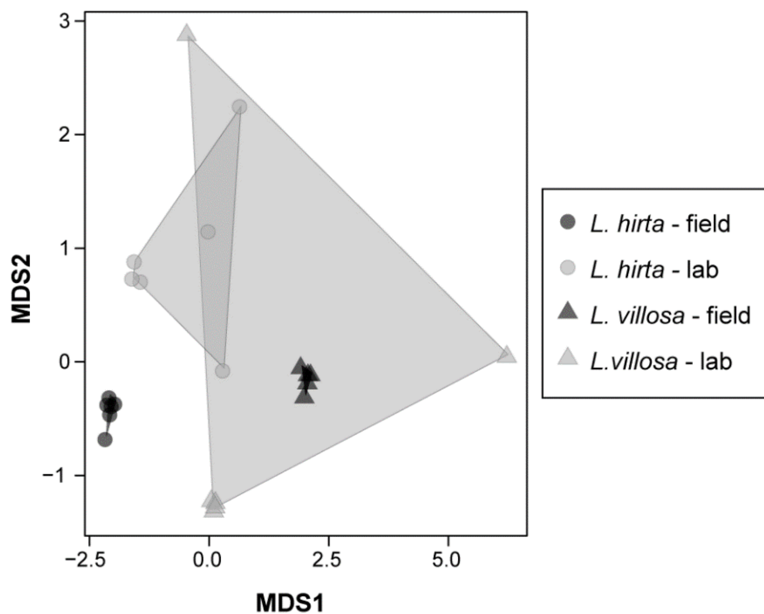

Figure 5. Non-metric multidimensional scaling (NMDS) on *Burkholderia* strain composition in *L. hirta* and *L. villosa* female accessory glands from field-collected and lab-reared individuals as assessed by minimum entropy decomposition analysis (MED) of 16S amplicon pyrosequencing data (see Fig. S4). Each data point represents a single replicate, species are represented by symbols, and environment (field or lab) is depicted in different grays. Statistical analyses indicate significant differences among all groups, as well as between lab and field individuals within each of the species (ANOSIM, $p < 0.01$ for all analyses).

Documenting the occurrence of coinfecting symbiotic strains and understanding the factors that determine their persistence in nature is highly pertinent for the fields of symbiosis and microbial ecology. The availability of high-resolution molecular tools appropriate for this purpose should now allow us to investigate symbiont mixed infections in more detail, and further address questions on the evolutionary and ecological factors that determine exclusivity or promiscuity in symbiotic partnerships, as well as the functional importance of strain diversity for the host. The *Burkholderia*-Lagriinae symbiosis is an example of a flexible yet persistent defensive symbiosis with multiple coinfecting strains. Due its experimental amenability, it represents a promising model system to study the dynamics of strain diversity as well as the molecular basis of symbiont specificity in facultative symbiosis.

5.4. Experimental Procedures

Insect collection and rearing

L. hirta adult specimens were collected in Jena, Freiburg and Berlin (Germany) between June and August of 2012, 2013 and 2014; and in Krakow (Poland) in July 2012. The beetles were reared in plastic containers (16.5x19x25cm) at temperatures between 20-25°C, and at 5°C for a three month diapause period corresponding to larval instars 3-5. The light regime was varied stepwise ranging from 16h/8h to 8h/16h (2 hour adjustment every month, except for winter and summer periods in which it was readjusted after 3 months). Autoclaved water was supplied in 50 mL centrifuge tubes covered with moist cotton, and a petri dish with soil or cotton was provided as an oviposition substrate. Adults were fed with fresh leaves from a variety of plants, including *Betula pendula, Tilia cordata, Tilia platyphyllos, Robinia pseudoacacia, Cornus sanguinea, Viburnum lantana, Acer platanoides* and *Acer campestre,* and with *Viburnum lantana* dry seeds. In *L. hirta*, the sex ratio was determined based on 744 specimens collected in natural populations from 2012-2014 and in 292 adults emerged in the laboratory in 2013. Since field and lab populations did not differ in adult sex ratio (χ^2 homogeneity test; df=1, n=292, χ^2=2.38, p=0.12), only the sex ratio in the natural population is shown in the results section. *L. villosa* individuals were collected in Brazil between January and March 2015 (SISBIO authorization Nr. 45742-1, CNPq process nº 01300.004320/2014-21). This species was kept under similar conditions, but not exposed to 5°C for diapause induction, and kept under a natural light regime. *L. villosa* adults were fed with *Glycine max* (soybean), *Brassica napus* (raps) and *Pisum sativum* (pea) leaves. Larvae of both species were reared under the same conditions as the adults and fed with dry leaves of the corresponding plant species.

Fluorescence in situ hybridization

FISH was used to localize *Burkholderia* on *L. hirta* eggs, as well as in larvae, male and female pupae, and adult females. To assess the presence of the symbionts on the egg surface, five eggs were washed in 0.1% SDS, and single drops of the washing suspension were dried on a glass slide and fixed with 50% ethanol. Larvae, pupae and a reproductive system dissected from an adult *L. hirta* female were fixed in a formaldehyde solution (4% in PBS) at 4°C overnight, washed three times for 20 min in PBS-Tx (0,3% Triton X-100 in PBS) and overnight in PBS + 6,8 % sucrose at 4°C. Samples were subsequently dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 90%, 96%, 99%) and 3x in isopropanol during 24h for each step. Dehydrated samples were embedded in Technovit 8100 cold polymerizing resin (Heraeus Kulzer, Germany) according to the manufacturer's instructions, cut into semithin sections (8µm) with a Microm HM355S microtome (Thermo Fisher Scientific, Germany) and mounted on glass slides coated with poly-L-lysine (Kindler, Germany). The tissue sections and the fixated egg-wash suspension were incubated for 90 min at 55°C in hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl (pH 8.0), 0.01 % SDS) containing 0.5 µM of the eubacterial probe EUB338 (Amann *et al.*, 1990) and the *Burkholderia*-specific probe Burk16S (modified from primer BKH1434Rw in Opelt et al. 2007) (Table 1); each labeled with either Cy5 or Cy3. DAPI (4′,6-diamidino-2-phenylindole) was used for host DNA counterstaining. After hybridization, samples were washed twice with buffer (0.1 M NaCl, 20 mM Tris/HCl (pH 8.0), 5mM EDTA, 0.01% SDS) and kept for 20 min at 55°C. Subsequently, the washing buffer was removed, distilled water was applied, and the samples were incubated at 55°C for 20 min before a last wash with water. Finally, samples were air-dried and covered with VectaShield® (Vector Laboratories, Burlingame, CA, USA). Images were acquired on an AxioImager.Z1 epifluorescence microscope (Carl Zeiss, Jena, Germany). In order to confirm the specificity of the Burk16S probe, the same procedure was carried out on cell suspensions obtained from *Achromobacter*, *Acinetobacter*, *Sphingobacterium*, *Ochrobactrum* and *E.coli* liquid cultures (negative controls) as well as symbiotic *B. gladioli* previously isolated from an *L. hirta* female (positive control).

3D reconstruction

An *L. hirta* larva and a dissected portion of a female reproductive system including the symbiont-bearing organs were fixated in Bouin solution (picric acid solution in water (1.2%), formaldehyde (37%) and acetic acid in a 19:6:1 volume ratio) at 4°C. Samples were dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 90%, 96%, 99%) and 3x in isopropanol during 24h for each step and embedded using the Epoxy Embedding Medium kit (Sigma-Aldrich, Germany). After infiltrating samples in aceton-epoxy solutions (2:1, 1:1 and 1:2 volume ratios) for two hours each, samples were transferred to a silicon form and covered with the epoxy embedding solution. Polymerization was performed following the manufacturer's instructions. Hardened epoxy blocks were cut in a rotation microtom (Mikrom HM355S) using a diamond knife (Diatome histo jumbo, Munich, Germany) to sections 2µm thick. These were placed on a silanized glass slide and dried for 24h at 60°C. Sections were stained with toluidine blue-pyrimidine solution (4.4 mg/mL toluidine blue, 1 mg/mL pyrimidine, 4.4 mg/mL sodium tetraborate in distilled water), humidified with xylol and covered with Entellan (Merck, Germany). Section images were acquired in an Axioimager.Z1 microscope (Carl Zeiss, Germany), and the 3D reconstruction was carried out using Amira 5.4.1. software.

DNA isolation, 16S rRNA gene amplification, cloning and sequencing

24 *L. hirta* live females were frozen at -20°C for 20 min, and each of the paired glandular structures associated to the ovipositor was dissected. For each individual, one of the organs was directly processed for bacterial cultivation as described below, and the second was stored at -80°C for nucleic acid extraction. For larvae, the complete bodies of 29 individuals between 12 and 57 days old were used for DNA extraction. After homogenizing each sample with liquid nitrogen, 300 µL of tissue and cell lysis solution (MasterPure™ complete DNA and RNA isolation Kit, Epicentre Technologies, Hessisch Oldendorf, Germany) and 4 µL lysozyme (100 mg mL^{-1}) were added. The samples were incubated at 37°C for 30 min, and the subsequent steps were carried out following the manufacturer's instructions. Isolated nucleic acids were resuspended in Low TE buffer (0.1 x TE) and stored at -20°C or -80°C. The 16S rRNA gene was amplified using the general eubacterial primers fD1 and rP2 (Weisburg et al., 1991. Table 1) on a Biometra TProfessional Thermocycler (Biometra, Jena, Germany) in 12.5 µL reaction volumes containing 1 µL template, 1 x PCR Buffer [100 mM Tris-HCl pH 8.3, 500 mM KCl and 15 mM $MgCl_2$], 0.5 mM $MgCl_2$, 0.24 mM dNTPs, 0.8 µM of each primer, and 0.5 U of Taq DNA polymerase (VWR, Dresden - Germany). The following parameters were used: 3 min at 94°C, followed by 32 cycles of 40 s at 94°C, 60 s at 65°C and 60 s at 72°C, and a final extension step of 4 min at 72°C. PCR products were purified using the InnuPREP PCRpure Kit (Analytik Jena-Biometra, Jena - Germany) following the manufacturer's instructions. Purified products were either sequenced directly using fD1 and rP2 primers, or first cloned into *E.coli* using the TOPO® TA Cloning® Kit (Invitrogen, Darmstadt, Germany). The cloning reaction was set up as described by the manufacturer. After mixing, the reaction was incubated for 20 min at room temperature and placed on ice. 2 µL of the cloning reaction mixture were subsequently added to a tube of thawed *E.coli* Strata Clone SoloPack Competent cells (Agilent Technologies, Frankfurt, Germany). The transformation mixture was placed on ice for 20 minutes and heat-shocked at 42°C for 45 s. After a 2 min incubation on ice, 250 µL of LB medium (pre-warmed at 42°C) were added and the cells were allowed to recover at 37°C with agitation for 1 h. 80 µL of each transformation mixture were then plated on LB-ampicilin plates previously spread with 40 µL of 2% X-gal (Zymo Research, Freiburg, Germany) and incubated at 37°C overnight. White colonies were used for plasmid insert sequencing using the M13 primer pair (Table 1).

Quantitative PCR

Quantitative PCR (qPCR) on DNA isolated from female accessory glands and larvae was carried out in a RotorgeneQ cycler (Qiagen, Hilden, Germany) following the protocol described for the Rotor-Gene SYBR Green PCR Kit and using the Burk16S_1F and Burk16S_1R primers (Table 1). This primer pair amplifies a 172 bp region of *Burkholderia* 16S rRNA, including all known symbiotic strains of *L. hirta* as confirmed *in silico*. PCR conditions were as follows: 95°C for 10 min, followed by 45 cycles of 95°C for 10s and 65°C for 30s. A melting curve was subsequently performed with a temperature ramp from 60°C to 99°C within 4.25 min.

Bacterial cultivation

The second accessory gland of each *L. hirta* female (N=22) was homogenized and suspended in 100 µL of sterile PBS and diluted at a factor of 10^{-5} in PBS. 100 µL of each dilution were plated on Nutrient Agar (5g/L peptone, 3g/L beef extract, 15 g/L agar), R2A Agar (Sigma Aldrich, Karlsruhe, Germany), and Actinomycete Agar (Sigma Aldrich, Karlsruhe, Germany) and incubated at 30°C for at least 7 days until distinct colony morphologies were identified. Biomass from colonies representing morphologically distinct types (a minimum of three per sample and per medium) was recovered and incubated in 100 µL of lysis solution (67 mM Tris–HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 5 mM beta-mercaptoethanol, 6.7 mM $MgCl_2$, 6.7 µM EDTA (pH 8) and 1.7 mM SDS) at 95°C for 5 min. Lysed colony suspensions were then used directly for PCR amplification as described above.

Bacterial identification and phylogenetic analysis

Sequences were curated manually in Geneious 6.0.5 (http://www.geneious.com) (62) and imported into Blast2go (63) for taxonomy assignment. 22 selected sequences were aligned together with 19 *Burkholderia* reference sequences and one *Ralstonia* sequence retrieved from GenBank (accession numbers listed in Supplementary Table 4) using the SINA alignment software (64). The phylogenetic reconstruction was based on an approximately-maximum-likelihood algorithm as implemented in FastTree 2.1.8 (65), and on Bayesian inference in MrBayes 3.1.2 (66) using a generalized time reversible (GTR) model in both cases. The Bayesian analysis was run for 20,000,000 generations sampling every 1,000 generations, and a 'burn-in' of 2,000 (=10%) was applied.

Microbial community analysis by high-throughput sequencing

Bacterial community profiles were characterized for individual accessory glands from 12 *L. hirta* females (six field collected and six reared in the laboratory) and eleven *L. villosa* (five field collected and six reared in the laboratory), as well as for two samples corresponding each to DNA pooled from six *L. hirta* female guts and six *L. hirta* male guts, respectively, from field-collected individuals. These sequence data have been submitted to the NCBI Sequence Read Archive (SRA) database under the BioProject ID PRJNA306502 and accession numbers SAMN04364612-37. Bacterial tag-encoded FLX amplicon pyroseqencing (bTEFAP) was performed by an external service provider (MRDNA Lab, Shallowater, TX, USA) using 16S rRNA primers Gray28F and Gray519R (Table 1). Sequencing was performed on a Roche 454 FLX based on company protocols. Processing and analyses of the sequences were carried out in QIIME (67). To ensure sequence quality, the split_libraries.py script was set to remove sequences below 200 or above 600 bp in length as well as those with more than one ambiguous base or one or more mismatches in the primer sequence. Additionally, a 50 bp quality score window was applied and no barcode errors were allowed. After quality filtering, between 7,737 and 73,151 high-quality reads per sample were available for analysis. To assess the taxonomic profile of the entire microbial community in the accessory glands, reads were clustered into operational taxonomic units (OTUs)

with a 97% similarity cut-off, using cd-hit (68) and uclust (69) algorithms. The most abundant sequence for each OTU was extracted as a representative sequence for taxonomy assignment, which was done using the uclust consensus taxonomic assigner against the Greengenes 13_5 database. For graphical representation, OTUs corresponding to the same genus were combined, and the percentage of reads in each genus relative to the total reads per sample was used to construct a heatmap in MultiExperiment Viewer software (MeV 4.9.0) (70).

To analyze the *Burkholderia* strain composition, taxonomy was assigned to every single read using the RDP classifier (Wang et al. 2007), based on the Greengenes core set (71). All original reads assigned to the genus *Burkholderia* (252,462) were retrieved and aligned with PyNast (72) using the Greengenes core reference alignment (73) with default settings in Qiime (67). The alignment was filtered without a lane mask and trimmed to 295 bp. Subsequently, oligotyping (24) and minimum entropy decomposition (MED) (26) pipelines were used to identify highly refined taxonomic units (oligotypes) within *Burkholderia*. For noise reduction, each oligotype was required to (i) occur in more than 0.1% of the reads for at least one sample, (ii) represent a minimum of 20 reads in all samples combined, and (iii) have a most abundant unique sequence with a minimum abundance of 20. After quality control, 98,8% of the reads were retained. For graphical representation, oligotypes containing less than 1% of the reads per sample were merged into a single category "Other *Burkholderia*". The corresponding heatmap was constructed in MeV 4.9.0 (70). Non-metric multidimensional scaling (NMDS) and analysis of similarities (ANOSIM) for *Burkholderia* strain data were carried out in R. 2.14.1 using the vegan package (74), and the corresponding figure was built using the ggplot2 package (75) in the same software.

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5.6. Supplementary Information

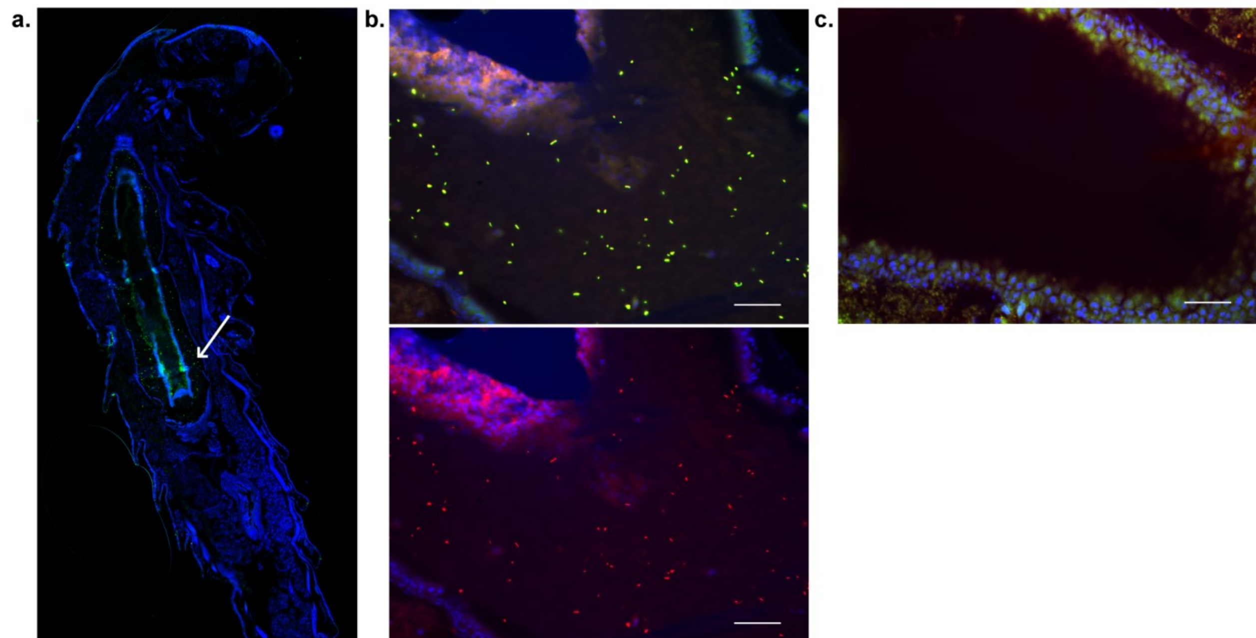

Figure S1. Fluorescence *in situ* hybridization (FISH) of *Burkholderia* symbionts in male and female *Lagria hirta* pupae. **(a)** Longitudinal section of a male at early pupation stage (day 1-2). The arrow indicates fluorescently labeled bacteria in the gut, mostly surrounding the larval gut that is shed during pupation. **(b)** Higher magnification of a male pupa (day 1-2) in the gut region, showing bacterial cells labeled with the general eubacterial probe EUB338-Cy5 in green (upper picture) and the *Burkholderia*-specific probe BKH1434-Cy3 in red (lower picture). **(c)** Female pupa (day 1-2) showing no bacteria in the gut region. Scale bars correspond to 50μm. Host cell nuclei were counterstained with DAPI (blue).

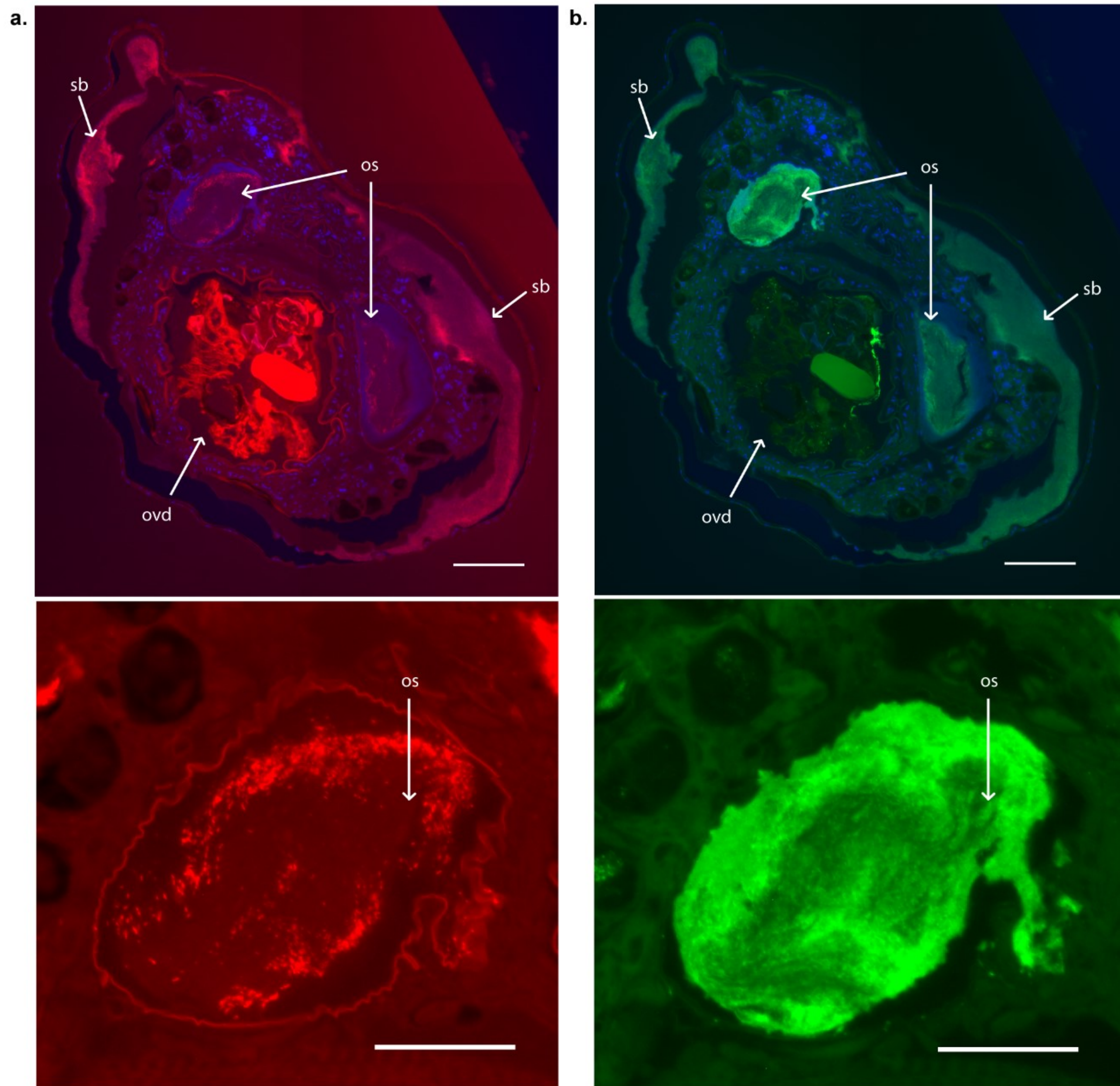

Figure S2. Localization of *Burkholderia* symbionts in a cross-section through the ovipositor of an adult *Lagria hirta* female collected from the field. Besides the sac-like accessory glands connected to the ovipositor (see main manuscript Fig. 1), a pair of structures (os) within the ovipositor and adjacent to the oviduct (ovd) also harbor symbionts, and additional bacterial cells are distributed in the area surrounding the ovipositor (sb) as revealed by FISH (**a**-**b**). (**a**) FISH using the *Burkholderia*-specific probe BKH1434-Cy3 (red). Although additional bacterial cells might be present in the ovipositor, high autofluorescence in this region hinder the unequivocal identification of bacteria. The inset (lower image) shows a magnification of one ovipositor-associated structure (os) labeled with the *Burkholderia*-specific probe. (**b**) FISH on the same section using the general eubacterial probe EUB338-Cy5 (green), suggesting the potential presence of additional non-*Burkholderia* bacteria in the ovipositor structures (os) and the surrounding region (sb). The inset (lower image) shows a magnification of one ovipositor-associated structure (os) labeled with the general eubacterial probe. Scale bars correspond to 100 µm (upper images) and 50 µm (lower images/insets). Host cell nuclei were counterstained with DAPI (blue).

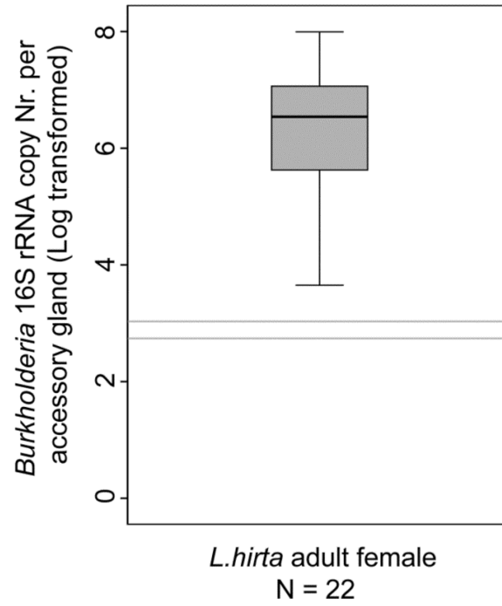

Figure S3. Quantification of *Burkholderia* symbionts in the accessory glands of 22 adult *L. hirta* females collected from the field. 16S rRNA gene copy numbers were obtained by quantitative PCR using *Burkholderia*-specific primers Burk16S1-F/R (Supplementary Table S5).

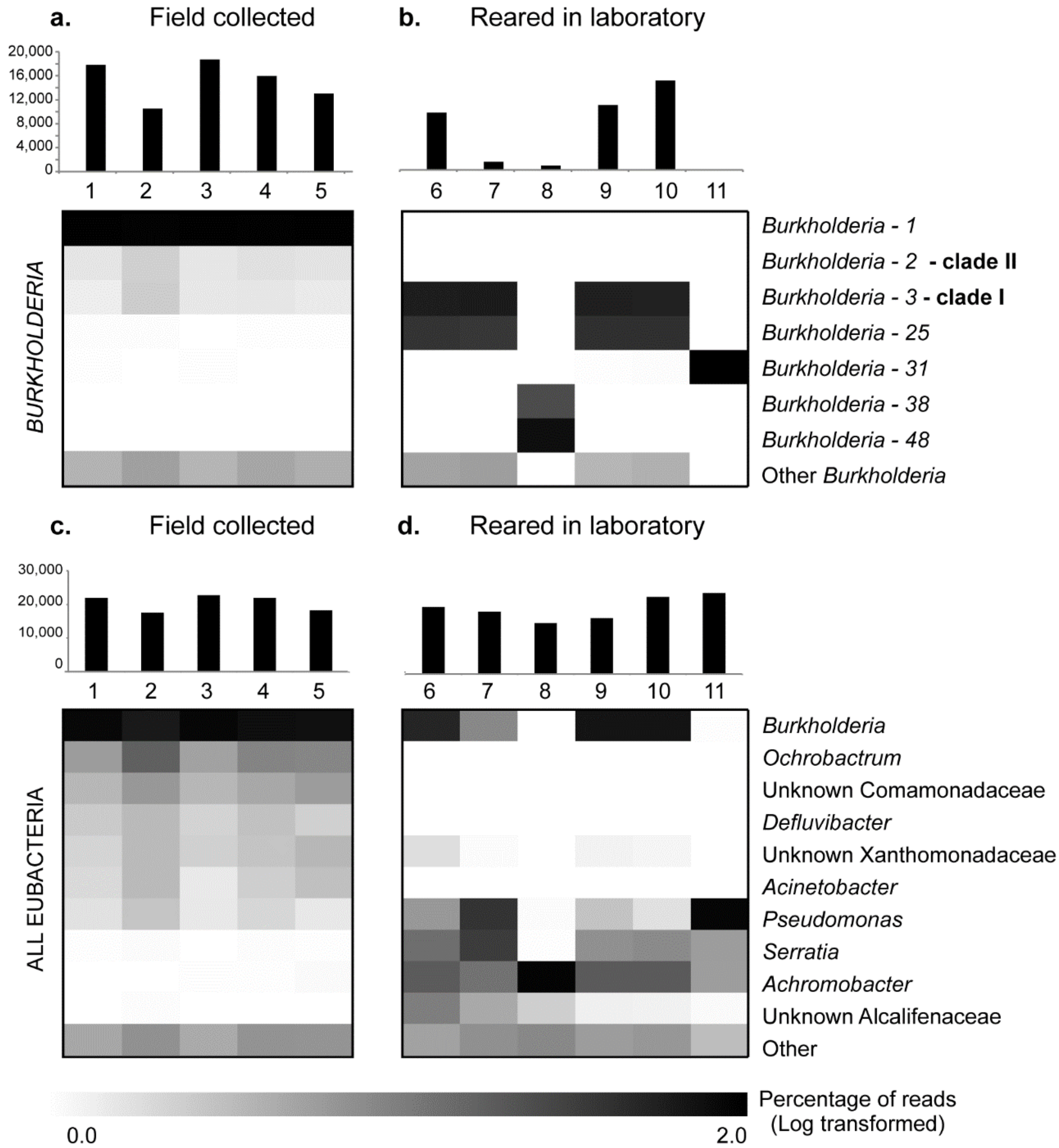

Figure S4. Bacterial community composition in the female accessory glands of *L. villosa* in natural and laboratory conditions as assessed by 454 pyrosequencing of 16S rRNA amplicons. (**a**-**b**) Total *Burkholderia* sequence numbers (bar graph – upper panel) and relative proportions of *Burkholderia* strains (log-transformed) in female accessory glands based on an oligotyping analysis on individuals collected in the field (**a**) and reared in the laboratory (**b**), respectively. Results show significant differences in *Burkholderia* strain composition between field-collected and lab-reared individuals (NMDS analysis and ANOSIM, p=0.01; Suppl. Fig 4).(**c**-**d**) Total eubacterial sequence numbers (bar graph – upper panel) and relative proportions (log transformed) of different bacterial genera per female accessory gland from individuals collected in the field (**c**) and reared in the laboratory (**d**), respectively.

Table S1. Natural infection rates of *Burkholderia* in adult *Lagria hirta* females from four different populations as assessed by culture-dependent and -independent methods.

Population	Cultivation		PCR/Cloning/ Sequencing		qPCR		Across methods*	
	***Burk*-pos./ screened**	***Burk*-positive (%)**	***Burk*-pos./ screened**	***Burk*-positive (%)**	***Burk*-pos./ screene d**	***Burk*-positive (%)**	***Burk*-pos./ screened**	***Burk*-positive (%)**
Jena	9/11	81.8	4/5	80.0	13/14	92.9	18/21	85.7
Berlin	1/2	50.0	2/2	100.0	3/4	75.0	3/3	100.0
Freiburg	1/1	100.0	1/1	100.0	1/1	100.0	1/1	100.0
Krakow	7/8	87.5	3/3	100.0	3/3	100.0	7/8	87.5
Total	**18/22**	**81.8**	**10/11**	**90.9**	**20/22**	**90.9**	**29/33**	**87.9**

*Individuals for which *Burkholderia* was present according to at least one method (Cultivation, PCR/Cloning/Sequencing or qPCR) were counted as positive.

Table S2. Gut bacterial community of field collected *L. hirta* adults as assessed by 454 pyrosequencing of 16S rRNA amplicons. Sequence processing and analyses are described in the methods section. OTUs were collapsed on the genus level. NCBI SRA accession numbers: SAMN04364636-37.

Taxon	**Adult male gut (%)**	**Adult female gut (%)**
Unknown Enterobacteriaceae	99.61	27.88
Unknown Entomoplasmatales	0.00	70.66
Burkholderia	0.00	0.25
Unknown Gammaproteobacteria	0.16	0.03
Unknown Pseudomonadaceae	0.08	0.06
Methylobacterium	0.01	0.08
Ca. *Liberibacter*	0.00	0.20
Sphingomonas	0.00	0.12
Other (less than 0,01%)	0.14	0.72

Table S3. Sequence numbers (total reads and *Burkholderia* sequences) per sample obtained by 454 pyrosequencing of the 16S rRNA gene for individual accessory glands from *L. hirta* and *L. villosa* females collected in the field or reared in the laboratory (see Fig. 4 and Supplementary Fig. 4).

Species	Individual	Field/Lab	Accession Nr.	Total Nr. of reads	*Burkholderia* reads	% *Burkholderia* reads
Lagria hirta	1	Field - Freiburg	SAMN04364612	13,211	10,966	83.01
	2	Field - Jena	SAMN04364613	19,475	14,742	75.70
	3	Field - Krakow	SAMN04364614	20,066	15,193	75.72
	4	Field - Jena	SAMN04364615	25,538	21,779	85.28
	5	Field - Jena	SAMN04364616	24,928	21,627	86.76
	6	Field - Jena	SAMN04364617	23,415	21,027	89.80
	7	Lab	SAMN04364618	12,214	21	0.17
	8	Lab	SAMN04364619	7,736	7,359	95.13
	9	Lab	SAMN04364620	68,479	54	0.08
	10	Lab	SAMN04364621	25,846	25,458	98.50
	11	Lab	SAMN04364622	73,022	195	0.27
	12	Lab	SAMN04364623	10,197	4,588	44.99
Lagria villosa	1	Field - Sta Lucia, Brazil	SAMN04364624	21,969	18,809	85.62
	2	Field - Sta Lucia, Brazil	SAMN04364625	17,583	11,364	64.63
	3	Field - Sta Lucia, Brazil	SAMN04364626	22,730	19,837	87.27
	4	Field - Sta Lucia, Brazil	SAMN04364627	21,991	16,965	77.15
	5	Field - Sta Lucia, Brazil	SAMN04364628	18,233	13,890	76.18
	6	Lab	SAMN04364630	19,969	10,217	51.16
	7	Lab	SAMN04364631	18,536	1,444	7.79
	8	Lab	SAMN04364632	15,191	3	0.02
	9	Lab	SAMN04364633	16,649	11,674	70.12
	10	Lab	SAMN04364634	22,949	16,111	70.20
	11	Lab	SAMN04364635	24,100	9	0.04

Table S4. Selected *L. hirta* symbiont and reference *Burkholderia* 16S rRNA sequences used for phylogenetic reconstruction (Fig. 3).

Sequence Id. (Labels Fig.2b)	Population[a]	Type[a]	Strain/clone	Accession no.
FreiburgF5_clone1	Freiburg, Germany	clone	F5_clone1	KU574036
FreiburgF5_clone2	Freiburg, Germany	clone	F5_clone2	KU574037
Jena132_clone3	Jena - Ammerbach, Germany	clone	132_clone3	KU574038
Jena132_clone8	Jena - Ammerbach, Germany	clone	132_clone8	KU574039
Jena161_clone1B	Jena - Ammerbach, Germany	clone	161_clone1B	KU574040
Jena183_clone5	Jena - Ammerbach, Germany	clone	183_clone5	KU574041
Jena183_clone8	Jena - Ammerbach, Germany	clone	183_clone8	KU574042
Krakow228_clone1B	Krakow, Poland	clone	228_clone1B	KU574043
Krakow228_clone3B	Krakow, Poland	clone	228_clone3B	KU574044
Berlin5_clone1B	Berlin, Germany	clone	5_clone1B	KU574045
Jena183_clone2	Jena - Ammerbach, Germany	clone	183_clone2	KU574046
Jena183_clone7	Jena - Ammerbach, Germany	clone	183_clone7	KU574047
JenaF2_clone1	Jena - Ammerbach, Germany	clone	F2_clone1	KU574048
JenaF2_clone3	Jena - Ammerbach, Germany	clone	F2_clone3	KU574049
Jena183_clone1	Jena - Ammerbach, Germany	clone	183_clone1	KU574050
Jena183_clone4	Jena - Ammerbach, Germany	clone	183_clone4	KU574051
Krakow228_clone4B	Krakow, Poland	clone	228_clone4B	KU574052
FreiburgF5_isolate0	Freiburg, Germany	cultured	Lh_F5-0	KU574031
Berlin7_isolate2-R2A	Berlin, Germany	cultured	Lh_7-2R2A	KU574032
Jena179_isolate1-R2A	Jena - Ammerbach, Germany	cultured	Lh_179-1R2A	KU574033
Krakow230_isolate1-AA	Krakow, Poland	cultured	Lh_230-1AA	KU574034
Krakow233_isolate1-NA	Krakow, Poland	cultured	Lh_233-1NA	KU574035
B.gladioli - CH-2	-	-	CH-2	AY500138
B.gladioli pv. alliicola	-	-	CFBP 2422	GU936679
B.gladioli - BSR3	-	-	BSR3	JF431409
B.gladioli - MS 102	-	-	MS 102	EU053154
B.gladioli - strain 3	-	-	strain 3	DQ090078
B.gladioli pv. gladioli	-	-	CFBP 2427	GU936677
B.gladioli - 321gr-6	-	-	321gr-6	DQ355169
B.gladioli pv. agaricicola	-	-	CFBP 3580	GU936678
B. plantarii	-	-	-	U96933
B. plantarii - NBRC104888	-	-	NBRC104888	AB682222.1
B. plantarii - 2396	-	-	2396	AB183679
B. glumae - PA27.4	-	-	PA27.4	EF193641.1
B. glumae - 336gr-1	-	-	336gr-1	DQ355164.1
B. glumae - 99gr-4b	-	-	99gr-4b	DQ355167

B. cepacia	-	-	ATCC 25416	U96927
B. caryophylli	-	-	ATCC 25418	AB021423
B. rhizoxinica	-	-	HKI 454	AJ938142
Burkholderia sp. - *Plinachtus bicoloripes* gut symbiont]	-	-	PBI_clone1 (Uncultured)	AB558203.1
B. terrae	-	-	KMY02	AB201285
Ralstonia pickettii	-	-	12J	NC010678

[a]Only for sequences of *L. hirta* symbionts obtained in this study.

Table S5. Primers and probes used for amplification, sequencing and fluorescence *in situ* hybridization of the 16S rRNA gene.

Name	Type	Sequence (5' → 3')	16S rRNA or rDNA target organism	Orientation	Reference
fD1	Primer	AGAGTTTGATCCTGGCTCAG	Most eubacteria	Fwd	Weisburg et al. 1991
rP2	Primer	ACGGCTACCTTGTTACGACTT	Most eubacteria	Rev	Weisburg et al. 1991
Burk16S_1F	Primer	GTTGGCCGATGGCTGATT	*Burkholderia* spp.	Fwd	This study
Burk16S_1R	Primer	AAGTGCTTTACAACCCGAAGG	*Burkholderia* spp.	Rev	This study
M13-F	Primer	TGTAAAACGACGGCCAGT	pSC-A (StrataClone cloning vector)	Fwd	StrataClone Cloning Kit, Agilent Technologies
M13-R	Primer	GGAAACAGCTATGACCATG	pSC-A (StrataClone cloning vector)	Rev	StrataClone Cloning Kit, Agilent Technologies
Gray28F	Primer	GAGTTTGATCNTGGCTCA	Most eubacteria	Fwd	Sun et al. 2011
Gray519R	Primer	GTNTTACNGCGGCKGCTG	Most eubacteria	Rev	Sun et al. 2011
EUB338	Fluorescent Probe (Cy3 or Cy5)	GCTGCCTCCCGTAGGAGT	Eubacteria	Rev	Amann et al. 1990
Burk16S	Fluorescent Probe (Cy3 or Cy5)	TGCGGTTAGACTAGCCACT	*Burkholderia* spp.	Rev	Modified from Opelt et al. 2007

CHAPTER 6

Impact of the defensive symbiont *Burkholderia gladioli* on the performance of *Lagria* beetles in the absence of natural enemies.

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6.1. Introduction

As highlighted in the previous chapters, most eukaryotes are associated with symbiotic microbes that play important roles for nutrition, defense, immune homeostasis, and reproductive success (1). However, harboring microbial symbionts can be metabolically demanding due to nutrient uptake, production of metabolic waste or toxins, or stimulation of the immune system. In mutualistic partnerships, the benefit conferred by the symbiont outweighs this burden, resulting in a net positive effect that favors a consistent association. Especially in protective symbioses, however, the cost to benefit ratio of maintaining an association can be highly variable in time and space, depending on the vulnerability of a particular life stage of the host or on the relative risk of encountering antagonists susceptible to the symbiont-conferred defense (see Chapter 2). The interplay between these changing selective pressures is decisive for the long-term stability of the association, and can determine, for example, symbiont infection frequency and distribution across host populations (2). Costs of harboring microbial symbionts and their implications have been studied in insects before, yet most of the work has been done in facultative and defensive symbionts of aphids (3–7).

The two previous chapters show that Lagriinae beetles engage in a defensive symbiotic association with multiple *B. gladioli* strains. Specifically in Chapter 4, we provided evidence that the bacteria protect the eggs of *L. villosa* from fungal pathogens. Given the evolutionary history of the association and the common characteristics of the symbiosis in *L. villosa* and *L. hirta*, a defensive basis is very likely in the latter as well. In this chapter, I evaluated the effects of the *B. gladioli* symbionts on overall performance (i.e. survivorship, development duration and weight) of *L. hirta* and *L. villosa* beetles in the absence of natural enemies. The ultimate goal of this approach was to assess the possibility of additional functional roles of the *B. gladioli* symbionts in *Lagria* (e.g. nutrient provisioning), or in the absence of one, evaluate the cost for the host of harboring the bacteria.

With this purpose, I experimentally manipulated the system and generated *Burkholderia*-free *Lagria* beetles by means of the egg surface-sterilization method described in Chapter 4. This procedure has been previously developed in hemipteran hosts (8), which - like *Lagria* beetles - acquire their bacterial symbionts from the egg surface every generation. In combination with this symbiont-elimination method, the availability of pure symbiont cultures allowed for direct and controlled reinfection of treated eggs, which serve then as a reference for evaluating symbiotic effects.

6.2. Materials and methods

6.2.1. Insect collection and rearing

L. hirta adult specimens were collected in Ammerbach – Jena (Germany) between June and August of 2012 and 2013. *L. villosa* individuals were collected in Brazil between January and March 2015 (SISBIO authorization Nr. 45742-1, CNPq process nº 01300.004320/2014-21). Adult beetles were reared in plastic containers (16.5x19x25cm) at temperatures between 20-25°C and a 16h:8h (*L. hirta*) or natural (*L. villosa*) light regime. Autoclaved water was supplied in 50 mL centrifuge tubes covered with moist cotton and a petri dish with soil was provided as a substrate for egg-laying. *L. hirta* adults were fed with fresh leaves from a variety of plants, including *Betula pendula, Tilia cordata, Tilia platyphyllos, Robinia pseudoacacia, Cornus sanguinea, Viburnum lantana, Acer platanoides* and *Acer campestre,* and with *Viburnum lantana* dry seeds. *L. villosa* adults were fed with *Glycine max* (soybean), *Brassica napus* (raps) and *Pisum sativum* (pea) leaves.

6.2.2. Bacterial cultivation

In order to isolate the symbiotic *Burkholderia*, an accessory gland of either an *L. hirta* or an *L. villosa* female was homogenized and suspended in 100 µL of sterile PBS and diluted at a factor of 10^{-5} in PBS. 100 uL of each dilution were plated on Nutrient Agar (5g/L peptone, 3g/L beef extract, 15 g/L Agar), R2A Agar (Sigma Aldrich, Germany), and Actinomycete Agar (Sigma Aldrich, Germany) and incubated at 30°C for 3 days. Biomass from colonies representing morphologically distinct types was recovered and incubated in 100 µL of lysis solution (67 mM Tris–HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 5 mM beta-mercaptoethanol, 6.7 mM $MgCl_2$, 6.7 µM EDTA (pH 8) and 1.7 mM SDS) at 95°C for 5 min. Lysed colony suspensions were used for amplification of the 16S rRNA gene using the general eubacterial primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (3'- ACGGCTACCTTGTTACGACTT-5') (9). PCRs were carried out on a Biometra TProfessional Thermocycler (Biometra, Germany) in 12.5 µL reaction volumes containing 1 µL template, 1 x PCR Buffer [100 mM Tris-HCl pH 8.3, 500 mM KCl and 15 mM $MgCl_2$], 0.5 mM $MgCl_2$, 0.24 mM dNTPs, 0.8 µM of each primer, and 0.5 U of Taq DNA polymerase (VWR). The following parameters were used: 3 min at 94°C, followed by 32 cycles of 40 s at 94°C, 60 s at 65°C and 60 s at 72°C, and a final extension step of 4 min at 72°C. PCR products were purified using the InnuPREP PCRpure Kit (Analytik Jena-Biometra, Germany) following the manufacturer's instructions, and sequenced. Pure cultures of colonies identified as *Burkholderia gladioli* were kept as glycerol stocks until further use. For the reinfection treatment in the egg-surface sterilization assays on *L. hirta* and *L. villosa*, either *B. gladioli* Lh-StG or Lv-StA, respectively, were recovered from glycerol stocks and cultured overnight in LB or King B medium (2g/L peptone, 1.5 g/L K_2HPO_4, 1.5 g/L $MgSO_4{\cdot}7H_20$, 15 g/L Agar). Bacteria were centrifuged and resuspended in sterile PBS at a concentration of 5 x 10^5 cells/µL for egg reinfection.

6.2.3. Egg-surface sterilization assays

To assess the impact of the *B. gladioli* symbionts on performance of both *L. hirta* and *L. villosa* beetles, we generated aposymbiotic (symbiont-free) individuals and compared these to individuals which underwent the same treatment but were reinfected with previously isolated *B. gladioli* symbionts, as well as to untreated controls. For this purpose, we adapted an egg-surface sterilization method that proved effective in other insects (8,10,11). Initially, each individual clutch was randomly divided into three equal-sized groups (sample sizes are presented in Table 6.1). The first group remained untreated (control), while the second and third were surface sterilized by submerging the eggs in 95% ethanol for 5 min, rinsing with autoclaved water, subsequently washing in 12% sodium hypochlorite for 30 s and again rinsing

with autoclaved water. Each group of eggs was placed in individual petri dishes (10 cm diameter) with moist filter paper. One of the surface-sterilized groups of eggs was reinfected with a suspension of *B. gladioli* in PBS, previously isolated from the corresponding host species (Lh-StG for *L. hirta* and Lv-StA for *L. villosa*) and prepared as described above. Only sterile PBS was added to the third group (aposymbiotic treatment), using 1.5 µL per egg. After hatching, Eppendorf tubes with moist cotton and dry leaves of the corresponding plant species were provided every second or third day (see *Insect collection and rearing* section). Survival was monitored and individuals were sacrificed at specific ages for nucleic acid extraction or freeze-drying depending on the experiment, as indicated in the next section and Table 6.1.

For *L. hirta*, two separate egg-surface sterilization assays were carried out. In the first one, all used egg clutches were laid by females that had been reared in the laboratory for at least one generation, whereas those from the second experiment were laid by field-collected females. In the case of *L. villosa*, only one assay with eggs from a lab-reared female was included in the analysis. Replicate numbers for all experiments following this procedure are specified in Table 6.1.

6.2.4. Nucleic acid extraction and quantitative PCR

In order to quantify *Burkholderia* in larvae that hatched from treated eggs, and thereby confirm the success of the surface sterilization and reinfection methods, respectively, we carried out a *Burkholderia*-specific qPCR on DNA extracted from the corresponding larvae, sacrificed between 8 and 12 days after hatching (same age within each clutch) or 40 days after hatching in the case of *L. villosa*. Whole larvae were either preserved in 70% ethanol or frozen shortly at -20°C and homogenized with liquid nitrogen. 300 µL of tissue and cell lysis solution (MasterPure™ complete DNA and RNA isolation Kit, Epicentre technologies, Germany) and 4 µL lysozyme (100 mg mL^{-1}) were added to each sample and these were incubated at 37°C for 30 min. The subsequent steps were carried out following the manufacturer's instructions for the MasterPure™ complete DNA and RNA isolation Kit (Epicentre technologies, Germany). Isolated nucleic acids were resuspended in Low TE buffer and stored at -20°C until use. qPCRs were carried out in a RotorgeneQ cycler (Qiagen, Hilden, Germany) following the protocol described for the Rotor-Gene SYBR Green PCR Kit and using the Burk16S_1F (3'-GTTGGCCGATGGCTGATT-5') and Burk16S_1R (3'-AAGTGCTTTACAACCCGAAGG-5') primers, which amplify a 172 bp region of *Burkholderia* 16S rRNA. PCR conditions were as follows: 95°C for 10 min, followed by 45 cycles of 95°C for 10s and 65°C for 30s. A melting curve was subsequently performed with a temperature ramp from 60°C to 99°C within 4.25 min.

6.2.5. Bacterial community analysis in *L. villosa* from egg-surface sterilization assay

We characterized the bacterial profiles of *L. villosa* individuals that developed from treated eggs and their untreated counterparts, as well as the bacteria present on the egg surface of the subsequent generation. Per treatment, two whole larvae were sampled and individual accessory glands were dissected from two females, representing individuals that developed from treated or control eggs. Additionally, three egg clutches corresponding to the next offspring of treated individuals were sampled for each treatment. DNA was isolated following the same procedure described above (*Nucleic acid isolation and quantitative PCR section*). 454 pyrosequencing and the corresponding microbial community analyses in Qiime (12) were carried out as described in Chapter 5. After quality filtering, between 6,510 and 71,051 high-quality reads per sample were available for analysis.

6.2.6. Statistical analyses

Since only a fraction of all the clutches that underwent the egg-surface sterilization and corresponding control treatments hatched, the reported results are based only on these clutches. *L. hirta* experiments were analyzed based on mixed models setting clutch as a random factor (accounting for subject-specific effects) and treatment as a fixed factor as specified in Table 6.1. Statistical significance of treatment effects for linear mixed models were evaluated based on Likelihood Ratio Tests against the corresponding null model, and a factor reduction approach was used to evaluate differences between treatment levels when pertinent. The data on *L. villosa* were obtained from the single clutch that hatched and therefore were analyzed without accounting for random effects. All statistical analyses were carried out in R 2.14.1 using the models and corresponding packages listed in Table 6.1. Additionally, survival probabilities were plotted based on Kaplan-Meier models using the rms package (13) in R 2.14.1.

Table 6.1. Summary of the experimental design and statistical analyses used for egg-surface sterilization assays on *L. hirta* and *L. villosa*.

			Statistical analyses					
Experiment	**Treated clutches**	**Clutches included in analysis**[a]	**$N_{Treatment}$**	**Dependent variable**	**Independent variable(s)**	**Model/test**	**R package**	**Ref.**
Lagria hirta - lab	13	6	N_{apo} =12 N_{reinf} =16 N_{con} =20	*Burkholderia* titer[b]	Treatment (fixed factor) Clutch (random factor)	Linear mixed model	lme4	(14)
			N_{apo} =80 N_{reinf} =82 N_{con} =169	Survival	Treatment (fixed factor) Clutch (random factor)	Cox mixed effects model	coxme	(15)
Lagria hirta - field	21	8	N_{apo} =199 N_{reinf} =200 N_{con} =189	Survival	Treatment (fixed factor) Clutch (random factor)	Cox mixed effects model	coxme	(15)
			N_{apo} =12 N_{reinf} =11 N_{con} =15	Dry weight[b] (Age: 76 days)	Treatment (fixed factor) Clutch (random factor)	Linear mixed model	lme4	(14)
Lagria villosa - lab	15	1	N_{apo} =6 N_{reinf} =6 N_{con} =6	*Burkholderia* titer[b]	Treatment	ANOVA - Tukey	stats	(16)
			N_{apo} =44 N_{reinf} =43 N_{con} =39	Survival	Treatment	Cox proportional hazards regression	Survival-rms	(13,17)
			N_{apo} =14 N_{reinf} =11 N_{con} =14	Larval stage duration[b]	Treatment	ANOVA - Tukey	stats	(16)
			N_{apo} =21 N_{reinf} =20 N_{con} =21	Wet weight[c] (Age: 32 days)	Treatment	ANOVA - Tukey	stats	(16)

[a]All other clutches did not hatch or no individuals survived the first week after hatching
[b]$Log_{10}(x)$ transformed data was used in the statistical model
[c]Sqrt(x) transformed data was used in the statistical model

6.3. Results and discussion

6.3.1. Symbiont elimination by egg-surface sterilization in *L. hirta*

The first experiment on *L. hirta* egg clutches, using offspring from lab-reared individuals, revealed that the egg surface-sterilization method effectively removed *Burkholderia* symbionts as assessed by quantitative PCR on the corresponding larvae. In the respective treatment (aposymbiotic), *Burkholderia* titers fell within the range of negative controls (Fig 6.1a). Additionally, we confirmed that the reinfection procedure was successful; implying that these individuals can acquire the applied *B. gladioli* Lh-StG and harbor them in the larval stage in amounts comparable to untreated controls (LMM, $p>0.05$) (Fig 6.1a). Highly significant differences in *Burkholderia* titers between the aposymbiotic individuals and both reinfected and untreated controls were statistically supported based on a factor reduction approach on the corresponding model (LMM, $p<0.001$ for both cases).

These larvae were monitored during the first nine days after hatching, revealing lower survival probability for aposymbiotic larvae as compared to untreated controls (Cox mixed effects model, $p<0.05$) and no statistically significant differences between survival of reinfected and untreated controls (Cox mixed effects model, $p>0.05$). If *B. gladioli* Lh-StG has a beneficial effect on the host it is expected that, in addition to the above, the survival probability of reinfected individuals would be statistically higher than that of aposymbionts. This was not the case (Cox mixed effects model, $p>0.05$), although there was a trend towards higher survival in the reinfected larvae (Fig. 6.1b). Survival data for a similar experiment on eggs from field-collected individuals, in which larvae were monitored for a longer period (42 days), also showed a lower survival probability for aposymbiotic larvae compared to untreated controls (Cox mixed effects model, $p<0.01$) (Fig 6.2a). Differing from the previous experiment, reinfected larvae were less likely to survive as compared to untreated controls ($p<0.05$) (Fig 6.2a); the interpretation of this difference is discussed in detail below. Also, survival probabilities of aposymbiotic and reinfected individuals were not statistically different from each other (Cox mixed effects model, $p>0.05$) arguing against a beneficial role of *B. gladioli* Lh-StG in the evaluated conditions. In summary, both experiments suggest that untreated controls have higher chances of surviving in comparison to treated, symbiont-free larvae. Yet, reinfection with *B. gladioli* Lh-StG had either a very weak beneficial effect or no impact on the survival probability of the treated larvae in this particular setting.

Several factors might explain these results. First, the egg-surface sterilization procedure itself could directly affect the health of the embryos and thus have detrimental consequences for both aposymbiotic and reinfected larvae. Yet, an additional effect due to reliance on the symbionts should still be possible to discern in the aposymbionts compared to the reinfected controls. As demonstrated in Chapter 4, there is strong evidence that *B. gladioli* symbionts indeed confer a benefit to the beetles, as they inhibit the growth of antagonistic fungi on the eggs of *L. villosa*. In the context of the present experiments, however, pathogen exposure is expected to be much lower in comparison to the natural soil environment or when intentionally included in the experimental setup (as in Chapter 4). Thus, given the absence of antagonists and the context-dependent nature of protective symbiosis, observing no significant differences in survival between treated larvae with or without the symbiotic *B. gladioli* is in line with defense being the prime advantage conferred by the bacteria. As to the possibility of additional nutritional provisioning by the symbionts, here we did not observe any effects of treatment on insect dry weight (Fig 6.2b), arguing against this functional role. However, given the natural presence of multiple symbiotic *B. gladioli* strains in *Lagria* beetles (Chapter 5), we cannot rule out the possibility that reinfection with only *B. gladioli* Lh-StG – or *B. gladioli* Lv- StA in the case of *L. villosa* discussed below- does not reflect the circumstances of the symbiosis in nature. Certainly, any or all of the following scenarios are possible: (i) this is not the optimal symbiont for the beetles used for experimentation in the lab (i.e. this particular symbiont and host are not coadapted), (ii) after *in vitro* cultivation, the *B. gladioli* Lh-StG strain exhibit different metabolic profiles which diverge from their symbiotic condition, or (iii) the presence of a consortium of strains is crucial for mutualism

with the insect. These could be confounding effects both in case of the known defensive role or a potential nutritional provision. Additionally, based solely on these data, there is no evidence of a cost of symbiosis with *B. gladioli* for *L. hirta*.

Considering the discrepancies in the *Lagria* - *Burkholderia* interaction between lab-reared and field-collected beetles (Chapter 5), it seems relevant to discuss the difference in survival patterns of reinfected offspring from lab-reared vs. field-collect females. Apparently, individuals from lab-reared mothers respond better to reinfection with *B. gladioli* Lh-StG than those from field-collected mothers (Fig. 6.1b and 6.2a, respectively). However, the monitoring time was different for both experiments and might affect the kind of conclusions we can draw from each. When statistically analyzing the data set of offspring from field-collected mothers considering only the equivalent time period (9 days after hatching), the survival probability pattern is very similar to that of the experiment with offspring from lab-reared mothers, resulting in the same statistical grouping of treatments (untreated control (a), reinfected (ab), aposymbiotic (b); Cox mixed effects model, $p<0.05$). Thus, the discrepancy in the patterns is likely not attributed to the original environment of the insects (lab vs. field). Importantly, these observations could imply that the impact on survival of having the symbionts is variable over time. This assumption remains speculative, however, especially given the very high mortality rate for all treatments, which hinders robust statistical analyses for data obtained more than two weeks after hatching.

Concordant with the hypothesis that the effect of the *Burkholderia* symbionts varies along host life cycle, during the earlier days after hatching there is a trend for lower aposymbiont survival compared to symbiotic individuals (either reinfected or untreated controls) in both experiments. Despite the lack of statistical support for differences between aposymbionts and reinfected individuals, this tendency is observed in offspring of lab-reared females (Fig 6.1b) and also -although more moderately- in those from field-collected mothers (Fig. 6.2a), suggesting that the *B. gladioli* Lh-StG symbionts are less detrimental or to some extent favorable for the host during this early phase. Certainly, the first days after hatching are a vulnerable stage for the insect, especially during the first larval instar (days 1-2) when the cuticle is still not melanized and the larvae have not started feeding yet. Despite the controlled conditions, susceptibility to the occasional presence of pathogenic microorganisms during these days, or a carry-over effect from inadvertent pathogen threat during the embryonic stage, could explain a stronger reliance on protective symbionts during early developmental phases.

As mentioned above, the overall high mortality rates during the first days after hatching, possibly aggravated in laboratory conditions, strongly reduced the number of monitored individuals in all groups affecting the likelihood of detecting treatment-associated effects. Thus, further work is necessary to identify rearing conditions that ameliorate early-stage mortality in *L. hirta* and thereby allow the direct and conclusive assessment of a functional role of the *Burkholderia* symbionts in this host species both with and without exposure to microbial antagonists.

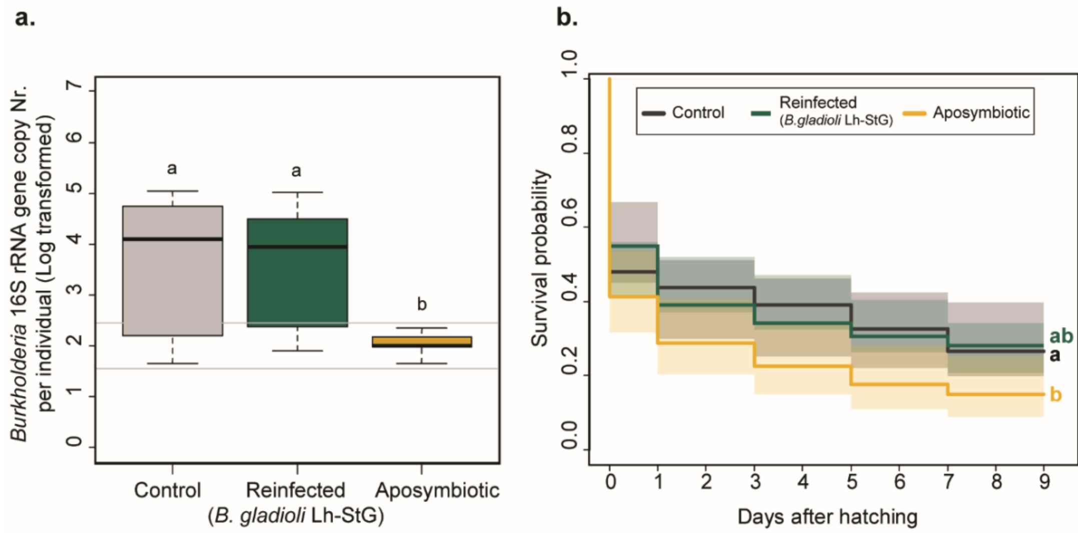

Figure 6.1. Egg-surface sterilization assay on six clutches laid by laboratory reared *L. hirta* to generate aposymbiotic individuals and assess the impact of *B. gladioli* symbionts on survival, in comparison to reinfected and untreated control individuals. (a) Quantification of *Burkholderia* in a subset of *L. hirta* larvae confirming the successful generation of aposymbiotic and reinfected individuals using this method. Gray lines indicate the maximum and minimum values of the negative controls and letters indicate groups with statistically significant differences ($p<0.05$). (b) Survival probability of *L. hirta* larvae corresponding to the aposymbiotic, reinfected and untreated control groups. Letters indicate groups showing statistically significant differences ($p<0.05$) and the shading represents the standard error. The number of individuals per treatment and the statistical model used for analysis in both (a) and (b) are specified in Table 6.1.

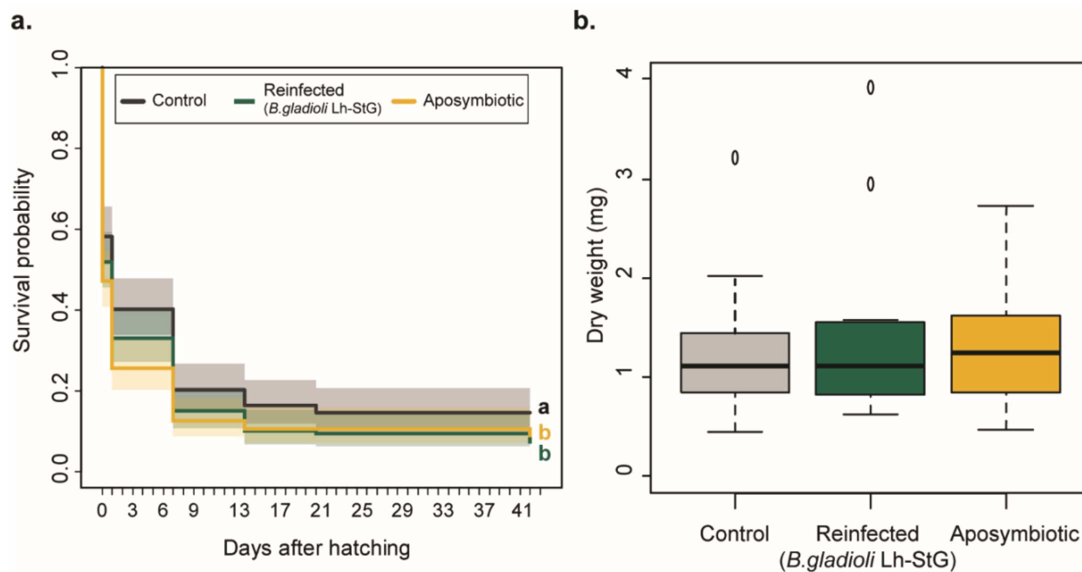

Figure 6.2. Egg-surface sterilization assay on eight clutches laid by field-collected *L. hirta,* evaluating the impact of *Burkholderia* symbionts on survival and dry weight of the larvae. (a) Survival probability of larvae corresponding to aposymbiotic, reinfected and untreated control groups. Letters indicate groups with statistically significant differences ($p<0.05$) and the shading represents the standard error. (b) Dry weight of a subset of *L. hirta* larvae sacrificed 76 days after hatching and corresponding to the same three treatments, revealing no significant effect of treatment on larval dry weight ($p>0.05$). The number of individuals per treatment and the statistical model used for analysis in both (a) and (b) are specified in Table 6.1.

6.3.2. Symbiont elimination by egg-surface sterilization in *L. villosa*

In lab reared *L. villosa* there was a considerably low proportion of egg clutches that hatched (4/15 treated). A single clutch was used in this case as it was the only one for which more than 10 % of the larvae survived after the first few days. As in *L. hirta*, we confirmed that egg-surface sterilization effectively eliminated *Burkholderia* from *L. villosa* beetles. *Burkholderia* abundance values obtained by qPCR for larvae that hatched from surface-sterilized eggs fell within the range of the negative controls and were significantly lower than both reinfected and untreated controls (Anova, $F(2,15)=92.046$, $p<0.001$; and Tukey post-hoc test, $p<0.001$). Also, applying an *in vitro* culture of a symbiotic strain (*B. gladioli* Lv-StA) on the surface of sterilized eggs resulted in uptake by the larvae. Reinfected individuals carried slightly lower *Burkholderia* titers than control individuals (Tukey test $p=0.0365$) (Fig 6.3a). This difference could be associated to a suboptimal colonization by bacterial cells migrating from the PBS suspension in comparison to the natural secretion, or to infection by a single strain instead of multiple *B. gladioli* strains, as would occur naturally.

Survival was monitored during 8 weeks, which included the complete duration of the larval stage for all individuals, and time from egg hatching until pupation was recorded for larvae that survived until this stage. The survival probability of individuals from the three treatments did not differ from each other (Cox proportional hazards regression, $p>0.05$) (Fig. 6.3b), however, there were significant differences in developmental time (ANOVA, $F(2,36)=29.26$, $p<0.001$) (Fig. 6.3c) and larval wet weight (ANOVA, $F(2,59)=34.47$, $p<0.001$) (Fig. 6.3d). The observation that both aposymbiotic and reinfected individuals took a longer time to reach pupation (Tukey test, $p<0.001$) and weighed less (Tukey test, $p<0.001$) than the untreated controls could be attributed, at least partially, to the impact of the egg-surface sterilization procedure as suggested for *L. hirta*. Interestingly, however, larval stage duration was longer in reinfected individuals than in their *Burkholderia*-free counterparts (Tukey test, $p<0.01$) and they weighed less than the latter at the same age (Tukey test, $p<0.001$). These results suggest that *L. villosa* larvae pay a metabolic cost for carrying the symbionts, or at least this strain, and also argues against nutritional provisioning. The observations are in line with defense as the main functional role of *B. gladioli* in *Lagria*, since such tradeoffs are known to occur and have been observed directly or indirectly in other protective symbioses (2). For example, the defensive symbiont *Hamiltonella defensa* compromises longevity in the black bean aphid (*Aphis fabae)* (6), and symbiont frequency declines in pea aphid (*Acyrthosiphon pisum*) host populations when not exposed to parasitoid enemies (4).

Still, as discussed for *B. gladioli* Lh-StG in *L. hirta*, potential failure to reproduce natural symbiotic conditions by reinfecting with an *in vitro B. gladioli* Lv-StA culture could be an alternative or additional explanation for the negative effects on development observed in reinfected individuals.

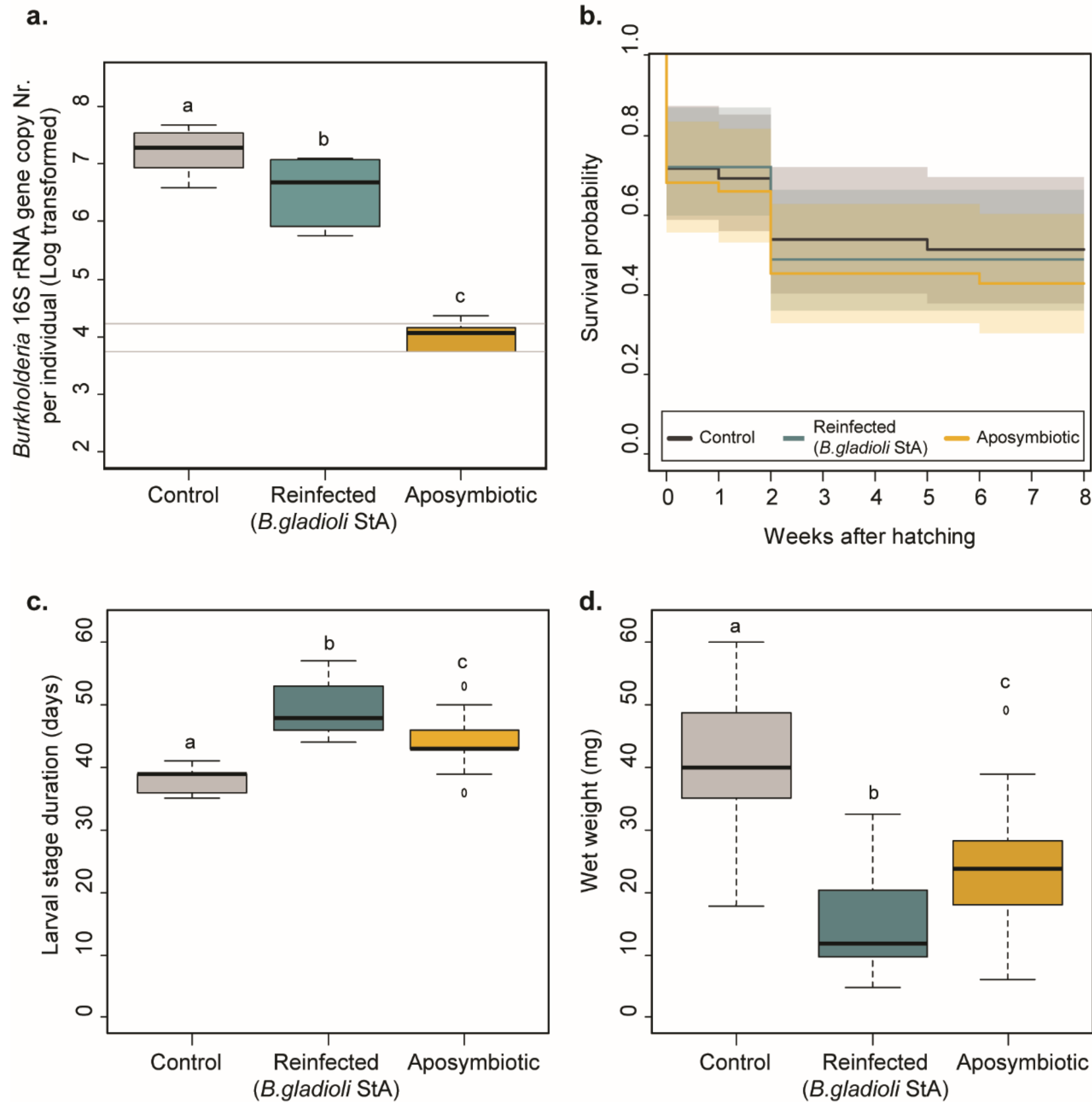

Figure 6.3. Egg-surface sterilization assay on offspring of laboratory reared *L. villosa* to evaluate the impact of *Burkholderia* symbionts on development and survival. (a) Titers of *Burkholderia* as assessed by quantitative PCR on the 16S rRNA gene in a subset of *L. villosa* larvae, confirming the successful generation of aposymbiotic and *B. gladioli* Lv-StA reinfected individuals. Gray lines indicate the maximum and minimum values of the negative controls. (b) Survival probability assessed in aposymbiotic, reinfected and control individuals, showing no significant differences between treatments ($p>0.05$). Shading represents the standard error. (c) Time from hatching until pupation for the same three treatments. (d) Wet weight of a subset of the corresponding larvae 32 days after hatching. In (a), (c) and (d) letters indicate groups with statistically significant differences ($p<0.05$). The number of individuals per treatment and the statistical model used for analysis in (a) through (d) are specified in Table 6.1.

In addition to evaluating insect performance, we monitored the microbial community of treated individuals across the different life stages of *L. villosa* in order to gain a better insight into the potential causes and consequences of our observations. We used 454 sequencing of bacterial 16S rRNA amplicons to characterize the bacterial profiles in (i) whole larvae that developed from treated eggs, (ii) accessory glands of females that developed from these larvae and (iii) a new generation of eggs laid by these females, as well as all of the above for the corresponding controls.

As expected, *Burkholderia* was overall the dominant and most consistent taxon in untreated individuals across life stages and it was nearly absent in aposymbiotic individuals. While *Burkholderia* also dominated the bacterial community of reinfected adults, one of the two sampled reinfected larvae showed a very low relative abundance of *Burkholderia* (2%). Since the *Burkholderia* titer in the same individual was also assessed by quantitative PCR and showed high and similar abundances to other reinfected individuals (Fig. 6.3a), it is plausible that an infection by an Enterobacteriaceae overshadowed *Burkholderia* despite being present in the symbiotic structures of the larva. Intriguingly, although there is a high probability that beetles that develop from reinfected eggs carry high *Burkholderia* cell numbers, this bacterial taxon represents less than 2% of the bacterial community on the eggs laid by these females. It is important to highlight that the pooled egg samples used for 454 sequencing were not necessarily laid by any of the sampled females, yet all mothers were genetically closely related (they hatched from the same clutch) and were treated and reared under the same conditions. Thus, these results raise the question whether *Burkholderia* acquired by artificial reinfection, or this specific strain, are not successfully transmitted to the next generation. Notably, the previously discussed results on the detrimental effects of reinfection with *B. gladioli* Lv-StA on development (Fig. 6.3c-d), as well as the lower titers of *Burkholderia* in reinfected as compared to untreated control larvae (Fig. 6.3a), could also be linked to difficulties in transmitting this strain vertically. It is possible, although presumable, that the host has mechanisms to selectively block transmission of a specific strain. A similar scenario of a partner choice mechanism has been observed in the European beewolf *Philanthus triangulum*, which fails to vertically transmit an actinobacteria different to its *Streptomyces philanthus* symbiont when it has been artificially reinfected with the nonnative strain (18). It is worth mentioning though, that *B. gladioli* Lv-StA has been isolated from a field collected *L. villosa* female, has been found in additional individuals, and is highly similar to other symbiotic strains of this, as well as other Lagriinae hosts, suggesting that it is associated to this beetle species in natural conditions. However, as mentioned for *L. hirta* and *B. gladioli* Lh-StG, this sole strain might not be optimal for the beetles used for experimentation, could have changed their metabolic profile after *in vitro* culture, or may be detrimental for the beetle if not part of a consortium of coinfecting strains. As discussed in Chapter 5, symbiont strain diversity and rearing under laboratory conditions are highly relevant when considering symbiont transmission and the stability of the *Lagria-Burkholderia* association. Thus, strain competition or the influence of the environment under laboratory conditions might help explain this observation, although the specific cause and mechanism behind it remain elusive.

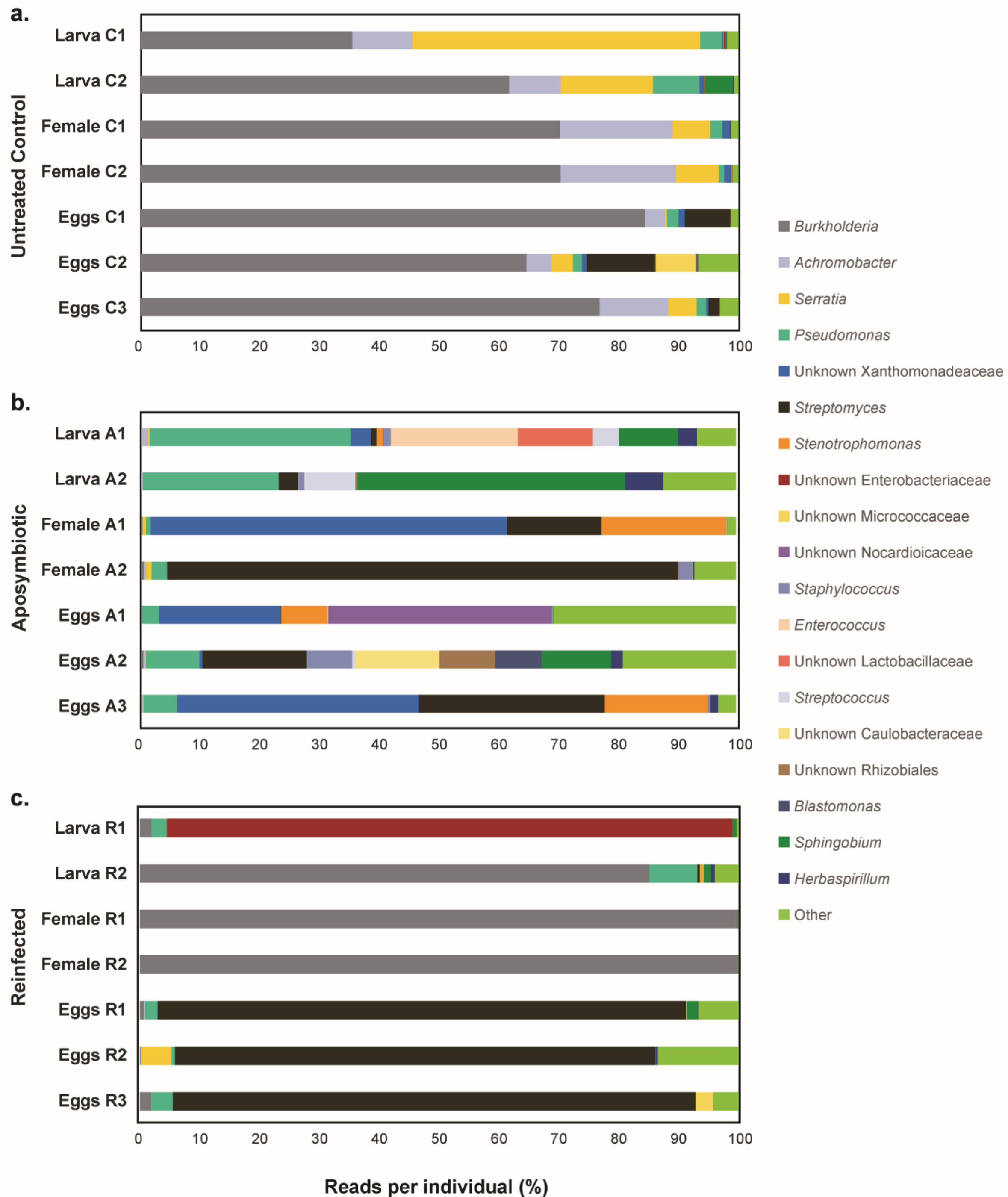

Figure 6.4. Bacterial community profiles of a subset of individuals from the egg-sterilization assay and their offspring. Samples specifically relate to: individual larvae that developed from treated eggs, individual accessory glands of females that developed from these larvae, eggs laid by this generation of females and correspond to the following groups: untreated controls (upper panel), aposymbionts (middle panel) and reinfected individuals (lower panel). Larvae and females do not represent the same individuals, and pooled egg samples were not necessarily laid by any of the sampled females, yet all mothers were genetically closely related and were treated and reared under the same conditions.

6.4. Concluding remarks

Taken together, the results suggest that a nutritional role for the *B. gladioli* symbionts in *Lagria* beetles is unlikely, and imply that harboring these bacteria represents a cost for the host, being mostly disadvantageous in the absence of antagonists. This scenario is consistent with the defensive basis of the symbiosis, considering the context-dependent nature of such associations. Still, further study is required to confirm whether reinfection with the isolated strains *B. gladioli* Lh-StG and Lv-StA is a valid representation of the situation in the field given the presence of multiple coinfecting symbiotic strains in nature.

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CHAPTER 7

Genome sequencing of two insect-associated *Burkholderia gladioli* strains isolated from *Lagria hirta* and *Lagria villosa* beetles.

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7.1. Introduction

Whole genome sequencing and comparative genomics have become powerful and increasingly accessible tools for exploring a wide array of biological questions, including the genetic basis and evolutionary implications of symbiosis (1–3). Especially the genomes of insect-associated bacteria have been studied extensively (2). In this context, genome evolution is particularly interesting given the potential restrictions imposed by a host associated lifestyle, and the profound implications that these constraints might have on genome architecture and functionality (3,4).

As discussed in earlier chapters (1 and 3), vertically transmitted symbionts with a high degree of tissue specialization or confinement are prone to suffer genome reduction over time, develop a nucleotide base composition bias towards adenine and thymine, and maintain a stable genome configuration (3). In contrast, many other symbionts are not restricted to cells or particular organs, are not obligate for the host, and are more likely involved in horizontal or mixed transmission mechanisms. The genomic characteristics of these bacterial symbionts are markedly different to those with a much tighter association with the host (3). It has been proposed that the genomes of some of these facultative symbionts resemble those of bacteria in an early stage of the transition to a host-restricted lifestyle, which tend to accumulate a high number of insertion sequences, and also exhibit an increased abundance of pseudogenes (4). It is thought that relaxed selection in the host environment, due to nutrient availability and a relatively stable environment, allows for the inactivation of multiple genes that could be superfluous in these conditions as compared to a less restricted lifestyle. Also, smaller symbiont effective population sizes decrease the efficiency of purifying selection due to genetic drift. Thereby, insertion elements and mutations that disrupt gene function can more easily become fixed in the bacterial population and often cause pseudogenization, as has been observed in a number of facultative symbionts of insects including *Sodalis* in tsetse flies (5), *Streptomyces* in beewolf digger wasps (6), *Hamiltonella* in aphids (7) and *Wolbachia* in *Drosophila* (8). Limited opportunity for horizontal exchange with other bacteria can further lead to gene loss, eventually resulting in a drastically reduced genome, as observed in symbionts that have transitioned completely to an obligate association (4).

Importantly, genomics also offers valuable information on the metabolic capabilities of a symbiont and is thereby a useful starting point to unveil a potential functional role (2,3). Additionally, knowledge on the genome composition of a symbiont is convenient for studying molecular interactions with the host and may facilitate targeted genetic manipulation of the symbionts.

Bearing this in mind, we have sequenced the genomes of two *B. gladioli* symbionts isolated from the accessory glands of *Lagria hirta* and *Lagria villosa*, respectively: *B. gladioli* Lh-StG and *B. gladioli* Lv-StA. Our purpose was to (i) study general genome characteristics (size, GC content, replicon number, coding density) and (ii) generate a database that substantiates targeted questions on the symbiosis. Specifically, further questions to be addressed in the near future concern the functional role of different *B. gladioli* strains as symbionts of *Lagria* and the molecular interactions underpinning the symbiosis.

7.2. Materials and methods

7.2.1. Genomic DNA isolation

The symbiotic strains *B. gladioli* Lh-StG and Lv-StA were isolated from the accessory glands of adult *L. hirta* and *L. villosa* females, as described in Chapters 4 and 6. Biomass from a single *B. gladioli* Lh-StG colony was inoculated in LB broth and grown at 30°C, 220 rpm for four days. 1.2 mL of the liquid culture at OD_{600} = 0.557 were used for genomic DNA isolation using the Qiagen DNeasy Tissue&Blood kit, following the manufacturer's instructions for Gram-negative bacteria (overnight lysis). For *B. gladioli* Lv-StA, biomass was grown in similar conditions as Lh-StG, but in King B broth (medium composition is reported in Chapter 5, *Experimental Procedures* section). In this case, genomic DNA was isolated using the Qiagen Genomic-tip 100/G Kit following the manufacturer's instructions for Gram-negative bacteria and using approximately 8 x 10^9 bacterial cells as starting material.

In both cases, the quality of the extracted genomic DNA was verified by visualization in a 0.8% agarose gel and the correct identity was confirmed by sequencing the 16S rRNA gene using the general bacterial primers fD1 and rP2 (9).

7.2.2. Whole genome sequencing, assembly and preliminary annotation

The genomes of *B. gladioli* Lh- StG and *B. gladioli* Lv-StA were sequenced by a commercial provider (Eurofins MWG Operon/Eurofins Genomics, Germany). Genome sequencing and analyses were carried out in collaboration with research partners from the Biomolecular Chemistry Department of the Leibniz Institute for Natural Product Research and Infection Biology.

B. gladioli Lh-StG: genome sequencing was carried out using a combination of GS FLX+ (shotgun) and Illumina HiSeq 2000 v3 (paired end: 2 x 100bp) technologies. A long jumping distance (LJD) protocol with an approximate insert size of 8 kb was applied for library construction. The assembly was conducted using an in-house pipeline (Eurofins MWG Operon) incorporating the software tool Newbler (v2.6) for assembly of 454 shotgun reads, mapping of the set of LJD pairs against the 454 contigs to infer insert size, and an iterative assembly in Velvet (v1.2.07) with all available Illumina (paired-end) and Roche 454 data. The results were revised manually and parameters were optimized for final scaffolds.

B. gladioli Lv-StA: genome sequencing was carried out using PacBio with Single Molecule, Real-Time (SMRT) technology. For de novo assembly (carried out by Eurofins Genomics), the HGAP pipeline was used (Hierarchical Genome Assembly Process). Briefly, a preassembly of long and accurate sequences was generated by mapping filtered subreads to so-called seed reads. Subsequently, the Celera assembler was used to generate a draft assembly using multi-kb long reads, which in this case rendered full genome closure. Finally, the Quiver algorithm was used to correct inDel and substitution errors.

Both genomes were annotated using the RAST online annotation service (10,11). To identify candidate biosynthesis gene clusters, antiSmash 2.0 (12,13) and the Artemis genome browser and annotation tools (14) were used by collaborators Dr. Claudia Ross and Dr. Kirstin Scherlach (Biomolecular Chemistry Department of the Leibniz Institute for Natural Product Research and Infection Biology).

7.3. Results and discussion

7.3.1. General genomic features

The assembly of the *B. gladioli* Lh-StG genome resulted in 157 scaffolds with a mean length of 55,052 bp (minimum length: 157 bp, maximum length: 4,084,973 bp), while that of the *B. gladioli* Lv-StA resulted in 4 contigs. This data set revealed that the two symbionts have multireplicon genomes, as is common in the members of the genus *Burkholderia* (15) (Table 7.1). Considering the genome size in bacteria with diverse lifestyles (Chapter 3, Fig. 3), the symbiotic strains *B. gladioli* Lh-StG and Lv-StA have relatively large genomes and high GC percentage. These characteristics, which are highly similar between the two strains (Table 7.1 and Fig. 7.1), resemble those of free-living bacteria or environmentally acquired bacterial symbionts and are also similar to other *Burkholderia* with diverse lifestyles. In other words, there are no evident signatures of genome reduction which could result from a host-associated lifestyle. Although the vertical transmission route of the *B. gladioli* symbionts of *L. hirta* and *L. villosa* involves a stage of presumably reduced effective population size (i.e. when entering the micropyle and colonizing the dorsal organs of the embryo), recurrent events of environmental acquisition should have an opposing effect on symbiont genome evolution. Indeed, it is known that even at low frequencies, horizontal transmission can have substantial influence on symbiont population structure and genome dynamics (3,16). Both symbiont cells and genes are likely replenished with horizontal transmission events of *B. gladioli* in Lagriinae, explaining the maintenance of a robust genome in at least some of the symbiotic strains. The fact that *B. gladioli* Lv-StA and Lh-StG can be readily cultured *in vitro* also suggests that these are not dependent on the host, and is coherent with a large and broadly functional genome. Whether or not the other symbiotic strains of *L. hirta* and *L. villosa* (Chapter 5) have suffered genome reduction at any level remains to be elucidated.

Table 7.1. General genome features of symbiotic strains isolated from *L. hirta* and *L. villosa* beetles.

Strain	Host/ Isolation source	Chromosome Nr.	Plasmid Nr.	Genome Size (Mb)	GC %	Coding sequences (CDS)[b]	16S rRNA gene copies[a]
***B. gladioli* Lh - StG**	*L. hirta*	2	-	8.64	67.8	7,590	6
***B. gladioli* Lv - StA**	*L. villosa*	2	2	8.56	67.9	7,468	5
***B. gladioli* BSR3**	*Oryzum sativum* (rice)	2	4	9.05	67.4	7,410	5

[a] Identical sequences among copies

[b] CDS number was determined using RAST software (10,11) for *B. gladioli* StG and StA, and as reported in the literature for *B. gladioli* BSR3 (17).

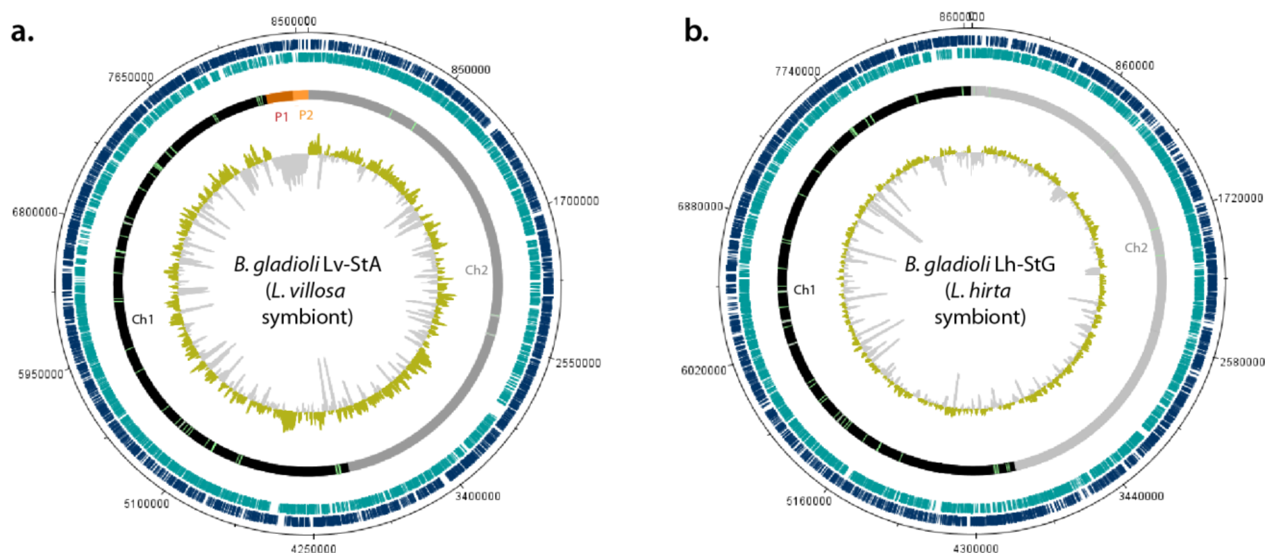

Figure 7.1. Genome representations displaying concatenated replicons of (a) *B. gladioli* Lv-StA isolated from *L. villosa* and (b) *B. gladioli* Lh-StG isolated from *L. hirta*. The two outer most rings in different tones of blue represent the coding sequences oriented in forward (dark blue) or reverse direction (lighter blue) and the next ring displays the corresponding replicon (Ch1/Ch2 = chromosome 1/2; P1/P2 = plasmid 1/2), with bright green sections corresponding to tRNA and rRNA genes. The inner most graph represents GC content (green = above average; light gray = below average) in the respective regions.

7.3.2. Preliminary annotation and secondary metabolite gene clusters

The genes predicted in the genomes of *B. gladioli* Lv-StA and Lh-StG are distributed similarly across functional categories, as suggested by the preliminary annotation carried out in RAST (Fig. 7.2.). This distribution of gene functions is also highly similar to that of two reference genomes from the same species, namely the rice pathogen *B. gladioli* BSR3 and the poisoned food isolate *B. gladioli* pv. *cocovenenans*, supporting little deviation in genome composition. Interestingly, however, genes associated to phages, prophages, transposable elements and/or plasmids are between 3.5 and 4.8 fold higher in the two symbionts of *Lagria* (Fig. 7.2) in comparison to the other two *B. gladioli*. This trend is in line with the generalized presence of many repetitive regions and unusually high numbers of mobile elements, phage and phage-derived genes across many of the facultative symbionts studied so far (3). As mentioned above, having an increased number of transposable elements and phages is thought to resemble an early genome erosion process. Although moderately, it is possible that relaxed selection and genetic drift in the *Lagria*-associated *B. gladioli* result in these features. Yet, as discussed in the previous section, frequent opportunities for gene exchange and horizontal acquisition of symbionts should avoid gene loss and subsequent genome reduction. Pseudogene identification as well as thorough manual annotation of the corresponding genes in *B. gladioli* Lv-StA and Lh-StG is necessary to draw strong conclusions from this observation in the context of symbiont genome evolution.

Considering the ability of the *B. gladioli* symbionts of *L. villosa* to protect the beetle eggs from fungal antagonists (Chapter 4), the presence of candidate gene clusters for the production of bioactive secondary metabolites by the symbiotic strains becomes of particular interest. As part of a collaborative work with members of the Biomolecular Chemistry Department of the Leibniz Institute for Natural Product Research and Infection Biology, Dr. Claudia Ross recognized a number of gene clusters suggesting high biosynthetic potential in the symbiotic strain *B. gladioli* Lh-StG. These included those for the production of four polyketide-synthases (PKS), nine non-ribosomal peptide synthases

(NRPS) and three PKS-NRPS hybrid gene clusters (18). From these, one PKS cluster is related -yet not identical- to a gene cluster of *Sorangium cellulosum* encoding the polyketide etnangien, a compound with antibacterial properties (19,20). This previously unknown metabolite is referred to as lagriene throughout the thesis. Additionally, two other gene clusters were identified which are almost identical to the genes for biosynthesis of toxoflavin and caryoynencin. These compounds have been formerly characterized from the plant pathogens *Burkholderia glumae* (21) and *Burkholderia caryophylli* (22), respectively, and also exhibit antibiotic activity (22,23,24). Further analyses on the genome of *B. gladioli* Lv-StA, from *L. villosa*, also confirmed the presence of these three biosynthetic gene clusters, stressing lagriene, toxoflavin and caryoynencin as promising candidates for causing the inhibitory effect. Specific details on the genes, gene length and corresponding putative proteins for all three gene clusters as well as evidence for their production by *B. gladioli* LvStA are presented in Chapter 4 (Supplementary Information).

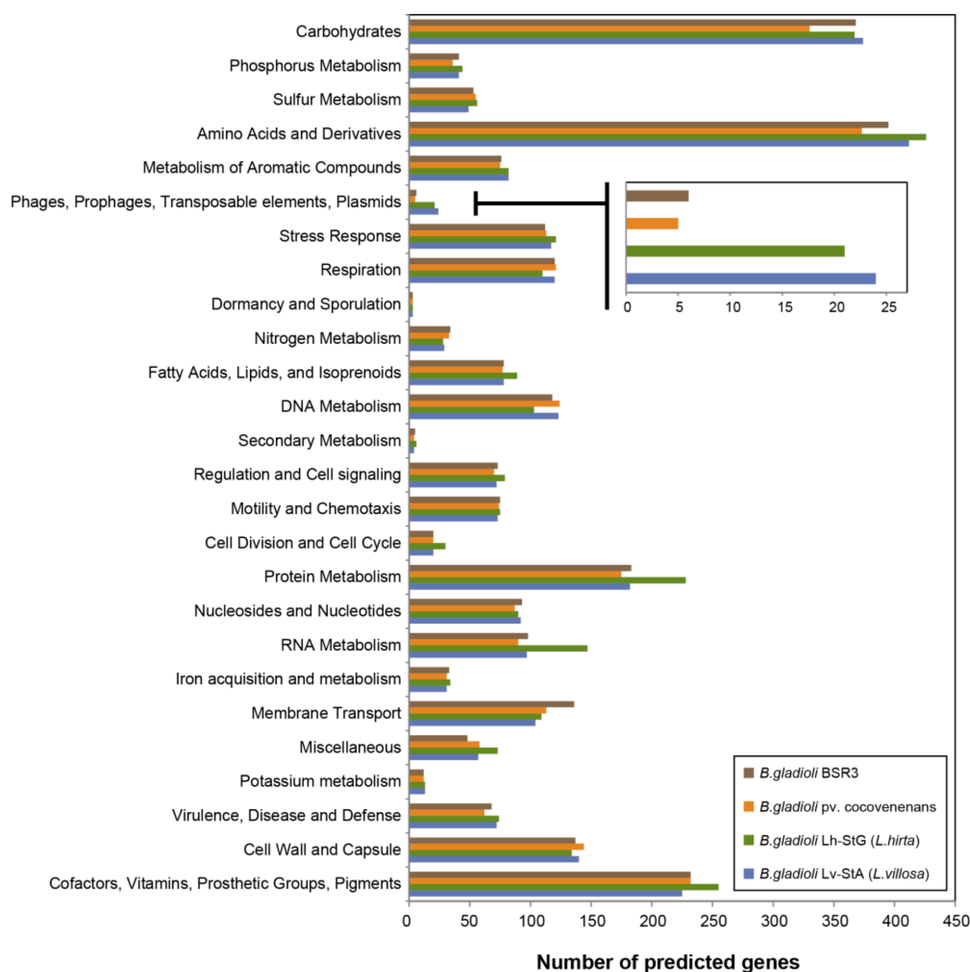

Figure 7.2. Distribution of genes in functional categories based on the genomes of *B. gladioli* symbionts of *L. hirta* and *L. villosa*, as well as the reference strains *B. gladioli* BSR3 (rice pathogenic) and *B. gladioli* pv. *cocovenenans* (causes human food-poisoning). Category assignment is based on an annotation carried out using the RAST online annotation service (10,11).

7.4. Concluding remarks and perspectives

Obtaining the whole genome sequences of two symbiotic *B. gladioli* strains is a preliminary, yet vital step for understanding the mechanistic basis of the symbiosis with Lagriinae beetles. Characteristics as size, base composition and coding density, as well as the high similarity among symbiotic and reference strains suggests a limited degree of specialization and host dependence, at least in the strains that are amenable to cultivation. In terms of symbiont functional role, finding gene clusters responsible for the production of the three mentioned secondary metabolites in the genomes (toxoflavin, caryoynencin and lagriene) is important for guiding future experiments to elucidate the chemical basis of defense by *B. gladioli* in Lagriinae beetles. For example, the generation of knock-out mutants for these genes will be a valuable tool to reveal the role of these compounds *in vivo*. In addition to their direct significance for antifungal or antibacterial protection on the eggs, these could potentially play a key role for partner choice as honest signals. The question of specific genes that are relevant for their interaction with a particular host remains to be addressed and the genomes will certainly be useful for this purpose in combination with manipulative experiments and transcriptomics. In the future, carrying out comparative analyses on the whole-genome level among additional symbiotic *B. gladioli* strains of Lagriinae beetles as well as other *B. gladioli* strains with different lifestyles will most certainly provide valuable insights into genes that are relevant for the symbiotic association with an insect.

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CHAPTER 8

General Discussion

To provide an overview of the Lagriinae - *Burkholderia* symbiosis within the general context of eukaryote-microbe interactions, two fundamental aspects will be discussed in the light of the concepts and results presented throughout chapters 1 to 7: (i) establishment and (ii) persistence of the association. These two matters ultimately address the questions of how this symbiosis could have evolved and why it has been maintained at an evolutionary timescale.

8.1. Establishment of the Lagriinae - *Burkholderia* symbiosis

8.1.1. Host morphological (pre)adaptations for symbiosis

By observing established inter-species partnerships in nature, it is evident that not any pair of organisms can intimately and persistently associate with each other. Indeed, the exclusivity of many symbioses suggests that the establishment of a long-term association usually depends on specific characteristics of both partners (1). These can comprise ecological, physiological or morphological pre-adaptations, as well as traits that are tailored by a cross talk between the partners operating over generations; that is, by a co-evolutionary process.

Morphological adaptations in the host can play several important roles for successful establishment and maintenance of symbiosis. Confining symbionts to specific structures can be advantageous for the host by locally restricting the symbionts to prevent systemic infection, and for the symbionts by avoiding exposure to host immune factors. Also, as discussed in Chapter 3, host morphological adaptations can be key for incorporating, as well as transmitting, bacterial symbionts. For some animal and plant hosts, the development of symbiont-bearing structures only takes place in the presence of a symbiont cue as occurs in *Euprymna* squids (2), or is triggered by complex signaling between partners as for root-nodule formation in legumes (3). In other cases, structures that are relevant for the symbiosis show morphological signs of having co-evolved with the symbiotic partner. For example, the specific morphology of the prostomata (domatia entrance) in plants from the *Leonardoxa africana* plant complex corresponds in size and shape to the head of the respective protective ant species that they associate with (4). In other cases, however, symbionts can be localized in host structures that most certainly evolved in a different context, like the gut. In the Lagriinae, there is no direct evidence that the development of the symbiotic organs in adults or larvae depend on the bacterial symbionts. However, results from this and previous studies allow us to generate a plausible hypothesis about the evolutionary origin of the symbiotic structures and their role in the establishment of the association.

Abdominal accessory glands developing from intersegmental cuticle are present in other groups of Coleoptera in addition to the Lagriinae, including several anobiids, cerambycids and curculionids (5,6), as well as other tenebrionids (7,8). Within the Tenebrionidae, these structures have been classified by Tschinkel and Doyen (1980) in four distinct groups based on morphological and developmental characteristics, yet all consist of cuticular reservoirs associated to secretory tissue. The "Lagriinae type" are musculated, as opposed to all others, and it is uncertain whether they are homologous to the most common type among Tenebrionidae (8). Although it has been suggested that the ancestral state of tenebrionids is the absence of accessory glands, there seems to be a strong tendency for intersegmental

membranes between the last abdominal segments (7/8 and 8/9) to become increasingly glandular and develop into reservoirs (8). As mentioned in Chapter 1, in the anobiids, cerambycids and curculionids that have similar glands, symbiotic microorganisms are harbored within the structures. However, among the tenebrionids the only reference to a symbiotic function of these reservoirs is that of Lagriinae (5), while in other cases across the family the structures are referred to as defense glands without mention of associated microorganisms (7–10). In the light of these observations, it is certainly possible that the symbiont-bearing organs of Lagriinae adult females evolved as glands with a different function, probably defense or egg coating production as in other insects (11), and were coapted for symbiont transmission. To evaluate this hypothesis, further information is needed in several fronts. First, thorough characterizations of accessory glands in Coleoptera are most readily available for males, since these were traditionally used for systematic studies (8) and were more often employed for the biochemical characterization of the related secretions (11). Therefore, corresponding descriptions of females would be useful to complement molecular studies and assess potential roles in symbiosis, especially for vertical transmission. Such characterization would also allow us to assess whether these structures were originally associated to host-derived production of defensive secretions and the equivalent function was later taken over by symbionts (see section 8.2.2.). Second, a solid phylogeny of the Lagriinae subfamily is lacking (12,13) and is fundamental to unequivocally determine the last common ancestor of current Lagriinae species that engaged in a symbiosis with bacteria. Finally, and in combination with the above, a more extensive molecular investigation on the presence and identity of bacterial symbionts in different Lagriinae genera would provide valuable insights on the evolutionary origin of the association.

An additional host trait that seems essential for symbiont transmission is the micropyle, a pore on the egg corion through which the symbiont cells enter and eventually access the developing dorsal invaginations in the embryo (5). The micropyle is, however, recurrent across insects to enable the entrance of sperm during fertilization. The structure itself is thus a preadaptation, and the evolution of permissiveness for specific bacteria to enter through the opening is presumably an adaptation in Lagriinae that facilitated the establishment of a vertical transmission route, as in other insect-microbe interactions (5).

Similar to the accessory glands of adult females, the symbiont-bearing structures in the larvae develop from intersegmental cuticle, yet between the four anterior-most segments of the body. The dorsal organs are also associated with secretory cells (5). As mentioned in Chapter 1, however, there are no described examples of similar structures in the larvae of any other insect (5,6). Notably, the presence of the symbionts –and presumably the structures- in both male and female larvae (Chapter 5) suggests that their maintenance at this stage is associated to a functional role of the symbionts in addition to egg defense. Although speculative, it is possible that these structures evolved based on a selective advantage of maintaining the symbionts during development and also sustaining a vertical transmission route.

8.1.2. Ecological and physiological characteristics of *B. gladioli* as potential symbionts.

As presented in Chapter 4, all the *Burkholderia* symbionts of the Lagriinae hosts investigated so far fall within the *B. gladioli* clade, whose members have been primarily described as plant pathogens (14,15). However, *B. gladioli* strains have occasionally also been found in fungal and animal hosts including humans (15) and mealy bugs (16,17), indicating that these bacteria can directly interact with fundamentally different eukaryotic hosts. Furthermore, these bacteria can persist in a range of different organs in their host, including leaves of a variety of plants, rice grains, rice panicles, onion bulbs (18,19), as well as coffee stems, seeds, berries and roots (20), and grass roots where they have been found intracellularly (21). Likewise, in humans *B. gladioli* can colonize various tissue types including the respiratory tract, abscesses or the blood stream (22). As discussed in Chapter 5, pronounced phenotypic flexibility in *Burkholderia* bacteria is thought to be associated to their markedly dynamic genomic composition. Recurrent genetic exchange is known to

occur in members of this genus, which allows for rapid acquisition of new metabolic capabilities (23). Thus, the scenario of a shared environment between a plant, this versatile bacterial group and a herbivorous insect likely enabled a host range expansion by *B. gladioli*. Specifically, the capacity to produce bioactive metabolites could have facilitated the transition from plant pathogenicity to defensive mutualism in Lagriinae as will be expanded on in section 8.2.2.

Symbiont motility might additionally play a role in symbiosis establishment, as has been demonstrated in other animal-bacteria symbioses. For example, the *Burkholderia* symbionts of the hemipteran *Riptortus pedestris* require motility to colonize the host midgut-crypts (24). Similarly, colonization of the light-organ in *Euprymna scolopes* squids by *Vibrio fischeri* is also strongly determined by symbiont motility (25,26). In tsetse flies, the obligate *Wigglesworthia* symbionts have maintained genes to synthesize a functional flagella despite their reduced genome. It has been demonstrated that motility is indeed relevant for the translocation of *Wigglesworthia* from the mother's milk glands to the intrauterine larvae as part of vertical symbiont transmission (27). In Lagriinae, invasion of the cuticular invaginations of the embryo likely requires active migration by the symbionts from the egg surface (5). This should also serve as a filtering step that contributes to partner specificity, since motile bacteria -including *Burkholderia*- will outcompete non-motile bacteria when colonizing the embryo. As has been accomplished in the bean bug (24) and squid (25) model systems, generating non-motile mutants and evaluating their capacity to initiate and maintain symbiosis would be a suitable approach to assess the specific role of motility in *Burkholderia* for establishing an association with *Lagria* beetles.

8.1.3. Partner encounter and establishment of a transmission mechanism

Initial and recurrent contact between *B. gladioli* and the beetles is most certainly explained by the plant-associated lifestyle of this bacterium, and/or its recurrent presence in the soil (28). *Lagria* eggs, larvae and pupae are also usually found on the soil, in contact with leaves and leaf litter, and the adults are either found in the same environment, or on the leaves of fresh plants. An initial encounter with the adults and the relevant organs could occur during mating or oviposition, when the ovipositor is protruded and can come into contact with leaves or soil. Additionally, extending the ovipositor can occur upon specific external stimuli, a behavior which is observed in *L. villosa* as a response to potential predators or similar threats. These are opportunities for *B. gladioli* bacteria to reach the ovipositor-associated structures and, considering their motility, possibly also the accessory glands.

Once in the ovipositor and/or accessory glands of a female *Lagria*, transfer of *Burkholderia* to the eggs is relatively straightforward as the bacterial cells are carried along with the secretion that is smeared on the egg surface during oviposition (5). It is also possible that free-living *Burkholderia* access the eggs once they have been laid, as successful reinfection with *in vitro* cultures of symbiotic *B. gladioli* (Chapters 4 and 6) suggest that direct application of the symbionts by the mother during oviposition is not indispensable for the embryos to acquire the symbionts. The motile symbionts should then reach and enter through the micropyle to colonize the dorsal invaginations.

Direct encounters between the larvae or pupae and *B. gladioli* are equally probable. There is experimental evidence that aposymbiotic *L. villosa* larvae can take up *B. gladioli,* which had been previously smeared on leaves from their environment, and incorporate these into the dorsal organs (29). The bacteria might enter the structures during molting, although the mechanism remains speculative. Notably, after adult emergence from these larvae, the bacteria are also found in the female accessory glands indicating successful integration with the host, at least upon completion of development (29). A similar process could have occurred during initiation of the symbiosis, at first only associated to the body surface and later with the evolution of cuticular invaginations in the embryos and eventually specialized dorsal structures. It is worth noting that current observations on *L. villosa* reveal that aposymbiotic individuals also develop the dorsal organs as larvae (29), suggesting that there is no direct stimulus from the symbionts triggering the formation

of these structures. However, the lack of phenotypic plasticity does not rule out that the organs might have evolved in a symbiotic context and under selective pressures favoring the accommodation of symbionts.

Given the dramatic restructuring of internal and external anatomy occurring during metamorphosis (30), relocation of symbionts might be challenging in holometabolous insects, as is the case of Coleoptera. In Lagriinae, it is possible that during pupation the *B. gladioli* symbionts mobilize outside the dorsal organs and into the molting fluid. There is in fact observational evidence that the organs might be exposed to the exterior during molting (see section 8.2.1. and Fig. 8.1). From there the bacteria could access the developing accessory glands, as these are also cuticular invaginations. This could have been the original mechanism for relocation and still be maintained in current host species, as was hypothesized by Stammer (1929) and Buchner (1965).

In summary, acquisition of *Burkholderia* from plants or their immediate environment can be envisioned for most life stages of Lagriinae and must have been key for the initial establishment of the symbiosis. Certainly, an overlap of reproductive interests through a vertical transmission route has also occurred in this system, and is expected to reinforce mutualism (31). Yet, along with the evolution of a vertical transmission route, opportunities for recurrently taking up symbionts from the environment have persisted over time (Chapters 4 and 5). The maintenance of flexible and complementary routes for symbiont transmission and the facultative nature of the association might represent advantages for host and symbiont. For example, environmental exposure of the symbionts enabling genetic replenishment can result in novel gene combinations that can be ecologically significant for both partners (32). This dynamic is from its very origin likely associated to the ecological context and functional basis that drive the Lagriinae-*Burkholderia* symbiosis, as will be discussed in the following sections.

8.2. Persistence at an evolutionary timescale

8.2.1. Defensive strategies during immature life stages in insects

Antagonistic species interactions, including competition, predation and parasitism, are important ecological drivers of evolutionary change (33,34). This involves effective defense strategies that increase survival probabilities in the presence of antagonists. In many animals, young and/or immobile life stages are particularly vulnerable to such threats. Eggs are a convenient substrate for pathogens and are often exposed to microbial infections in humid microhabitats. Especially if laid in clusters, eggs also face the risk of parasitoid attack (35), and are both a rich meal and a potential "easy-catch" for various animals with oophagic habits (36). Therefore, effective protection mechanisms are crucial during this stage. Egg defensive strategies can include an innate immune response against invading pathogens (37,38), as well as camouflage, and several forms of chemical and physical protection or defensive behaviors of the mother which usually entail substantial cost (39).

An early immune response has been demonstrated in eggs of the model species *Tribolium castaneum*, and involves the expression of genes participating in Toll and IMD signaling, melanization, production of reactive oxygen species and antimicrobial peptides in the extraembryonic serosal epithelium (38). Although this membrane is absent in a small group of Diptera, it is present in all other insects (40), suggesting that innate immunity operates as an anti-pathogen egg protection across insects. To date, however, experimental work on the role of the serosa is limited to *T. castaneum* and there is no direct evidence from natural settings in this or any other insect including members of Lagriinae.

As to mother-derived protection in insect eggs, physical barriers like the chorion, and in some cases also oothecae, scales, silk or spumaline, can reduce direct contact with antagonists (35). The architecture and size of the egg mass has

also been shown to have an effect on parasitism, and behavioral adaptations like parental guarding also exist (35). In addition to these, numerous examples of chemical defense occur in insects, particularly among Lepidoptera, Hemiptera, Orthoptera and Coleoptera (41,42), often in the form of female-applied coatings (43). The protective substances can be obtained in different ways. These can be transferred by the male to the female, for example as a nuptial gift, which occurs with cantharidin in fire-colored beetles (44,45) and blister beetles (46). The toxins can also be produced *de novo* in the female like the glycosides provided by *Chrysolina* leaf beetles (47), or the miriamides with antifungal properties in the cabbage butterfly *Pieris brassicae* (48). Finally, the compounds can be sequestered from the diet as is the case for the pyrrolizidine alkaloids obtained by *Utetheisa ornatrix* moths from *Crotalaria* spp. plants (49,50). Notably, mother-provided egg coatings can have additional functions like protection against desiccation, release of oviposition pheromones, or symbiont transmission (11). In fact, transmission of symbionts via the egg surface, or "egg-smearing" is a common strategy across multiple insect orders, as expanded on in Chapter 3. Bearing in mind the high biosynthetic potential of microorganisms and the afore-mentioned vulnerability of the egg stage, an interesting ecological scenario comes about, in which egg smearing can be relevant not only for transmission, but also for symbiont-mediated defense at the egg stage. In the Lagriinae-*Burkholderia* system, covering the eggs with antibiotic-producing *B. gladioli* increases the probability of (i) offspring survival and (ii) maintenance of the symbiosis across generations. Therefore, it stands out as a pivotal aspect of this symbiosis and its long-term stability.

Symbiont-mediated defense on eggs or young life stages has been demonstrated in other animal-microbe symbioses involving other insects (51,52), marine invertebrates (53,54), and hoopoe birds (55) (see Chapter 2); and is likely recurrent in nature. In *Lagria* beetles, experimental evidence indicates that the *Burkholderia* symbionts inhibit fungal pathogens which come in contact with the eggs. Yet, the effects on survival were first evident in the larvae that hatched from these eggs; at least when *Purpureocillum lilacinum* was the antagonist (Chapter 4: Fig. 2e and Extended Data Fig. 5b). It is possible that *P. lilacinum* infection develops slowly enough so that the effects on survival arise only after hatching. In fact, a similar retarded effect has been reported in the silverleaf whitefly *Bemisia argentifolii*, in which egg inoculation with the entomopathogenic fungus *Verticillium lecanii* does not have an impact on hatching rate, but causes higher mortality in the hatching nymphs (56). In this case the rate of infection depends on the timing of fungal inoculation and on the virulence of the inoculated strain (56), which might also explain the effects of *P. lilacinum* infection on aposymbiotic eggs of *L. villosa*.

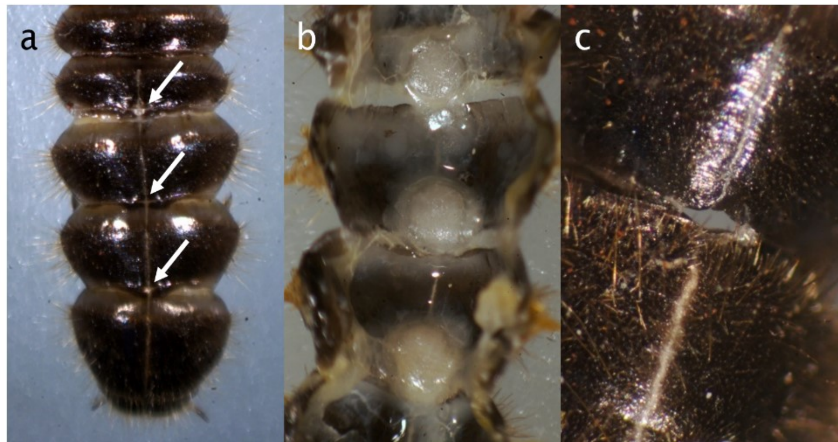

Figure 8.1. Dorsal organs of an *L. villosa* larva located in between body segments 1-4 and co-localizing with the middorsal line. (a) Dorsal view of the three structures. (b) View from an internal perspective after removing internal organs. (c) View of an opening in the cuticle after removing a dorsal organ.

An alternative explanation for these results (Chapter 4: Fig. 2e and Extended Data Fig. 5b) is that the *Burkholderia* symbionts protect the larvae from the fungi that are still present in the surrounding environment. However, evidence for a direct protective role in the larval stage is currently lacking. As mentioned in section 8.1. and Chapter 5, however, this hypothesis would be in line with the maintenance of the symbionts in both male and female larvae. Notably,

observations on the dorsal structures of *L. villosa* larvae indicate that these compartments can connect to the exterior, apparently at least in the pre-molting phase, as small openings form in the cuticle exactly where these three symbiotic organs are located (Fig. 8.1). As suggested for the migration of the bacterial symbionts from these organs to the adult accessory glands (Chapter 1 and section 8.1.3), it is possible that the *Burkholderia* -and the bioactive substances produced by them- reach the molting fluid which accumulates between the old and new cuticle. Considering that insects remain unmelanized for some time and are particularly vulnerable to predation and infection immediately after ecdysis (57), this could be a remarkable strategy for defense. Experimentally addressing this question is indeed an exciting next step for describing this defensive symbiosis.

8.2.2. Symbiosis as a source of ecological and evolutionary innovation for *Lagria* and *B. gladioli*

What are the advantages of symbiont-mediated defense over host-derived protection? Certainly, protection mechanisms can be complimentary, and it is evident that in many organisms there is a combination of strategies. It is also possible that there are trade-offs between one mechanism and the other, but changing ecological conditions or host energy investment patterns could make reliance on a single strategy unfavorable. For example, leaf-cutter ants use both secretions from their metapleural glands (58)(59) and symbiont-derived compounds (60,61) for pathogen defense. Likewise, the solitary European beewolf and related digger wasps employ hydrocarbons from the postpharyngeal gland (62) as well as antibiotics from associated *Streptomyces* for antifungal protection (51). Although protective symbiosis is only one of a diverse range of defense mechanisms, the realization that it is recurrent in nature and widespread across many metazoan taxa (Chapter 2) suggests that there must be particular factors promoting this phenomenon in nature.

The genetic repertoire of a symbiotic microbe is an additional source of novel traits affecting the host's phenotype. In the case of heritable microbial symbionts, evolutionary innovation within the context of common reproductive interests can result in immediate benefits for both partners. It is difficult to unequivocally assure that bacterial symbionts are in general a more rapid source of evolutionary innovation than eukaryotic hosts based solely on genetic change, since a complex combination of factors (e.g. generation time, effective population size, DNA repair mechanisms, lifestyle, cell number) influence evolutionary rates across organisms (63,64). However, ecological innovation can occur at multiple levels and through different mechanisms for which the acquisition or replacement of symbionts opens a new spectrum of possibilities. Indeed, there is strong evidence that symbiosis provides key opportunities for niche expansion (65) and can aid in facing environmental challenges (66).

In particular, many bacteria and fungi are well known for their potential to produce bioactive secondary metabolites (67,68), a characteristic which can be exploited by insects (69) or other eukaryotic hosts (Chapter 2). In this context, the specific features of *B. gladioli* make their association with Lagriinae beetles an interesting example of ecological innovation mediated by symbiosis. For the insect, associating with a plant pathogen that evolved genes for the production of toxins seems an opportune ecological adaptation in the presence of antagonists that are susceptible to the same compounds. Toxoflavin, for example, is a very potent electron carrier which can alter normal respiration processes and can also lead to the production of hydrogen peroxide under aerobic conditions (70). These characteristics are thought to make it toxic for many types of cells, including those constituting both plants (71) and microorganisms (70). Similarly, caryoynencin was originally isolated from the plant pathogen *Burkholderia caryophylli* (72), but its special molecular arrangement of multiple conjugated triple bonds is generally essential for a broad bioactivity (73), including antifungal and antibacterial properties (74). Bistramide A, which is structurally highly similar to lagriamide, has an antiproliferative effect on eukaryotic cells by binding to actin and impairing the progress of the cell cycle (75). Finally, etnangien, a compound closely related to lagriene, affects nucleic acid polymerases in gram-negative bacteria and

eukaryotes. This capacity to affect a broad range of cell types emphasizes the extensive ecological potential of microbial-derived compounds and specifically those from *B. gladioli* as a potential symbiont. It also highlights, however, that toxicity becomes a potential risk for the insect host. Therefore, corresponding resistance or avoidance mechanisms in the insect are required, and costs of harboring the symbionts are not unexpected (Chapter 6).

Additionally, symbioses in which the microbes experience recurrent exposure to the external environment are an opportunity for acquiring foreign genes associated to novel and relevant ecological traits. As referred to in Chapters 2 and 5, this represents a swift and versatile mechanism in the context of an arms-race with coevolving natural enemies for the host. In fact, our findings on the potential production of lagriamide by the symbiotic bacteria, and its similarity to compounds presumably produced by cyanobacterial symbionts of tunicates, favor the hypothesis of horizontal gene transfer as a powerful mechanism in the *Burkholderia*- Lagriinae defensive symbiosis (Chapter 4).

Although there are advantages of outsourcing chemical defense to microbial symbionts, prolonging this benefit in an evolutionary timescale depends on multiple factors. It is fundamental that (i) effective partner choice and/or fidelity mechanisms exist, (ii) the interaction with the microbe is not overall detrimental to host health, and (iii) a permanent association is viable -or at least not fatal- for the microbe as well. While points (i) and (ii) allude to issues discussed in the following sections, (iii) emphasizes a relevant and often disregarded aspect of plant- and animal-microbe symbiosis in the context of evolutionary ecology. That is, the microbe's take on a symbiotic lifestyle (76).

How does defense operate in the context of the symbiont's evolutionary interests? I will start by considering the specific case of *B. gladioli* on *Lagria* eggs. There, it is easy to envision members of the bacterial community on an egg surface interacting among each other and competing for limited nutrients present on the egg, as they would do on a different substrate. In this context, antibiotic production by the symbiont can be seen as a direct antagonistic action, that is, as a mechanism to outcompete other microorganisms (77,78). Alternatively, it has been argued that antibiotics primarily serve as cell-signaling molecules regulating gene expression in natural microbial communities (79,80). At sub-inhibitory concentrations, antibiotics produced by the symbionts in this environment could interfere with cell-cell communication, affect stress response factors, developmental programs, biofilm formation or even virulence in other microorganisms (80). Although these responses depend largely on the type of molecule and the microbial species, it is certainly conceivable that *B. gladioli* are taking part in such molecular dynamic on the egg surface. Ultimately, any of these two scenarios has the potential of increasing nutrient accessibility for *B. gladioli*, or aiding it to thrive in this competitive environment. In addition to nutrient accessibility, the ecological value for *B. gladioli* of succeeding to associate with the insect might lie in the opportunity for dispersal to other hosts, specifically plants. At least in the early stages of symbiosis, protecting the host from infections by pathogens likely emerged as a side-effect of this microbial interaction. On the long-run, heritability might enhance the efficiency of defense for the host, yet the mechanistic basis of *Burkholderia's* interaction with the microbial communities could still be comparable to that occurring in other environments. This is especially likely given the dynamic nature of this symbiosis, in which the bacteria or at least some of the symbiotic strains (see Chapter 5 and section 8.2.3) seem to readily switch between lifestyles and there is no sign of genome reduction or host dependence (Chapter 7).

8.2.3. How to select and maintain cooperating symbiont(s)?

Although cooperation through antibiotic production can be a byproduct of *B. gladioli*'s ecological and metabolic traits, the maintenance of the association over evolutionary timescales suggests that additional mechanisms exist to ensure successful acquisition and proliferation of beneficial strains. In this sense, the fact that six different members of this subfamily all carry symbionts within the *B. gladioli* clade (Chapter 4) reflects specificity and stability of the symbiosis

over considerable time. From Chapters 4 and 5, it is clear that *Burkholderia* are both vertically and horizontally transferred in Lagriinae beetles. Typically in vertically transmitted symbionts, partner fidelity mechanisms become relevant to maintain cooperation, as recurrent interaction between symbiont and host enables feedback and reinforces the association over generations (81,82). In the beetles it is possible that some of the *B. gladioli* strains are consistently vertically transmitted, as discussed in Chapter 6. Thus, partner fidelity might play a role in maintaining a functional mutualism with these particular strains. Environmental acquisition, on the other hand, requires mechanisms to –actively or passively- sort out the right symbiont (81,82). Given the apparently frequent opportunity for horizontal transfer of the *B. gladioli* symbionts in Lagriinae, it is likely that partner choice mechanisms are most relevant in this system.

Partner choice can operate via honest signaling, in which symbiont-derived molecules are recognized by the host. This signal can be the same compound that provides a symbiotic benefit, or it can be an indirect signal of a cooperative phenotype (83). Importantly, the signal must correspond to partner quality or else mutualism will easily break down due to the vulnerability to cheating (84). The fact that *B. gladioli* interact with other eukaryotic hosts including plants and humans could be linked to a means of signaling, however, to date there is no information on whether any of the bioactive compounds produced by *B. gladioli* are recognized by Lagriinae beetles.

Alternatively to signaling, screening has been proposed as a partner choice mechanism in symbiosis (85). An interesting scenario has been suggested for defensive symbiosis, and particularly for the Actinomycete-attine ant symbiosis, which could very well apply to the *Burkholderia*-Lagriinae association. Based on a theoretical model, Scheuring and Yu (86) put forward the hypothesis that, given a high initial population density of antibiotic-producers colonizing by either vertical transmission or environmental acquisition, resource provision by the host can stimulate competition by enhancing antibiotic production. This would result in control of non-producing microbes, or pathogens, and dominance by high-quality defensive symbionts in the microbial community. Although the host does not directly assess symbiont performance, the competition itself screens for the best-performing ones. In the case of *B. gladioli* and *Lagria*, the egg surface would represent a clear scenario for intense competition against potential invaders, with an initial high number of *B. gladioli* cells provided in the secretion applied by the mother.

In addition, based on the same model proposed by Scheuring and Yu (86), it has been argued that the initial presence and dominance of antibiotic-producers facilitates the recruitment of additional antibiotic-producing lineages due to priority effects. This occurs because it is unlikely that the beneficial strains (antibiotic-producers) or the non-beneficial ones (non-producers) can invade a population already dominated by the others. In other words, there are two alternative stable states; reaching one or the other equilibrium depends on which bacterial type was initially most abundant (86). For the particular case of *Burkholderia* in Lagriinae, this hypothesis requires further studies on the ecological dynamics accompanying antibiotic production by the symbionts (e.g. antibiotic concentrations and resistance in each strain). If substantiated, it could help explain the presence of multiple coinfecting *B. gladioli* lineages in *L. villosa* and *L. hirta*. As discussed in Chapter 5, coinfecting symbiotic strains might provide an ample range of different antibiotics and thus a more generalized defensive mechanism, which would reinforce recurrent environmental acquisition of additional strains in the described scenario. Furthermore, the potential horizontal gene transfer from environmentally acquired strains to vertically transmitted ones might also favor a dynamic and enriched defensive armory, thus supporting a certain degree of permissiveness during partner choice.

8.2.4. Context dependence and dynamics of a multipartite interaction

The ecological underpinnings of the *Lagria-Burkholderia* association go beyond those of a one to one relationship, which is true for many symbioses. This is particularly relevant in this case though, as the host plant, fungal antagonists, the

insect itself and at least three bacterial symbiont strains seem to be fundamental for the ecological platform that sustain the symbiosis. In this section, I will discuss the impact and role of the main players in the interaction, emphasizing the dynamics of the cost to benefit ratio in response to the ecological context.

Most probably, the interaction between *B. gladioli* and various plants is the most prevalent and oldest among the partners involved in the multipartite interaction. As discussed earlier, pathogenicity is the predominant fitness impact that has been observed and described for this bacterium in plants (14,19). However, *B. gladioli* strains have also been found to have antibiotic activity against phytopathogens *in vitro* (17,87). It is also true that despite the negative impact observed on the reproductive fitness of soybean plants (Chapter 4), infection does not cause drastic disease symptoms as in other plants. This demonstrates that the impact of *B. gladioli* infection varies for different plants, which is not surprising. It is also expected that pathogenicity depends largely on environmental conditions as predicted by the disease triangle dogma, a classic concept in plant pathology (88). Thus, despite the cost of *B. gladioli* infection, some plant hosts might gain a net benefit if they are, for example, exposed to a highly virulent fungal phytopathogen that is susceptible to *B. gladioli's* bioactivity. This hypothesis remains to be tested for the symbiotic strains of *L. villosa* and *L. hirta* in different plants, and would be interesting to observe in natural conditions in which the fitness implications of symbiotic associations can be strikingly different from the laboratory.

For the insect host, we have shown that carrying the symbionts has a defensive benefit. However, we have also observed that this advantage is context dependent and that symbiont maintenance entails a cost. In Chapter 6 we show that, for *L. villosa,* carrying at least one of the strains (*B. gladioli* Lv-StA) results in slower development and decreased weight. Importantly, the symbionts do not seem to compromise survival, but in the absence of pathogenic threats the association seems disadvantageous for the beetle. This is additionally supported by results in Chapter 4 (Extended Data Fig. 5b), which show that aposymbiotic larvae hatching from eggs without fungal growth are those with the best chances of surviving the earlier larvar instars (about 90% survival probability), while symbiotic individuals have a lower probability to survive in the absence of fungal growth on the egg (between 70 and 80%). This difference indicates a cost for carrying the symbionts. Yet, the cost pays off, since those affected by fungal infection have in general better chances of surviving if they are symbiotic. Under these conditions aposymbionts do worse than all other treatments (Untreated controls, Reinfected with *B. gladioli* Lv-StA or Reinfected with the natural egg microbiota), with a close to 40% probability of surviving within the first days after hatching. In a natural scenario, contact with antagonists is likely common, but variability in the occurrence of these threats (e.g. *P. lilacinum* or other entomopathogens) should cause shifts in the cost to benefit ratio of maintaining the symbiosis.

As discussed in Chapter 5, harboring multiple symbiotic strains might entail a cost due to competition. However, as mentioned in the previous section, coinfecting strains could also be advantageous in the context of a generalized defense mechanism. Indeed, results presented in Chapter 4 suggest that a range of different compounds (toxoflavin, caryoynencin, lagriene and lagriamide) produced by at least two of the symbiotic strains of *L. villosa* (*B. gladioli* LvStA and LvStB) are likely to take part in a broad-spectrum protection. These could also be synergistic, which would clearly point to a functional benefit of accepting and maintaining multiple strains.

For *B. gladioli*, an intimate association with *Lagria* beetles during the larval stage could involve limited access to nutrients and increased competition, when they are restricted to the dorsal organs for prolonged time periods. Here the bacteria might face stressful conditions, similar to the *Burkholderia* symbionts of the bean bug *Riptortus pedestris* within the midgut crypts (89). This implies that either it pays off for *B. gladioli* to inhabit these structures due to a specific benefit (e.g. dispersal), or exploitation by the host is occurring without any profit for the bacteria. This condition would rather resemble imprisonment, which has been discussed previously in the context of microbe-eukaryote symbioses (76). Although it is challenging to experimentally address this question by assessing the advantages for the bacteria, a first

approach would be to evaluate metabolic changes and replication dynamics of the *B. gladioli* in host-restricted conditions. In particular, this could reveal context dependence of mutualism in relation to host life stage, also from the symbiont´s perspective.

The coexistence of multiple bacterial strains is a fundamental aspect in the ecological dynamics of the interaction. As stressed above, intense competition is predicted by traditional models of symbiosis in cases of mixed infections (1,90). Nutrient availability has a strong influence on competition between microbial strains –as for other organisms- (78), which should vary in different host life stages and might be regulated by the host. Furthermore, the spatial configuration of the symbiotic community on the egg surface might be important for the dynamics of competition or potential cooperation. In biofilms, which could resemble the secretion-covered egg, non-mixing of different genotypes might reduce competition and enhance within-genotype cooperation (91). Along the same lines, the emergence of cheaters is less likely in such a spatially structured setting (91). Then again, strain mixing could occur in other host life stages affecting microbial interactions, but further investigation is needed to understand the ecological dynamics of symbiont strain diversity during the life cycle of *Lagria* beetles.

In light of the complexity of the *Lagria-Burkholderia* symbiosis, it is noteworthy that there are diverse ecological factors regulating this dynamic interaction. Despite a far from stable arrangement, the association persists and is widespread in the Lagriinae subfamily; at the same time, *B. gladioli* conserves its ability to interact with a plant host. An important lesson from these findings is that categorizing an interaction as pathogenic or mutualistic can limit our understanding on its full ecological background. These circumstances are even more complex in a natural setting, and thus it will be a challenging yet fascinating endeavor to further tease apart key aspects of this system and their overall relevance for symbiosis research.

8.3. Research perspectives on the Lagriinae-*Burkholderia* symbiosis

This first approach to understand the ecological and evolutionary basis of the Lagriinae-*Burkholderia* symbiosis has come with new research questions that are pertinent to this particular association, but also interesting in the more general context of symbiosis. A first relevant set of questions relates to how and why symbiont strain diversity is maintained. Is the coexistence of multiple symbiotic strains dependent on natural enemy occurrence or host life stage? Can the host discriminate between strains and does this play a role in partner choice and partner fidelity? Does the host benefit from the dynamic nature of the symbiosis? Is carrying multiple strains advantageous through better or more versatile antibiotic defense? These questions certainly apply to other symbiotic associations as well, and are interesting in the wider context of defensive symbiosis.

On another front, insect-microbe molecular interactions on the egg and particularly the role of the host immune system during this stage are only starting to be understood (37,38). In the case of *B. gladioli* in *Lagria* beetles, gaining access to the insect embryo is arguably one of the crucial steps for establishing and maintaining the association. This requires not only active migration of the bacterial symbionts into a specific organ, but also crossing the extraembryonic serosa. As mentioned in section 8.2.1, this is the first cellular epithelium at the interface between the external environment and the yolk with the developing embryo, where a fully-functional set of immune genes are expressed upon infection with certain microbes (38). How do multiple (at least three) strains of *B. gladioli* bypass this control mechanism? Is there a synergistic or complimentary effect between the symbiont-mediated protection and host immunity?

A third aspect, which could be relevant from the point of view of agricultural impact, is the role of Lagriinae beetles in vectoring *B. gladioli* between plants. We have shown that beetle to plant transmission and *vice versa* are possible in laboratory conditions (Chapter 4), but there is no information on the frequency and impact of vectoring in the field. *L. villosa* beetles occur in high abundances in a variety of crops in Brazil, and have been reported to cause damage due to herbivory (92–94). However, no research has been carried out on the correlation between *L. villosa* occurrence and bacterial or fungal plant infections, which could both be related to the symbiosis with *B. gladioli,* as discussed previously.

Indeed, there is still much to learn from the Lagriinae-*Burkholderia* symbiosis. Importantly, this system offers several advantages for manipulative experimentation that should facilitate addressing these questions. Cultivability of at least one of the symbiotic *Burkholderia* strains of *L. villosa* and *L. hirta*, respectively, and existing knowledge on genetic manipulation of *B. gladioli* (95) and other *Burkholderia* (96–98) are clear advantages. Additionally, the possibility to generate aposymbiotic *Lagria* beetles (Chapters 5 and 6) opens promising opportunities for hypothesis-driven approaches.

8.4. References

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9. Summary

Symbiosis is ubiquitous in nature and can play an important role in shaping the biology of both eukaryotes and prokaryotes. In particular, defensive symbiosis is an emerging topic in the field. Yet, thorough characterizations addressing the mechanistic, ecological and evolutionary basis of an animal-microbe protective association are limited to a few systems, especially in insects.

This dissertation focuses on the symbiotic association between Lagriinae beetles (Coleoptera: Tenebrionidae) and *Burkholderia* (β-proteobacteria), a genus exhibiting remarkable metabolic and ecological versatility. The bacteria are extracellularly located in accessory glands of the female reproductive system and can be vertically transmitted in a secretion smeared by the mother on the egg surface. From there, the symbionts migrate into unusual compartments that develop dorsally as cuticular invaginations in the embryo. The aims of the project were to (i) molecularly characterize the *Burkholderia* symbionts and their transmission route, (ii) assess if the symbiosis is present in multiple Lagriinae hosts and is thus evolutionarily ancient, and (iii) investigate the functional and ecological significance of *Burkholderia* in these insects.

Using *Lagria hirta* and *Lagria villosa* beetles as model systems, the symbionts were identified as *Burkholderia gladioli*, a bacterium mostly known for its plant pathogenic traits. I localized these bacteria on adults, larvae and eggs of both species confirming the proposed vertical transmission route. The presence of *B. gladioli* in these and four other Lagriinae host species from geographically distant origins (Germany, Brazil, Japan and Australia) suggested that the symbiotic association is relatively ancient and that it evolved within this phytopathogenic bacterial group. However, the *B. gladioli* symbionts of the different Lagriinae do not form a monophyletic clade. They instead cluster together with plant-associated *B. gladioli* strains, indicating that environmental acquisition also occurs, and also highlighting plants as potential alternative hosts for the beetle symbionts. Indeed, a symbiotic *B. gladioli* strain isolated from *L. villosa* can successfully infect soybean plants, a food source for this beetle species, and has a negative effect on the plant's reproductive output (i.e. seed number). This implies that, at least this symbiotic strain of the beetle conserves the ability to intimately interact with a plant host and stresses the flexible transmission dynamics of the *B. gladioli* symbionts.

Presumably, the potential of plant pathogenic *B. gladioli* bacteria to produce potent bioactive substances was also essential for establishing a protective mutualism with the insect. In *L. villosa* beetles, I could show that the presence of *B. gladioli* on the surface of eggs inhibits the growth of antagonistic fungi as compared to symbiont-free eggs, which has a significant effect on the survival of the corresponding larvae. Thereby, I demonstrate a symbiont-mediated defense, which could be highly advantageous at the nutrient-rich and immobile egg stage. Furthermore, based on genome sequencing of an isolated symbiotic *B. gladioli* strain and chemical analyses of symbiotic *L. villosa* egg extracts, it was possible to elucidate four compounds (toxoflavin, caryoynencin, lagriene and lagriamide) that could be responsible for the protective effect.

Contributing to the complexity of the system, *L. hirta* and *L. villosa* beetles each carry at least three different *B. gladioli* strains that can coexist in a single host individual. This finding is of particular interest in the context of a defensive symbiosis; it is possible that production of complementary or synergistic protective substances by the different strains confers a broad-spectrum protection, or higher flexibility for responding to varying occurrence of natural enemies. Interestingly, despite having a vertical transmission route in addition to the possibility of horizontal acquisition, laboratory conditions cause shifts in *B. gladioli* strain composition and occasional complete loss of *B. gladioli* symbionts. This observation highlights potential pitfalls when studying facultative microbial symbionts in the laboratory.

Given the context-dependent nature of defensive symbiosis, it is relevant to consider what kind of impact the symbionts have on the host in the absence of antagonists. In these circumstances, removal of *Burkholderia* in both *L. hirta* and *L. villosa* and reinfection with an *in vitro* culture of a single *B. gladioli* symbiotic strain either does not have a significant effect (*L. hirta*) or causes slower development and reduced weight in the host (*L. villosa*). This result could be associated to a metabolic cost of carrying the symbionts, which can become evident in the absence of a pathogenic threat if defense is the sole benefit conferred by the *Burkholderia*. Alternatively, it is possible that infection with this single strain is not representative of the effects caused by multiple coinfecting strains in natural conditions.

The symbiosis between Lagriinae beetles and *B. gladioli* constitutes an ecologically complex and evolutionarily dynamic defensive mutualism. The findings on this system contribute to our knowledge on defensive symbiosis in several ways. First, it exemplifies how an insect defensive partner can evolve from a plant pathogenic bacterium owing to its metabolic potential and versatility. Second, it is the first direct demonstration of symbiont-mediated defense at the egg stage of an insect. This phenomenon might be more common in nature than previously thought given that symbiont vertical transmission *via* the egg surface is a frequent strategy in insects and other arthropods. Third, the fact that multiple symbiotic strains coexist in the host brings about interesting questions regarding the potential advantages of strain diversity in defensive symbiosis and the evolutionary dynamics supporting their long-term maintenance. These aspects of the Lagriinae-*Burkholderia* symbiosis substantiate this as a promising and fascinating system to further explore animal-microbe interactions.

10. Zusammenfassung

Symbiosen sind weitverbreitet in der Natur und können einen wichtigen Einfluss auf die Biologie eukaryotischer und auch prokaryotischer Organismen ausüben. Inbesondere Verteidigungssymbiosen sind ein spezielles, neu aufkommendes Thema in diesem Bereich. Gründliche Beschreibungen, die sich mit den mechanistischen, ökologischen und evolutionären Grundlagen von Mikroben als Beschützer von Tieren befassen, sind jedoch auf nur wenige Systeme beschränkt.

Die vorliegende Arbeit untersucht die symbiotische Assoziation von Lagriinae Käfer (Coleoptera: Tenebrionidae) mit *Burkholderia* (β-proteobacteria), einer Gattung mit außergewöhnlicher metabolischer und ökologischer Vielseitigkeit. Die Bakterien befinden sich extrazellulär innerhalb von Drüsen des weiblichen reproduktiven Systems und können vertikal übertragen werden, in einem Sekret, das von der Mutter auf die Oberfläche der Eier geschmiert wird. Von dort wandern die Symbionten zu ungewöhnlichen Strukturen, die sich als Einstülpungen der Cuticula am dorsalen Körperteil der Larven entwickeln. Auf Basis dieser Beobachtungen, wurden die folgende drei Ziele für dieses Projekts gesetzt: (i) eine molekulare Charakterisierung der *Burkholderia* Symbionten und die Identifikation ihrer Übertragungsroute durchzuführen, (ii) die Präsenz dieser Symbiose in verschiedenen Lagriinae-Wirten zu testen um zu beurteilen, ob diese evolutionär alt ist, und (iii) die funktionale und ökologische Bedeutung der *Burkholderia* für diese Insekten zu untersuchen.

Mithilfe der *Lagria hirta*- und *Lagria villosa*-Käfer als Modelsysteme wurden die Symbionten als *Burkholderia gladioli* identifiziert - Bakterien, die meistens wegen ihrer pathogener Wirkung auf Pflanzen bekannt sind. Ich habe diese Bakterien in Erwachsenen, Larven und Eiern beider Arten lokalisiert und damit die vermutete vertikale Übertragungsroute bestätigen können. Die Anwesenheit von *B. gladioli* in diesen und vier anderen Lagriinae-Arten von geographisch weit voneinander entfernten Herkunftsorten (Deutschland, Brasilien, Japan und Australien) weist darauf hin, dass die symbiotische Assoziation relativ alt ist und dass sie innerhalb dieser Gruppe von Phytopathogenen entstanden ist. Die Symbionten verschiedener Lagriinae formen jedoch keine monophyletische Klade. Stattdessen gruppieren sie sich mit *B. gladioli* Stämmen, die mit Pflanzen assoziiert sind. Dies lässt vermuten, dass die Übernahme von Symbionten auch aus der Umgebung erfolgen kann, denn Pflanzen könnten potenzielle alternative Wirte für die Symbionten der Käfer sein. Tatsächlich haben wir festgestellt, dass einer von *L. villosa* isoliterter *B. gladioli*-Stamm Sojabohnenpflanzen, eine Futterquelle dieser Käfer, nicht nur erfolgreich infizieren kann sondern auch eine negative Wirkung auf die Reproduktion der Pflanze (d.h. Anzahl der Sammen) hat. Dies deutet darauf hin, dass dieser symbiotische Stamm mit einer Wirtspflanze eng interagieren kann und betont die Flexibilität der Übertragungsdynamik der *B. gladioli*-Symbionten.

Das Potenzial von pflanzenpathogener *B. gladioli* bioaktive Substanzen zu produzieren war vermutlich auch entscheidend für die Etablierung eines beschützenden Mutualismus mit dem Insekt. Mit *L. villosa* Käfer konnte ich beweisen, dass *B. gladioli* auf der Oberfläche der Eiern das Wachstum von antagonistischen Pilzen im Vergleich zu Eiern ohne Symbionten unterdrücken, und damit einen bedeutenden Effekt auf die Überlebenswahrscheinlichkeit der ausschlüpfenden Larven haben. Dadurch wies ich eine durch Symbionten vermittelte Verteidigung nach, die sehr vorteilhaft für das nährstoffreiche und immobile Eier-Stadium sein kann. Darüber hinaus, ist uns durch die Genomanalyse eines isolierten symbiotischen *B. gladioli*-Stammes und durch chemische Analysen von *L. villosa* Eier-Extrakten mit Symbionten gelungen, vier Substanzen (Toxoflavin, Caryoynencin, Lagriene und Lagriamide) zu identifizieren, die voraussichtlich verantwortlich für die beschützenden Effekte sind.

L. hirta- and *L. villosa*-Käfer tragen mindestens drei verschiedene *B. gladioli*-Stämme, die gleichzeitig in dem selben Wirt leben können. Dieser Befund ist besonders interessant in dem Kontext von Verteidigungssymbiosen. Möglicherweise wird durch die Produktion von komplementären oder synergistischen beschützenden Substanzen von den verschiedenen Stämmen ein Breitbandschutz erreicht, oder eine höhere Flexibilität um gegen das wechselnde Auftreten natürlicher Feinde zu reagieren. Interessanterweise treten unter Laborbedingungen starke Änderungen der Zusammensetzung der *B. gladioli*-Stämme oder der vollständige Verlust der Symbionten auf, trotz einer Vertikalübertragungsroute zusätzlich zu der Möglichkeit der horizontalen Einnahme. Diese Beobachtung zeigt die Schwierigkeiten, die bei Untersuchungen fakultativer mikrobieller Symbionten im Labor auftreten können.

Da Verteidungssymbiose kontextabhänging sind, ist es relevant, die Einwirkung der Symbionten auf den Wirt ohne Begegnung mit Antagonisten zu berücksichtigen. Unter diesen Umständen hat die Entfernung der *Burkholderia* von *L. hirta* und *L. villosa* und die Reinfektion mit einer *in vitro*-Kultur eines einzigen *B. gladioli* symbiotischen Stammes entweder keinen Effekt (*L. hirta*) oder ergibt eine längere Entwicklungszeit und ein verringertes Körpergewicht des Wirtes (*L. villosa*). Dies könnte ein Hinweis dafür sein, dass die Symbionten metabolische Kosten verursachen. Wenn eine Verteidigungsfunktion der einzige von *Burkholderia* gewährte Vorteile ist, sollte dieser Aufwand ohne die Bedrohung von Pathogene offensichtlich werden. Anderenfalls ist es möglich, dass die Infektion mit einem einzigen Stamm die Einwirkung von mehreren Stämmen nicht darstellt.

Die Symbiose zwischen Lagriinae-Käfern und *B. gladioli* ist ein ökologisch komplexer und evolutionär dynamischer Verteidigungsmutualismus. Die Befunde aus diesem System tragen zur generellen Kenntnis der Verteidigungssymbiose in verschiedener Weise bei. Erstens verdeutlicht es einen Weg, wie sich ein Verteidigungspartner eines Insekts aus einem Pflanzen-Pathogen entwickeln kann, aufgrund seines metabolischen Potentials und seiner Vielseitigkeit. Zweitens sind unsere Ergebnisse der erste direkte Beweis für Symbiont-vermittelte Protektion von Insekteneiern. Dieses Phänomen könnte häufiger in der Natur sein als bisher angenommen, denn die Vertikalübertragung von Symbionten durch die Oberfläche der Eier ist eine verbreitete Strategie bei Insekten und anderen Arthropoden. Drittens wirft die Koexistenz mehrerer symbiotischer Stämme in einem Wirt interessante Fragen bezüglich der Vorteile von Stammvielfalt für die Verteidigungssymbiose und der evolutionären Dynamik auf, die deren langfristige Erhaltung unterstützt. Diese Aspekte machen die Lagriinae-*Burkholderia*-Symbiose zu einem aussichtsreichenden und faszinierenden System zur weiteren Erforschung von Interaktionen zwischen Tieren und Mikroorganismen.

Appendix

Chapter 2 - Table S1: Defensive microbial symbionts in animals. Associations involving eukaryotic symbionts are highlighted with grey background.

Host phylum	Host taxon	Environment	Protected life stage of the host	Symbiont taxon	Symbiont localization	Means of defense	Chemistry of defense	Biosynthesis	Antagonist against which the defense is active	Evidence for defensive symbiosis	Ref.
Porifera	*Halichondria okadai*	Marine	Adult	*Alteromonas* sp.	Unknown	Secondary metabolite production	Ubiquinones	Terpenoid	Predatory barnacles, e.g. *Balanus amphitite*	*In vitro* settlement inhibition of isolated compounds	[1]
Porifera	*Lissodendoryx isodictyalis*	Marine	Adult	*Winogradskyella poriferum*	Unknown	Secondary metabolite production	Polyether	Other	Biofilm forming bacteria that attract barnacle larvae, predatory barnacles, e.g. *Balanus amphitite* & *Hydroides elegans*	*In vitro* settlement inhibition of crude extracts and isolated compounds, *in vitro* antibacterial activity of isolated cultures, *in vitro* inhibition of bacterial biofilm formation	[2, 3]
Porifera	*Reniera japonica*	Marine	Adult	*Flexibacter* sp.	Unknown	Radical protection	3R,3'R-zeaxanthine	Terpenoid	Singlet oxygen, free radicals	*In vitro* production of zeaxanthine	[4]
Porifera	*Dysidea herbacea*	Marine	Adult	*Oscillatoria spongeliae*	Extracellular endosymbiont	Secondary metabolite production	Polychlorinated diketopiperazides, e.g. Herbadysolide**,** Dihydrodysamide C, Demethyl-dihydrodysamide C, 13-Demethylisodysidenin, Didechlorodihydrodysamide C	Other	Fish predators	Isolated compounds deter predatory fish in feeding assays with spiked food.	[5-7]
Porifera	*Dysidea herbacea, Lendenfeldia chondrodes, Phyllospongia papyracea*	Marine	Adult	*Oscillatoria spongeliae*	Extracellular endosymbiont	Secondary metabolite production	Polybrominated biphenylethers	Other	Fish predators, bacteria	Deter predatory fish in feeding assays with spiked food. *In vitro* antibacterial activity of extracted compounds	[7-9]
Porifera	*Dysidea herbacea*, *Lamellodysidea herbacea*	Marine	Adult	*Oscillatoria spongeliae*	Extracellular endosymbiont	Secondary metabolite production	dysidin, dysidenin, neodysidenin & related compounds	NRPS-PKS	Unknown	Phospholipase inhibition	[10-14]
Porifera	*Dysidea herbacea*	Marine	Adult	*Synechocystis sp*	Extracellular endosymbiont	Secondary metabolite production	Dysiherbaine	Other	Unknown	*In vitro* neurotoxic activity of extracted compounds	[15, 16]
Porifera	*Pseudoceratina clavata*	Marine	Adult	*Salinispora sp.*	Unknown	Secondary metabolite production	Rifamycin B & SV	PKS	Bacteria	*In vitro* antibacterial activity of isolated cultures, Rifamycins are known antibiotics against Gram-posivive bacteria	[17, 18]

Porifera	*Plakortis simplex*	Marine	Adult	*Sphingomonas sp.*	Extracellular endosymbiont	Secondary metabolite production	Plakortins, Plakosides, Simplexides, Crasserides, Bacteriohopanoids	PKS Other Other Other terpenoid	bacteria, fish predators (e.g. *Carassius aurata*)	*In vitro c*ompound production by bacterial isolate *In vitro* antimicrobial & cytotoxic (antimalarial) activity, *In vitro* immunosuppressive activity, *In vitro* immunosuppressive activity, *In vitro* feeding deterrent	19-27
Porifera	*Psammocinia* aff. *bulbosa*	Marine	Adult	Unknown	Unknown	Secondary metabolite production	Psymberin (=ircinastatin)	*trans* AT PKS	Unknown	*In vitro* cytotoxic activity of extracted compound	28, 29
Porifera	*Mycale hentscheli*	Marine	Adult	Unknown	Unknown	Secondary metabolite production	Mycalamide A	*trans* AT PKS	Unknown	*In vitro* cytotoxic (antimalarial) activity of extracted compound	29-31
Porifera	*Theonella swinhoei*	Marine	Adult	Unicellular heterotrophic bacteria	Extracellular endosymbiont	Secondary metabolite production	Swinholide A	*trans* AT PKS	Unknown	*In vitro* cytotoxic activity of extracted compound	32, 33
Porifera	*Theonella swinhoei*	Marine	Adult	*Candidatus* Entotheonella palauensis	Extracellular endosymbiont	Secondary metabolite production	Theopalauamide, Theonellamide A	(NRPS?)	Unknown	*In vitro* antimycotic activity of extracted compound	33-37
Porifera	*Theonella swinhoei*	Marine	Adult	*Candidatus* Entotheonella sp	Extracellular endosymbiont	Secondary metabolite production	Polytheonamides Onnamides Pseudoonamide A Keramamides Nazuamide A Cyclotheonamide A Theopederins	Ribosomal NRPS-PKS	Unknown	*In vitro* antimycotic, antiviral and cytotoxic activity of some of the extracted compounds	38-44
Porifera	*Discoderma calyx*	Marine	Adult	*Candidatus* Entotheonella sp?	Extracellular endosymbiont	Secondary metabolite production	Calyxamides A&B	(NRPS?)	Unknown	*In vitro* cytotoxic activity of extracted compound	45
Porifera	*Discoderma calyx*	Marine	Adult	*Candidatus* Entotheonella sp?	Extracellular endosymbiont	Secondary metabolite production	Calyculins	NRPS-PKS	Unknown	*In vitro* cytotoxic activity of extracted compound	46
Cnidaria: Anthozoa	*Pseudopterogorgia* spp. (*P. elisabethae* and *P. bipinnata*)	Marine	Mostly Adults	Dinoflagellates: *Symbiodinium* spp.	Intracellular endosymbiont	Secondary metabolite production	Pseudopterosins, and kallolide family of diterpenes	Terpenes	*Thalassoma bifasciatum* (fish)	Localization of compounds in symbiont cell fraction; labeling experiments; coral extracts unpalatable to fish predators	47-49
Cnidaria: Anthozoa	*Pseudopterogorgia americana*	Marine	Adults	Dinoflagellates?	Intracellular endosymbiont	Production of precursors of toxic compounds	Secosterols, including 9,11-secogorgosterol and 9,11-secodinosterol	Sterols	*Thalassoma bifasciatum* (fish)	Isolated dinoflagellates from several marine organisms produce gorgosterol and dinosterol; deterrence of secosterols to fish predator	50-52

Cnidaria: Anthozoa	unspecified soft coral	Marine	Adult?	Actinobacteria: *Micromonospora* sp. ACM2-092	Unknown	Secondary metabolite production	Thiocoraline	Other	Antimicrobial and cytotoxic activity	*In vitro* bioactivity of compounds	[53]
Cnidaria: Hydrozoa	*Hydra vulgaris*	Limnic	Adults	Complex community; mostly Beta-Proteobacteria	Ectosymbiont and extracellular endosymbiont	Unknown	Unknown	Unknown	Fungal pathogens	Experimental manipulation of microbiota affects protection against a fungal pathogen; *in vitro* antifungal effects	[54]
Bryozoa	*Bugula neritina, B. simplex*	Marine	Mostly larvae and Adult reproductive tissue	Gamma-Prot.: '*Candidatus* Endobugula sertula'	Extracellular endosymbiont	Secondary metabolite production	Bryostatins 1-20	*Trans*-AT PKS	Multiple predators	Co-localization of bryostatins and symbionts *in vivo*; decrease in bryostatin concentrations after antibiotic treatment; identification of bryostatin biosynthesis gene cluster; deterrence of bryostatins against various predators	[55-67]
Bryozoa	*Sessibugula translucens, Bugula dentata*	Marine	Adult	*Pseudoalteromonas tunicata*	Extracellular endosymiont	Secondary metabolite production	Tambjamines	Other	Fish predators	Tambjamines isolated from bryozoans; deterrence against fish predators; identification of tambjamine biosynthesis gene cluster in *Pseudoalteromonas tunicata*; isolation of *P. tunicata* from some bryozoans	[67-72]
Bryozoa	*Amathia spp.*	Marine	Adult	Unidentified	Unknown	Secondary metabolite production	convolutamines, convolutindole	Other	Parasitic nematodes?	Intraspecific variability in bryozoans chemistry; structure of some of the compounds related to microbial metabolites; *in vitro* nematocidal activity	[73]
Bryozoa	*Amathia spp.*	Marine	Adult	Rod-shaped bacterium	Ectosymbiont	Secondary metabolite production	Amathamides and related compounds	Other	Unknown	Localization of amathamides correlated with bacteria	[64, 74-76]
Bryozoa	*Biflustra perfragilis*	Marine	Adult	Unknown	Unknown	Secondary metabolite production	Perfragilins	Other	Unknown	Similarity to microbial metabolites; *in vitro* cytotoxic activity	[77]
Bryozoa	*Flustra foliacea*	Marine	Adult	Unknown	Unknown	Secondary metabolite production	brominated indoles and diterpenoids	Other and terpenes	Unknown	Intraspecific variation in chemical composition of *F. foliacea*	[78, 79]

Bryozoa	*Phidolopora pacifica, Diaperoecia californica, Heteropora alaskensis, Tricellaria ternata,* and *Hippodiplosia insculpta*	Marine	Adult	Unknown	Unknown	Secondary metabolite production	phidolopins and other nitrophenols	Other	Fouling organisms	Occurrence in taxonomically diverse sympatric bryozoans; *in vitro* activity of compounds	[80]
Tunicata	*Atapozoa* sp. (=*Sigillina signifera*)	Marine	Adult	*Pseudoalteromonas tunicata*	Extracellular endosymbiont	Secondary metabolite production	Tambjamines	Other	Fish predators	Tambjamines isolated from *Atapozoa* sp.; deterrence against fish predators; identification of tambjamine biosynthesis gene cluster in *Pseudoalteromonas tunicata*; isolation of *P. tunicata* from some tunicates	[67, 70-72, 81, 82]
Tunicata	*Trididemnum solidum, Aplidium albicans*	Marine	Larvae	Cyanobacteria: *Synechocystis trididemni*	Ectosymbiont	Secondary metabolite production	Didemnins	NRPS/PKS	Fish predators	Didemnin B found in two phylogenetically distant ascidians; plasmid-localized didemnin gene cluster in a marine Alpha-Proteobacteria; deterrent activity against predatory fish	[83-90]
Tunicata	*Lissoclinum patella*	Marine	Adult	Cyanobacteria: *Prochloron didemni*	Ectosymbiont	Secondary metabolite production	Cyanobactins (Patellamides, Trunkamide, Patellins, and others)	Ribosomal	Unknown	Co-occurrence of cyanobactins and *Prochloron* symbionts; cyanobactin gene clusters identified in symbiotic and free-living cyanobacteria; *in vitro* cytotoxicity	[87, 91-97]
Tunicata	*Lissoclinum patella*	Marine	Adult	Alpha-Prot.: *'Candidatus* Endolissoclinum faulkneri	Intracellular endosymbiont	Secondary metabolite production	Patellazoles	*Trans*-AT PKS	Unknown	Inconsistent occurrence across *L. patella* individuals; metagenomic and genomic analyses and identification of the patellazole gene cluster in the otherwise eroded symbiont genome; *in vitro* cytotoxicity	[98-102]
Tunicata	*Lissoclinum bistratum*	Marine	Adult	Cyanobacteria: *Prochloron didemni*	Ectosymbiont	Secondary metabolite production	Bistramides	PKS	Unknown	Enrichment of bistramides in *Prochloron* cell fraction; *in vitro* cytotoxicity	[103-106]

Tunicata	*Synoicum adareanum*	Marine	Adult	Unknown bacterium	Unknown	Secondary metabolite production	Palmerolides	*Trans*-AT PKS/NRPS	Unknown	Putative bacterial palmerolide PKS fragments sequenced; *in vitro* cytotoxicity	[107]
Tunicata	*Ecteinascidia turbinata*	Marine	Adult	'*Candidatus* Endoecteinascidia frumentensis'	Intracellular endosymbiont	Secondary metabolite production	Ecteinascidin-743	NRPS	Unknown	Bacterial NRPS cluster likely responsible for ET-743 production assigned to the symbiont genome by analysis of codon usage; *in vitro* cytotoxicity of ET-743	[108-110]
Tunicata	*Eudistoma toealensis*	Marine	Adult	Possibly actinobacterial associates	Unknown	Secondary metabolite production	Staurosporines	Other	Unknown	Production of staurosporines by some terrestrial and marine Actinobacteria (*Streptomyces* spp.); presence of Actinobacteria (*Salinispora* and *Verrucosispora*) in ascidian tissue	[111]
Acoelomorpha: Convolutidae	*Amphiscolops spp.*	Marine	Unknown	Dinoflagellates: *Amphidinium* spp.	Unknown	Secondary metabolite production	Amphidinolides A-H	PKS	Predators?	*In vitro* production of cytotoxic amphidinolides by isolated dinoflagellates	reviewed in [112]
Acoelomorpha: Convolutidae	*Amphiscolops spp.*	Marine	Unknown	Dinoflagellates: *Symbiodinium* sp.	Unknown	Secondary metabolite production	Zooxanthellatoxins A and B	PKS?	Unknown	*In vitro* production of vasoconstrictive zooxanthellatoxins & cytotoxic symbiodinolide by isolated dinoflagellates	reviewed in [112]
Nemertea	*Cephalothrix simula*	Marine	Adult	*Bacillus sp.*	Extracellular(?) endosymbiont	Secondary metabolite production	Tetrodotoxin	Unknown	Predators	In vitro cytotoxic activity of extracted compound	[113-117]
Mollusca, Gastropoda	*Crysomallon squamiferrum*	Marine	Adult	δ- and ε-proteobacteria	Ectosymbiont	Mineral armor	Pyrite and greigite	Other	Predators	Hardening of scales	[118-120]
Mollusca, Gastropoda	*Conus pulicarius* & *Conus sp.*	Marine	Adult	*Streptomyces sp*	Intracellular endosymbiont	Secondary metabolite production	Aerugine, Pulicatins, Watasemycin	Other	Unknown	*In vitro* compound production by bacterial isolate *In vitro* antimicrobial *In vitro* anti-inflamatory *In vitro* anti-hypotensive	[121, 122]
Mollusca, Gastropoda	*Conus tribblei* & *Chicoreus nobilis*	Marine	Adult	*Streptomyces sp.*	Unknown	Secondary metabolite production	Nobilamides	(NRPS?)	Unknown	*In vitro* compound production by bacterial isolate *In vitro* anti-inflamatory	[123]

Mollusca, Gastropoda	*Conus circumcisus* & *Conus sp*	Marine	Adult	*Gordonia sp*	Unknown	Secondary metabolite production	Circumcins	Unknown	Unknown	*In vitro c*ompound production by bacterial isolate *In vitro* neuroactive	[124]
Mollusca, Gastropoda	*Conus tribblei* & *Conus rolani*	Marine	Adult	*Nocardiopsis alba*	Unknown	Secondary metabolite production	Nocapyrones	PKS	Unknown	*In vitro c*ompound production by bacterial isolate Structurally related compounds are shown to be defensive	[125, 126]
Mollusca, Gastropoda	Teredinidae (“shipworms”)	Marine	Adult	Teredinibacter turnerae	Ectosymbiont	Secondary metabolite production	Tartrolons	*Trans* AT PKS	Bacteria, Boron	*In vitro* antimicrobial activity of isolated compounds, (controlling boron concentration, which is cytotoxic in high levels?)	[127-132]
Mollusca, Gastropoda	*Lienardia totopotens*	Marine	Adult	*Streptomyces sp.*	Unknown	Secondary metabolite production	Lobophorins	PKS	Bacteria, Predators(?)	*In vitro c*ompound production by bacterial isolate *In vitro* antibacterial *In vitro* cytotoxic	[133]
Mollusca, Cephalopoda	*Hapalochlaena* sp.	Marine	Adult	Variable bacteria	Variable locations	Secondary metabolite production	Tetrodotoxin	Unknown	Predators	Extraction and structure elucidation of cytotoxic tetrodotoxin derivatives, *In vitro* production of tetrodotoxin by variable isolates	[116, 134, 135]
Mollusca, Cephalopoda	*Euprymna scolopes*	Marine	Adult	*Vibrio fisherii*	Ectosymbiont	Light emission	Luziferase	Other	Predators and prey	Camouflage through counterillumination	[136-139]
Mollusca, Cephalopoda	*Loligo* sp., *Sepia* sp., *Euprymna scolopes*	Marine	Egg	α- and γ -proteobacteria, *Bacteroidetes* bacteria (esp. *Roseobacter* in *Loligo* and *Sepia*, *Phaeobacter* in *Euprymna*)	Unknown endosymbiont	Secondary metabolite production	Unknown	Unknown	Unknown	*In vitro a*ntimicrobial activity of extracts from symbiont populated glands and eggs	[140-151]
Crustacea	*Palaemon macrodactylus*	Marine	Egg	*Alteromonas* sp.	Ectosymbiont	Secondary metabolite production	2,3-indolinedione (isatin)	Other	Fungal pathogen: *Lagenidium callinectes*	Aposymbiotic eggs are susceptible but isolated compound restores protective effect upon application to aposymbiotic eggs	[152]
Crustacea	*Homarus americanus*	Marine	Egg	Rod-shaped, Gram-negative bacteria	Ectosymbiont	Secondary metabolite production	4-hydroxyphenethyl alcohol (tyrosol)	Other	Fungal pathogen: *Lagenidium callinectes*	*In vitro* inhibition of *L. callinectes* growth by extracted compound	[153]
Crustacea	Isopoda: *Santia spp.*	Marine	Adult	Cyanobacteria: *Synechococcus, Prochlorothrix* and *Synechocystis*	Ectosymbiont	Secondary metabolite production	Unknown	Unknown	Fish predators	Aposymbiotic isopods not rejected, extract from symbiotic animals restores protection	[154]

Nematoda	various *Heterorhabditis* spp. and *Steinernema* spp. (Rhabditida)	Terrestrial , within Insect	All life stages	Various *Photorhabdus luminescens* and *Xenorhabdus nematophila*	Ectosymbiont	Secondary metabolite production	Rhabduscin	Other	Potential activity against fungi	Defensive compound elucidated	155
Nematoda	various *Heterorhabditis* spp. (Rhabditida: Heterorhapditidae)	Terrestrial , within Insect	All life stages	Various *Photorhabdus* spp.	Ectosymbiont	Secondary metabolite production	Carbapenem (beta-lactam antibiotic)	Other	Activity against distantly related bacteria, yeasts, fungi	Defensive compound elucidated, *in vitro* defensive activity	156-158
Nematoda	various *Heterorhabditis* spp. (Rhabditida: Heterorhapditidae)	Terrestrial , within Insect	All life stages	*Photorhabdus luminescens*	Ectosymbiont	Secondary metabolite production	Carbapenem-like molecule	Other	Broad-spectrum activity against Gram-negative bacteria	Defensive compound elucidated, *in vitro* defensive activity	157
Nematoda	various *Heterorhabditis* spp. (Rhabditida: Heterorhapditidae)	Terrestrial , within Insect	All life stages	*Photorhabdus luminescens*	Ectosymbiont	Secondary metabolite production	Genistine	Other	Unknown	Defensive compound elucidated	159
Nematoda	various *Heterorhabditis* spp. (Rhabditida: Heterorhapditidae)	Terrestrial , within Insect	All life stages	*Photorhabdus luminescens* and others	Ectosymbiont	Secondary metabolite production	Isopropylstilbenes (e.g. 3,5-dihydroxy-4-isopropylstilbene)	Other	Activity against Gram-positive and Gram-negative bacteria, fungi, nematodes	Defensive compounds elucidated, *in vitro* defensive activity	160-166
Nematoda	various *Heterorhabditis* spp. (Rhabditida: Heterorhapditidae)	Terrestrial , within Insect	All life stages	*Photorhabdus luminescens*	Ectosymbiont	Secondary metabolite production	Anthraquinones (red pigments color cadaver) (e.g. 1,3,8-trihydroxy-9,10-anthraquinone)	PKS	Activity against microorganisms assumed,[167] tested only against *E. coli*	Defensive compounds elucidated	159, 161, 162, 168
Nematoda	*Heterorhabditis bacteriophora* (Rhabditida: Heterorhapditidae)	Terrestrial , within Insect	All life stages	*Photorhabdus luminescens*	Ectosymbiont	Secondary metabolite production	Photobactin	Other	Antibacterial	Defensive compounds elucidated, *in vitro* defensive activity	158
Nematoda	*Heterorhabditis* sp. (Rhabditida: Heterorhapditidae)	Terrestrial , within Insect	All life stages	*Photorhabdus aeruginosa*	Ectosymbiont	Secondary metabolite production	Lumicins	Unknown	Broad-spectrum bacteriocins to outcompete other *Photorhabdus* spp. and strains as well as other bacteria	Defensive compounds elucidated, *in vitro* defensive activity	169, 170
Nematoda	various *Heterorhabditis* spp. (Rhabditida: Heterorhapditidae)	Terrestrial , within Insect	All life stages	Various *Photorhabdus* strains	Ectosymbiont	Secondary metabolite production	Photorhabdicins (bacteriophage-related R- and F-type pyocins)	Unknown	Broad-spectrum bacteriocins to outcompete other *Photorhabdus* spp. and strains as well as other bacteria	Defensive compounds elucidated, *in vitro* defensive activity	169, 171
Nematoda	various *Heterorhabditis* spp. (Rhabditida: Heterorhapditidae)	Terrestrial , within Insect	All life stages	*Photorhabdus luminescens*	Ectosymbiont	Secondary metabolite production	Small, extracellular, possibly nonproteinacious compound(s)	Unknown	Ants	Defensive compounds elucidated, *in vivo* fitness benefits	172-175
Nematoda	various *Steinernema* spp. (Rhabditida : Steinernematidae)	Terrestrial , within Insect	All life stages	*Xenorhabdus budapestensis* and *X. szentirmaii*	Ectosymbiont	Secondary metabolite production	Fabclavines (peptide-polyketide-polyamino hybrids)	PKS/NRPS	Broad-spectrum activity against bacteria, yeasts and protozoa	Defensive compounds elucidated, *in vitro* defensive activity	176

Nematoda	various *Steinernema* spp. (Rhabditida : Steinernematidae)	Terrestrial , within Insect	All life stages	*Xenorhabdus bovienii* and *X. nematophilus*	Ectosymbiont	Secondary metabolite production	Xenorhabdins (dithiolopyrrlones; member of pyrothine family)	Unknown	Activity against Gram-positive bacteria, little effect against Gram-negative bacteria, some with activity against fungi	Defensive compounds elucidated, *in vitro* defensive activity	156, 162, 177
Nematoda	various *Steinernema* spp. (Rhabditida : Steinernematidae)	Terrestrial , within Insect	All life stages	Various *Xenorhabdus* spp.	Ectosymbiont	Secondary metabolite production	Xenorxides (oxidized products of Xenorhabdins)	Unknown	Broad-spectrum activity against Gram-positive bacteria, yeast, fungi	Defensive compounds elucidated, *in vitro* defensive activity	178
Nematoda	*Steinernema* sp. (Rhabditida : Steinernematidae)	Terrestrial , within Insect	All life stages	*Xenorhabdus* sp. PB30.3	Ectosymbiont	Secondary metabolite production	Xenobactin (hexadepsipeptide)	(NRPS?)	Activity against protozoa and Gram-positive bacteria, not effective against Gram-negative bacteria	Defensive compounds elucidated, *in vitro* defensive activity	179
Nematoda	*Steinernema carpocapsae* (Rhabditida : Steinernematidae)	Terrestrial , within Insect	All life stages	*Xenorhabdus nematophilus* and others	Ectosymbiont	Secondary metabolite production	Xenocoumacins (peptide-polyketide origin ; benzopyran-1-one derivatives)	NRPS-PKS	Activity against Gram-positive bacteria	Defensive compounds elucidated, *in vitro* defensive activity	180
Nematoda	*Steinernema carpocapsae* (Rhabditida : Steinernematidae)	Terrestrial , within Insect	All life stages	*Xenorhabdus nematophilus*	Ectosymbiont	Secondary metabolite production	Xenematide (cyclodepsipeptide)	NRPS	Antibacterial activity	Defensive compounds elucidated, *in vitro* defensive activity	181, 182
Nematoda	*Steinernema carpocapsae* (Rhabditida : Steinernematidae)	Terrestrial , within Insect	All life stages	*Xenorhabdus nematophilus*	Ectosymbiont	Secondary metabolite production	Benzylideneacetone (monoterpenoid)	Terpenoid	Specific activity against Gram-negative bacteria	Defensive compounds elucidated, *in vitro* defensive activity	183
Nematoda	*Steinernema carpocapsae* (Rhabditida : Steinernematidae)	Terrestrial , within Insect	All life stages	*Xenorhabdus nematophilus*	Ectosymbiont	Secondary metabolite production	Nematophins (indole derivatives)	Other	Activity against Gram-positive and Gram-negative bacteria as well as a few fungi	Defensive compounds elucidated, *in vitro* defensive activity	160, 184
Nematoda	*Steinernema carpocapsae* (Rhabditida : Steinernematidae)	Terrestrial , within Insect	All life stages	*Xenorhabdus nematophilus*	Ectosymbiont	Secondary metabolite production	Xenorhabdicins (phage related R-type pyocin)	Unknown	Narrow-spectrum bacteriocins to outcompete *Xenorhabdus* spp., *P. luminescens* and sister taxon *Proteus*	Defensive compounds elucidated, *in vitro* defensive activity, in vivo fitness benefits	169, 185-187
Nematoda	*Steinernema carpocapsae* (Rhabditida : Steinernematidae)	Terrestrial , within Insect	All life stages	*Xenorhabdus nematophilus*	Ectosymbiont	Secondary metabolite production	ant-deterent factor (ADF)	Unknown	Deter ants	Defensive compounds elucidated, *in vivo* fitness benefits	175
Insecta, Blattodea,	Fungus-farming Macrotermitinae	Terrestrial	Fungal cultivars	*Termitomyces* sp.	Ectosymbiont	Secondary metabolite production	Unknown mycocins	Unknown	Related cultivar species and strains	*In vitro* defensive activity	188

Insecta, Blattodea	Fungus-farming termites (Termitidae)	Terrestrial	Fungal cultivars	Various Actinobacteria	Ectosymbiont	Secondary metabolite production	Unknown	Unknown	Competing fungi (*Pseudoxylaria*, *Trichoderma*)	*In vitro* defensive activity	189
Insecta, Blattodea	Fungus-farming *Microtermes* sp.	Terrestrial	Fungal cultivars	*Streptomyces* sp.	Ectosymbiont	Secondary metabolite production	Microtermolides A and B	PKS-NRPS	Unknown	Defensive compounds elucidated	190
Insect, Orthoptera	Locust: *Schistocerca gregaria*	Terrestrial	Adult	*Pantoea agglomerans* (γ -proteobacteria)	Ectosymbiont	production of bioactive compound	Quinines	Other	Pathogenic fungi (*Metarhizium anisopliae)*	*In vivo* fitness benefits, in vitro defensive activity and elucidation of defensive compounds	191-193
Insecta, Hemiptera	Whitefly: *Bemisia tabaci*	Terrestrial	Adult	*Rickettsia* sp.	Intra- and extracellular endosymbiont	Unknown	Unknown	Unknown	Bacterial pathogen (*Pseudomonas syringae)*	*In vivo* fitness benefits in laboratory conditions	194
Insecta, Hemiptera	Aphid: *Myzus persicae* (Aphididae)	Terrestrial	Nymph and Adult	*Regiella insecticola* (γ -proteobacteria)	Intra- and extracellular endosymbiont	Unknown	Unknown	Unknown	Parasitoid wasp (*Aphidius colemani*)	*In vivo* fitness benefits	195
Insecta, Hemiptera	Aphids: *Acyrthosipon pisum, Aphis craccivora* and *Aphis fabae* (Aphididae)	Terrestrial	Nymph and Adult	*Hamiltonella defensa* (γ -proteobacteria) (APSE bacteriophage encoded genes)	Intra- and extracellular endosymbiont	production of bioactive compound, and possibly complementary mechanisms	Shiga-like toxin, cytolethal distending toxin, and YD-repeat toxins (possibly others as well)	(Ribosomal peptide?) (Ribosomal peptide?) (Ribosomal peptide?)	Parasitoid wasps (*Aphidius ervi* , *Aphidius eadyi, Lysiphlebus fabarum)*	*In vivo* fitness benefits	196-202
Insecta; Hemiptera	Pea aphid: *Acyrthosipon pisum* (Aphididae)	Terrestrial	Adult	*Regiella insecticola, Rickettsiella* sp. (γ - proteobacteria) *Rickettsia* sp. (α-proteobacteria)	Intra- and extracellular endosymbiont	Unknown	Unknown	Unknown	Fungal pathogen (*Pandora neoaphidis*)	*In vivo* fitness benefits	203-205
Insecta, Hemiptera	Asian citrus psyllid: *Diaphorina citri* (Psylidae)	Terrestrial	Adult	*Candidatus* Profftella armatura (β-proteobacteria)	Intracellular endosymbiont	production of bioactive compound	Diaphorin (polyketide)	*trans*-AT PKS	Unknown	Defensive compound elucidated and present in 100% of individuals at potentially cytotoxic concentrations	206
Insecta, Hemiptera	Red gum lerp psyllid: *Glycaspis brimblecombei* (Psylidae)	Terrestrial	Adult	*Arsenophonus* sp.	Intracellular endosymbiont	Unknown (proposed: phage-mediated)	Unknown	-	Parasitoids	Positive correlation between presence of symbiont and natural enemy in the field	207
Insecta, Coleoptera	Some *Euops* Leaf-rolling weevils (Attelabidae)	Terrestrial	Larva, Pupa	*Penicillium herquei*	Ectosymbiont	Secondary metabolite production	Scleroderolide	PKS	Fungal and bacterial pathogens	Defensive compounds elucidated, *in vitro* defensive activity, *in vivo* fitness benefits	208-210
Insecta, Coleoptera	*Dendroctonus brevicomis* (Curculionidae)	Terrestrial	All life stages	*Ogataea pini* (Saccharomycetales: Saccharomycetaceae)	Ectosymbiont	Volatile organic compound production	Ethanol, carbon disulfide (CS2), delta-3-carene	Other Other Terpene	*Beauvaria bassiana* insect pathogen	Defensive compounds elucidated, *in vitro* defensive activity	211

Insecta, Coleoptera	*Dendroctonus frontalis* (Curculionidae)	Terrestrial	Fungal cultivars	*Streptomyces thermosacchari* (Actinobacteria)	Ectosymbiont	Secondary metabolite production	Mycangimycin (polyene)	Other (Polyene)	Competing fungi	Defensive compounds elucidated, *in vitro* defensive activity	212, 213
Insecta, Coleoptera	*Dendroctonus frontalis* (Curculionidae)	Terrestrial	Fungal cultivars	*Streptomyces* sp.	Ectosymbiont	Secondary metabolite production	Frontalamides A and B	PKS-NRPS	Unknown	Defensive compounds elucidated	214
Insecta, Coleoptera	Fungus-farming *Euwallacea validus* (Curculionidae)	Terrestrial	Fungal cultivars	Unknown cultivar fungus	Ectosymbiont	Secondary metabolite production	Cerulenin, Helvolic acid	Other Terpenoid	Fungal pathogens of cultivars	Defensive compounds elucidated, *in vitro* defensive activity	215
Insecta, Coleoptera	Alfalfa weevil: *Hypera postica* (Curculionidae)	Terrestrial	Not specified	*Wolbachia* (α-proteobacteria) (and/or others)	Intracellularendosymbiont	Unknown	Unknown	Unknown	Parasitoid wasp (*Microctonus aethiopoides*)	*In vivo* fitness benefits (specificity of association not confirmed)	200, 216
Insecta, Coleoptera	Rove beetles: *Paederus* spp. (Staphylinidae)	Terrestrial	Larvae (potentially others)	*Pseudomonas* sp. (γ -proteobacteria)	Extracellular endosymbiont	Production of bioactive compound	Pederin (polyketide)	*trans*-AT PKS	Wolf spiders (Araneae: Lycosidae, Salticidae)	*In vivo* fitness benefits (decreased predation)	217-224
Insecta, Hymenoptera	*Microplitis demolitor* and other parasitoid wasps (Braconidae, Ichneumonidae)	Terrestrial	Egg and larva	*Brachovirus* and *Ichnovirus* (Polydnaviruses)	Endosymbiont	Immunosuppression in parasitized host	Apoptosis induction, haemocyte clumping, phenoloxidase inhibition (various genes)	-	Parasitized host immune system (usually Lepidoptera larvae)	*In vivo* fitness benefits, characterization of genes responsible for protective function	225-227
Insecta, Hymenoptera	Various fungus-farming Attine species (Formicidae)	Terrestrial	Fungal cultivars	*Pseudonocardia* spp. (Actinobacteria)	Ectosymbiont	Secondary metabolite production	Dentigerumycin, five angucyclines and a nystatin-like compound	(NRPS-PKS?) (depsipeptide)	Specific activity against pathogen of fungal cultivars(*Escovopsis* sp.)	Defensive compounds elucidated, in vitro defensive activity, in vivo fitness benefits	228-236
Insecta, Hymenoptera	Various fungus-farming Attine species (Formicidae)	Terrestrial	Fungal cultivars	*Streptomyces* spp., *Amycolatopsis* spp. (Actinobacteria)	Ectosymbiont	Secondary metabolite production	Streptomyces: Candicidin	PKS	Competing fungi	Defensive compounds elucidated, *in vitro* defensive activity	235, 237-240
Insecta, Hymenoptera	*Allomerus* ants (Formicidae)	Terrestrial	Fungal cultivars	*Streptomyces* spp. *Amycolatopsis* spp. (Actinobacteria)	Ectosymbiont	Secondary metabolite production	Unknown	Unknown	Competing fungi	defensive compounds elucidated, *in vitro* defensive activity	241
Insecta, Hymenoptera, Formicidae	Fungus-farming *Atta sexdens, Atta texana* (Formicidae)	Terrestrial	Fungal cultivars and all life stages	Various killer yeasts (Basidiomycota, Ascomycota)	Ectosymbiont	Secondary metabolite production	Unknown mycocins	Unknown	Fungal pathogens of cultivars and insects	*In vitro* defensive activity	242, 243
Insecta, Hymenoptera	Fungus-farming *Cyphomyrmex costatus* (Formicidae)	Terrestrial	Fungal cultivars	*Lepiota* sp.	Ectosymbiont	Secondary metabolite production	Lepiochlorin	Other	Bacteria and fungi?	Defensive compounds elucidated	244
Insecta, Hymenoptera	Fungus-farming *Cyphomyrmex minutus* (Formicidae)	Terrestrial	Fungal cultivars and all life stages	*Tyridiomyces formicarum*	Ectosymbiont	Secondary metabolite production	Several diketopiperazines	Other (peptide)	Fungal pathogens of cultivars and insects	Defensive compounds elucidated	245
Insecta, Hymenoptera	Fungus-farming *Atta colombica* (Formicidae)	Terrestrial	Fungal cultivars	*Leucocoprinus gongylophorus* (Lepiotaceae,	Ectosymbiont	Secondary metabolite production	Unknown	Unknown	Endophytic fungi within plant	*In vitro* defensive activity	246

				Basidiomycota)					substrate of fungal cultivars		
Insecta, Hymenoptera	*Philanthus* spp., *Trachypus* spp., *Philantinus* spp. (Crabronidae)	Terrestrial	Larva, Pupa	*Streptomyces philanthi* (Actinobacteria)	Ectosymbiont	Secondary metabolite production	Cocktail containing streptochlorin and eight piericidin derivatives	PKS PKS	Fungal and bacterial pathogens	Defensive compounds elucidated, in vitro defensive activity, in vivo fitness benefits	247-251
Insecta, Hymenoptera	Honey bees: *Apis* spp. (Apidae)	Terrestrial	Larvae and Adult	*Lactobacillus* spp., *Bifidobacterium* (α - proteobacteria)	Ectosymbiont	Production of bioactive compounds	Organic acids (lactic, formic, and acetic acids), hydrogen peroxide and various volatiles (benzene, toluene, octane, ethylbenzene and nonane), 3-OH fatty acids, 2-heptanone and various peptides	Other Lipid Other Peptides	Bacterial and fungal pathogens (*Melissococcus plutonius, Paenibacillus larvae Pseudomonas*, Enterobacterioce a, *Bacillus* and *Candida*)	*In vitro* defensive activity and In vivo fitness effects	252-259
Insecta, Hymenoptera	Honey bees: *Apis* spp. (Apidae)	Terrestrial	Larvae and Adult	*Bacillus* spp. (Firmicutes)	Ectosymbiont	Unknown (Proposed: production of bioactive compounds)	Unknown	-	Pathogenic fungi Chalkbrood causative agent (*Ascosphaera apis*)	*In vitro* defensive activity	253, 260, 261
Insecta, Hymenoptera	Honey bees: *Apis* spp. (Apidae)	Terrestrial	Adult	Mucorales, Aspergilli, Penicilli	Ectosymbiont	Unknown (Proposed: production of bioactive compounds)	Unknown	-	Pathogenic fungi (*Ascosphaera apis*)	*In vitro* defensive activity	252
Insecta, Hymenoptera	Bumble bees: *Bombus* spp. (Apidae)	Terrestrial	Adult	*Gilliamella apicola*	Ectosymbiont	Unknown (Proposed: colonization resistance)	Unknown	-	Gut trypanosomatid parasite (*Crithidia bombi*)	Negative correlation between presence of symbiont and parasite in wild populations	262-268
Insecta, Diptera	Mosquitoes: *Aedes* spp. (Culicidae)	Terrestrial	Adult	*Wolbachia pipientis* (α-proteobacteria)	Intracellular endosymbiont	Stimulation of immune system	Induces Toll pathway and antimicrobial peptide production in host	-	Pathogenic viruses	*In vivo* reduction of virus titers and reduced transmission capacities	269, 270
Insecta, Diptera	Mosquitoe: *Culex quinquefasciatus* (Culicidae)	Terrestrial	Adult	*Wolbachia pipientis* (α-proteobacteria)	Intracellular endosymbiont	Unknown	Unknown	-	Pathogenic viruses	*In vivo* reduction of virus titers and reduced transmission capacities	271
Insecta, Diptera	Fruit flies: *Drosophila* spp. Drosophilidae)	Terrestrial	Adult	*Wolbachia pipientis* (α-proteobacteria)	Intracellular endosymbiont	Unknown	Unknown	-	Pathogenic viruses	*In vivo* fitness benefits	270-274
Insecta, Diptera	Fruit fly: *Drosophila hydei* (Drosophilidae)	Terrestrial	Larva and Adult	*Spiroplasma* sp. (Mollicutes)	Intra- and extracellular endosymbiont	Unknown	Unknown	-	Parasitoid wasp (*Leptopilina heterotoma*)	*In vivo* fitness benefits	275
Insecta, Diptera	*Drosophila melanogaster* (Drosophilidae)	Terrestrial	Food substrate	Various yeasts	Ectosymbiont	Secondary metabolite production	Unknown	Unknown	Aspergillus nidulans food competitor	*In vivo* fitness benefits	276
Insecta, Diptera	Fruit fly: *Drosophila neotestacea* (Drosophilidae)	Terrestrial	Adult	*Spiroplasma* sp. (Mollicutes)	Intra- and extracellular endosymbiont	Unknown	Unknown ribosomal-inactivating protein	-	Nematode (*Howardula aoronymphium*)	*In vivo* fitness benefits	274, 277

Insecta, Diptera	House fly: *Musca domestica* (Muscidae)	Terrestrial	Larva	*Klebsiella oxytoca* (γ -proteobacteria), *Bacillus cereus* (Firmicutes)	Ectosymbiont	Unknown (proposed: colonization resistance or production of bioactive compounds)	Unknown	-	Fungi	*In vivo* fitness benefits	[278]
Chordata, Tetraodontiformes	Atlantic salmon *Salmo salar*, gilthead seabream *Sparus aurata*, flathead mullet *Mugil cephalus* and other fish species	Limnic/Marine	Adult (gut)	*Carnobacterium, Lactobacillus* and other Lactic acid bacteria (Firmicutes)	Ectosymbiont	Colonization resistance, stimulation of immune system, production of bioactive compounds	Bacteriocins (Carnocin UI49, Piscicocin V1, Divercin V41) and organic acids (lactic and acetic acid)	Ribosomal peptides , Other	Bacterial pathogens	*In vitro* defensive activity, *in vivo* fitness benefits, defensive compounds elucidated	[279-282]
Chordata, Tetraodontiformes	Puffer fish: *Spheroides rubripes* (Tetraodontidae) and several other marine organisms	Marine	Stage not specified	*Vibrio, Pseudomonas*, Actinomycetes and others (Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes)	Unknown	Secondary metabolite production	Tetrodotoxin	Other	Predators	Defensive compound elucidated	[113, 115, 117]
Chordata,	Newts and Salamanders: *Taricha* spp., *Cynops* spp. and others (Caudata, Ambystomatidae and Salamandridae) Frogs: *Atelopus* spp. and other anurans (Anura: Brachycephalidae, Dendrobatidae, Bufonidae and Rhacophoridae)	Limnic/Terrestrial	Adult, eggs and larvae (some species)	Unknown (bacterial origin uncertain)	Unknown	Secondary metabolite production	Tetrodotoxin	Other	Snake predators	Defensive compound elucidated, active secretion upon predator encounter, toxicity to predators, coevolutionary arms race with snake predators	[113, 283-288]
Chordata	Red back salamander: *Plethodon cinereus* (Caudata: Salamandridae)	Limnic/Terrestrial	Adult (skin and gut)	*Janthinobacterium lividum* (β-proteobacteria) *Lysobacter gummosus* (γ -proteobacteria)	Ectosymbiont	Secondary metabolite production	Indole-3-carboxaldehyde and violacein 2,4-diacetylphloroglucinol	Other	Fungal pathogen (*Batrachochytrium dendrobatidis*)	*In vitro* defensive activity , defensive compounds elucidated, co-occurrence of defensive compounds and symbiotic bacteria on host	[289-293]
Chordata	Mountain yellow-legged frog: *Rana muscosa*	Limnic/Terrestrial	Adult (skin)	*Janthinobacterium lividum* (β-proteobacteria)	Ectosymbiont	Secondary metabolite production	Violacein	Other	Fungal pathogen (*Batrachochytrium dendrobatidis*)	*In vivo* fitness benefits, defensive compound elucidated	[294]

	(Anura: Ranidae)										
Chordata	Mountain yellow-legged frog: *Rana muscosa* (Anura: Ranidae)	Limnic/Terrestrial	Adult (skin)	*Pseudomonas fluorescens* (γ -proteobacteria)	Ectosymbiont	Synergistic effect of compound with host immune system	2,4-diacetylphloroglucinol	Other	Fungal pathogen Batrachochytrium dendrobatidis)	*In vitro* defensive activity , defensive compounds elucidated	295
Chordata	Hoopoe birds: *Upupa epops* (Coraciformes: Upupidae)	Terrestrial	Adult (feathers), and eggs	*Enterococcus faecalis* (Firmicutes)	Ectosymbiont	Secondary metabolite production	Bacteriocins (enterocin MR10 and AS-48) and mixture of volatiles (main: Butanoic acid, 2-methyl butanoic acid, 4-methyl pentanoic acid, indole, 3-phenyl propanoic acid and 4-chloro indole)	Ribosomal peptides , Other	Bacterial pathogens (*Bacillus licheniformis*, *Staphylococcus* and several Enterobacteriaceae)	*In vivo* fitness benefits, *in vitro* defensive activity , defensive compounds elucidated	296-301
Chordata	Human: *Homo sapiens* (Primates: Hominidae)	Terrestrial	Stage not specified (skin and nasal cavity)	*Staphylococcus epidermis* (Firmicutes)	Ectosymbiont	Secondary metabolite production, stimulation of immune system, colonization resistance	Epidermin, Pep5, epilancin K7 , modulin PSMγ and PSMδ, (thiolactone-containing pheromone AMPs), ESP serine protease	Ribosomal peptides	Pathogenic microbes (*Staphylococcus aureus*)	*In vitro* defensive activity, defensive compounds elucidated, rate of pathogen colonization decreased in presence of symbiont.	302-306
Chordata	Human: *Homo sapiens* (Primates: Hominidae)	Terrestrial	Stage not specified (intestine)	*Bacteroides fragilis* (Bacteroidetes)	Ectosymbiont	Modulation of immune system suppresses inflammatory response)	Polysaccharide A (PSA)	Other	Opportunistic bacterial pathogen (*Helicobacter hepaticus*)	*In vivo* fitness benefits, defensive compounds elucidated	307
Chordata	Laboratory mice *Mus musculus* (strain C57BL/6J) (Rodentia: Muridae)	Terrestrial	Not specified	Herpesvirus	Endosymbiont	Stimulation of immune system	Effect in immune system: prolonged production of the antiviral cytokine interferon-c and systemic activation of macrophages	-	Bacterial pathogens (*Listeria monocytogenes* and *Yersinia pestis*)	*In vivo* fitness benefits	308

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"Think left and think right and think low and think high.
Oh, the things you can think up if only you try!"

Dr. Seuss

Originality statement / Eigenständigkeitserlärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Jena, den 11. November, 2016

Laura Victoria Flórez

CURRICULUM VITAE

L A U R A   V I C T O R I A   F L Ó R E Z   P A T I Ñ O

EDUCATION

2011 – 2016 Ph.D. within the IMPRS program in the Max Planck Institute for Chemical Ecology – Friedrich-Schiller-Universität. Jena, Germany. Project title: "*Burkholderia* as defensive bacterial symbionts of Lagriinae beetles"

2004 – 2011 B.Sc. Biology. Universidad de Los Andes. Bogotá, Colombia

2004 – 2011 B.Sc. Chemistry. Universidad de Los Andes. Bogotá, Colombia
Minor degree in German Language and Culture. Universidad de Los Andes. Bogotá, Colombia

2010 Undergraduate Thesis. Max Planck Institute for Polymer Research. Mainz, Germany. Project Title: "Characterization of ellipsoidal polystyrene nanoparticles and their uptake in human mesenchymal stem cells".

2009 – 2010 Academic Exchange year at Johannes-Gutenberg-Universität Mainz, Germany

1999 – 2004 Bilingual High School Degree with emphasis in Science and Technology. Colegio La Quinta del Puente. Bucaramanga, Colombia

COMPLEMENTARY EDUCATION

2015 Workshop on Data Visualisation: Principles and Applications. Science Craft, Jena, Germany.

2015 Workshop on Adobe Illustrator. Max Planck Institute for Chemical Ecology, Jena, Germany.

2014 Collection of Volatiles Course. Max Planck Institute for Chemical Ecology, Jena, Germany.

2013 Course: Introduction into the statistical program R. Max Planck Institute for Chemical Ecology, Jena, Germany

2013 Compact Course: Higher Education in natural sciences. Friedrich Schiller University, Lehrelernen Servicestelle, Jena, Germany

2012 Workshop on Microbial Evolution. University of Basel, Institute of Zoology and Evolutionary Biology, Basel, Switzerland

2012 Genomics Workshop. Institute for Genome Sciences, University of Maryland - School of Medicine. Baltimore, USA

2012 Introducing R as a flexible tool for statistical analyses. Friedrich Schiller University, Jena, Germany

2011 Research Internship. Insect Symbiosis Group, Max Planck Institute for Chemical Ecology. Jena, Germany. Project: "Symbiosis between the darkling beetle *Lagria hirta* (Coleoptera: Tenebrionidae) and bacteria of the genus *Burkholderia*"

2007 Research Training Program (RTP). National Museum of Natural History, Smithsonian Institution, Washington D.C., U.S.A. Project: "DNA Barcoding of the ant genus *Pheidole* nominal species and morphospecies from Guyana leaf-litter biodiversity surveys"

AWARDS AND ACADEMIC ACHIEVEMENTS

2015 ESEB Outreach Initiative. Project: "Snapshots of adaptation: what nature's pictures tell us about evolution". European Society for Evolutionary Biology.

2015 Best poster award at the Gordon Research Conference - Animal-Microbe Symbioses, Waterville Valley, USA.

2014 2nd place in oral presentation category, III Congress of the Latin American Association of Chemical Ecology (ALAEQ). Bogotá, Colombia.

2014 Best talk at the 4th International Student Conference on Microbial Communication. Jena, Germany

2014 IMPRS travel award for the best talk at the 13th IMPRS Symposium. Dornburg, Germany.

2013 Best poster at the DZG Meeting Evolutionary Biology. Göttingen, Germany.

2011 Graduate Research Fellowship, International Max Planck Research School. Jena, Germany.

2010 National third place in the ECAES test (Exam for the Quality of Superior Education in Colombia) for the Biology program.

2004 National first place in ICFES test (SAT equivalent, applied to every Colombian high school student and requirement for superior studies in any discipline).

2004 Academic merit scholarships:

"Alberto Magno" (granted by Los Andes University, Bogotá, Colombia)
"Mario Galán Gómez" (granted by ECOPETROL, Colombian Petroleum Company)
Excellence Scholarship (granted by Banco de la República, Bogotá, Colombia)

PUBLICATIONS

2016 Klein, A., Schrader, L., Gil, R., Manzano-Marín, A., **Flórez, L. V**., Wheeler, D., Werren, J. H., Latorre, A., Heinze, J., Kaltenpoth, M., Moya, A., Oettler, J. A novel intracellular mutualistic bacterium in the invasive ant *Cardiocondyla obscurior*. *The ISME Journal*, 10(2), 376-388.

2015 **Flórez, L. V**., Biedermann, P., Engl, T., Kaltenpoth, M. Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. *Natural Product Reports.*

2015 Salem, H., **Flórez, L. V**., Gerardo, N., Kaltenpoth, M. An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. *Proceedings of the Royal Society B: Biological Sciences*, 282: 20142957

2012 **Flórez, L.**, Herrmann C., Cramer JM., Hauser CP., Koynov K., Landfester K., Crespy D., Mailänder V. How shape influences uptake: interactions of anisotropic polymer nanoparticles and human mesenchymal stem cells. *Small.* 8(14): 2222-30.

CONFERENCE CONTRIBUTIONS

Oral Presentations

2014 Flórez, L. V. and Kaltenpoth, M. A symbiotic strategy for chemical egg defense in insects: the *Lagria* - *Burkholderia* case. III Congress of the Latin American Association of Chemical Ecology (ALAEQ), Bogotá, Colombia.

2014 Flórez, L. V. and Kaltenpoth, M. Outsourcing protective tasks? Bacteria-mediated defense in lagriid beetle eggs. Xth European Congress of Entomology (ECE), Royal Entomological Society, University of York, York, United Kingdom.

2014 Flórez, L. V. and Kaltenpoth, M. Bacteria-mediated egg protection? Transmission and defensive function of symbiotic bacteria in lagriid beetles. MiCom 2014 - 4th International Student Conference on Microbial Communication, Jena School for Microbial Communication, Jena, Germany.

2014 Flórez, L. V. and Kaltenpoth, M. Outsourcing protective tasks? Bacteria-mediated defense in lagriid beetle eggs. 13th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany.

Poster Presentations

2015 Flórez L. V., Gaube P., Kaltenpoth M. (2015). Egg defense in Lagriid beetles and the many talents of *Burkholderia* symbionts. Gordon Research Conference - Animal-Microbe Symbioses, Waterville Valley, USA.

2014 Flórez L. V. Kaltenpoth M. Bacteria-mediated egg protection? Transmission and function of *Burkholderia* endosymbionts in lagriid beetles. Keystone Symposia Meeting: Mechanisms and Consequences of Invertebrate-Microbe Interactions, Tahoe City, USA.

2014 Flórez, L. V., Seng S., Kaltenpoth M. The hitchhiker's guide to symbiosis: a plant pathogen as an insect mutualist. SAB Meeting 2014, MPI for Chemical Ecology, Jena, Germany.

2013 Flórez, L. V. and Kaltenpoth, M. Deciphering an enigmatic partnership: prevalence, transmission and genomics of Burkholderia symbionts in lagriid beetles. ICE Symposium, MPI for Chemical Ecology, Jena, Germany

2013 Flórez, L. and Kaltenpoth, M. Evolving as allies? Ecology and Evolution of the symbiosis between Burkholderia bacteria and tenebrionid beetles (Lagria spp.). 14th Congress of the European Society for Evolutionary Biology, Lisbon, Portugal.

2013 Flórez, L. and Kaltenpoth, M. Burkholderia bacteria as insect endosymbionts: The fully equipped bacterial partners of Lagria hirta tenebrionid beetles. 12th IMPRS Symposium, MPI for Chemical Ecology, Jena, Germany.

2013 Flórez, L. and Kaltenpoth, M. Burkholderia as insect endosymbionts: the fully equipped bacterial partners of Lagria hirta tenebrionid beetles. 18th DZG graduate meeting „Evolutionary Biology", Deutsche Zoologische Gesellschaft, Göttingen, Germany.

2012 Flórez, L. and Kaltenpoth, M. Burkholderia sp. as endosymbionts in the darkling beetle Lagria hirta: Deciphering a story of alliance. 7th International Symbiosis Society Congress, International Symbiosis Society, Kraków, Poland.

2012 Flórez, L. and Kaltenpoth, M. Deciphering the symbiosis between Burkholderia sp. (ß-proteobacteria) and Lagria hirta (Coleoptera: Lagriidae): function, life history and molecular interactions. 11th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany.

TEACHING AND WORK EXPERIENCE

Academic Tutor at Universidad de Los Andes, Bogotá – Colombia, in the areas of:

2009 Foundations of Analytical Chemistry. Chemistry Department.
2008 Molecular Biology. Department of Biological Sciences.
Global Climate. Chemistry Department.
2006 General Chemistry. Chemistry Department.

2008-2011 Private tutoring for elementary and high school students.

Laboratory Assistant in:

2007 Vertebrate Evolution Laboratory, Universidad de Los Andes, Bogotá, Colombia

MEMBERSHIP IN PROFESSIONAL SOCIETIES

2013 - European Society for Evolutionary Biology (ESEB)