Dynamics and functionality of a multipartite defensive symbiosis in immature *Lagria* beetles

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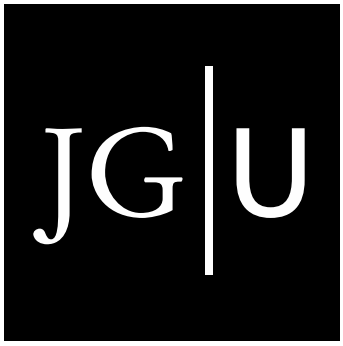

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"… those who learned to collaborate and improvise most effectively have prevailed."

Charles Darwin

SUMMARY

Almost all macroorganisms engage in partnerships with microorganisms, which facilitated not only their origin and evolution but have also great impact on their ecology. Microbial symbionts play important roles in the web of life, by taking over tasks essential for survival or development of their hosts. Of those, defense against natural enemies is a challenge for all animals on Earth, including insects. Although insects have evolved numerous traits to cope with different kinds of natural enemies on their own, their association with symbiotic microbes opened up new opportunities to adapt to challenges occurring with antagonistic encounters.

This dissertation focuses on the defensive symbiosis between darkling beetles of the genus *Lagria* (Coleoptera: Tenebrionidae) and several defensive symbionts including *Burkholderia* bacteria (Pseudomonadota: Betaproteobacteria). *Lagria* beetles accommodate their symbionts throughout development. They begin their journey in accessory glands of the adult female reproductive system, after which they are applied within a secretion onto the egg surface during oviposition and colonize three peculiar dorsal cuticular invaginations in the larvae. On the eggs, symbionts produce antimicrobial compounds to protect the egg stage against fungal infections from the environment. In larvae both sexes carry the symbionts, while male adults do not contain symbionts, indicating that the symbionts fulfill an important function also in post-hatch stages, which might be antifungal defense as well.

With this hypothesis, the aim of this thesis was to investigate the symbiosis in the developing life stages of two *Lagria* species, *L. villosa* and *L. hirta*. Therefore, I intended to (i) elucidate the abundance, composition, and localization of several symbiont strains across host development, (ii) investigate the defensive potential of several members of the symbiont community in different life stages, and (iii) examine the morphological modifications of the beetles for symbiont transmission and maintenance throughout molting and metamorphosis. Bacterial community analyses via 16S amplicon sequencing (Illumina) and qPCR of *L. villosa* and *L. hirta* revealed that the beetles are consistently associated with their symbionts throughout all life stages. Moreover, they harbor a diverse community, which predominantly consists of *Burkholderia* strains, while also symbionts of other families are consistently present in lower abundance. In *L. villosa*, one *Burkholderia* strain (LvStB) is highly abundant in the community of all life stages. Despite its reduced genome and metabolic capacities, and its putative immotility compared to other *Burkholderia* strains, it dominates and is often found as the only *Burkholderia* strain. Its ability to produce the antifungal compound lagriamide is therefore a potential key factor for its stability and leading role in the beetle. Meanwhile in *L. hirta*, the two strains LhStH and LhStG dominate the community in varying abundances across the different life stages, proving that also closely related symbiont strains can coexist within single individuals, likely through functional complementation of defensive traits. Histological sections, fluorescence *in situ* hybridizations (FISH), light sheet microscopy, and micro-computed tomography (µCT) revealed that the symbionts are housed in three pouches of the dorsal thorax in larvae, which are needed for colonization and undergo morphological changes in the pupal stage. The symbiotic organs are formed through invaginations of the cuticle but remain open to the outer surface through a channel and are maintained throughout larval development. Through this channel, symbionts can be internalized after hatching from the egg, ensuring a vertical transmission route, but they can also be released to the outer surface. In pupae, a morphological dimorphism of the organs in females and males leads to a decline of

symbionts during pupation in males and ultimately to the complete loss in male adults. For female pupae, the localization on the outer surface enables successful vertical symbiont transmission independent of the reorganization process of the internal tissue during metamorphosis. *In vitro* and *in vivo* assays, FISH and MALDI-imaging revealed that the symbionts and lagriamide are not only present inside the symbiotic organs, but also on the surface, where they protect young larvae against pathogenic fungi. On the surface, they inhibit fungal infestation on pupae and larvae, leading to higher larval survival probability. Keeping ectosymbionts accessible to the surface illustrates an effective defense strategy for the beetles against infections from fungal enemies. Furthermore, HPLC-MS, *in vivo* assays, and genome analyses revealed that in addition to its importance and consistent production in *L. villosa*, a close relative of lagriamide, namely lagriamide II, seems to be similarly responsible for the egg protection in *L. hirta*. However, lagriamide and their producers are not the only defensive symbionts, but also other *Burkholderia* strains protect the larval (LvStA) or egg stage (LvStA, LhStG) and have the metabolic capabilities for various protective compounds. Additionally, at least three non-*Burkholderia* symbionts (*Acinetobacter*Lv1, *Luteibacter*Lv2, *Variovorax*Lv3) showed protective capabilities on the eggs and through the presence of candidate biosynthetic gene clusters in their genomes their potential for defense.

With this thesis, I explored missing links in the developmental stages of the *Lagria* host and contributed to the general knowledge of defensive symbioses through several aspects: By shedding light on the structure, development, and function of the symbiotic organs, a unique morphological adaptation for insects to tie and maintain symbionts throughout development and host generations was revealed. The open structure of the cuticular invaginations housing antibiotic-producing ectosymbionts exemplifies an effective defense strategy for insects against fungal infections, especially during vulnerable phases of molting and metamorphosis. Moreover, their open structure allows for the horizontal acquisition of other putative symbionts, keeping the association flexible to potentially respond quickly to changing environments. This flexibility is furthermore reflected by the presence and coexistence of multiple protective symbionts next to dominant strains with different metabolic capabilities. Whether multiple symbiont infections are advantageous for the host and symbiont, and how they can persist in a long-term relationship are evolutionary interesting questions that can be further addressed in the *Lagria* symbiosis.

ZUSAMMENFASSUNG

Nahezu alle Makroorganismen gehen in der Natur Partnerschaften mit Mikroorganismen ein. Diese Verbindungen ermöglichten nicht nur die Entstehung und Evolution verschiedenster Arten, sondern können sich auch auf deren Ökologie auswirken. Mikrobielle Symbionten spielen deswegen eine wichtige Rolle im Netz des Lebens, indem sie für ihren Wirt überlebens- oder entwicklungsnotwendige Aufgaben übernehmen. Hierbei ist die Verteidigung gegen natürliche Feinde eine Herausforderung, der sich alle Tiere auf der Erde stellen müssen, einschließlich der Insekten. Und obwohl Insekten selbst zahlreiche Eigenschaften entwickelt haben, um mit verschiedenen Arten von Antagonisten umzugehen, eröffneten Partnerschaften mit symbiotischen Mikroorganismen neue Möglichkeiten, sich an verschiedenste natürliche Feinde anzupassen.

Diese Dissertation konzentriert sich auf die Abwehrsymbiose zwischen Wollkäfern der Gattung *Lagria* (Coleoptera: Tenebrionidae) und mehreren Verteidigungssymbionten, einschließlich *Burkholderia*-Bakterien (Pseudomonadota: Betaproteobacteria). Die Symbionten begleiten ihre Wirtstiere während der gesamten Entwicklung vom Ei bis zum adulten Käfer. Sie beginnen ihre Reise im weiblichen Käfer in akzessorischen Drüsen des Fortpflanzungssystems, wonach sie während der Eiablage in einem Sekret auf die Eioberfläche geschmiert werden und in den Larven drei einzigartige dorsale Einstülpungen der Kutikula besiedeln. Auf den Eiern produzieren sie antimikrobielle Verbindungen, um die Eier vor Pilzinfektionen aus der Umwelt zu schützen. In Larven tragen beide Geschlechter die Symbionten, während erwachsene männliche Käfer keine Symbionten mehr haben. Dies deutet darauf hin, dass die Symbionten womöglich auch nach dem Schlüpfen aus dem Ei eine wichtige Funktion für ihren Wirt erfüllen, nämlich weiteren Schutz gegen Pilzbefall aus der Umwelt.

Mit dieser Hypothese war das Ziel dieser Arbeit, die Symbiose in den weiteren Lebensstadien zweier Wollkäferarten, *L. villosa* und *L. hirta*, zu untersuchen. Daher wollte ich (i) die Häufigkeit, Zusammensetzung und Lokalisation mehrerer Symbiontenstämme über verschiedene Lebensstadien des Wirts hinweg analysieren, (ii) das Verteidigungspotenzial mehrerer Mitglieder der Symbiontengemeinschaft in verschiedenen Lebensstadien untersuchen und (iii) die morphologischen Anpassungen der Käfer für die Übertragung und Aufrechterhaltung der Symbionten während der Häutung und Metamorphose betrachten. Analysen der Bakteriengemeinschaft mittels 16S-Amplikon-Sequenzierung (Illumina) und qPCR zeigten, dass die Käfer durchweg in allen Lebensstadien mit ihren Symbionten assoziiert sind. Darüber hinaus beherbergen sie eine vielfältige Gemeinschaft, die überwiegend aus *Burkholderia*-Stämmen besteht, wobei auch Symbionten anderer Familien in geringeren Mengen vorhanden sind. Bei *L. villosa* kommt ein *Burkholderia*-Stamm (LvStB) besonders häufig vor. Trotz seines reduzierten Genoms und seiner vermeintlichen fehlenden Fähigkeit zur Motilität dominiert er in Anwesenheit anderer *Burkholderia*-Stämme und ist oft als einziger *Burkholderia*-Stamm im Wirt zu finden. Seine Fähigkeit, den antimykotischen Wirkstoff Lagriamid zu produzieren, ist vermeintlich ein potenzieller Schlüsselfaktor für seine Konstanz und führende Rolle im Käfer. Währenddessen dominieren bei *L. hirta* zwei Stämme, LhStH und LhStG, die Gemeinschaft, obgleich in unterschiedlicher Häufigkeit in den verschiedenen Lebensstadien. Dies beweist, dass auch eng verwandte Symbiontenstämme innerhalb einzelner Individuen koexistieren können, wahrscheinlich durch funktionelle Ergänzung der unterschiedlichen Abwehrsubstanzen. Histologische Schnitte, Fluoreszenz-in-situ-Hybridisierungen (FISH), Lichtscheibenmikroskopie und

Mikro-Computertomographie (µCT) zeigten, dass die Symbionten in drei Einstülpungen des dorsalen Thorax in Larven untergebracht sind. Die symbiotischen Organe werden durch Einstülpungen der Kutikula gebildet, bleiben aber durch einen Kanal zur Außenfläche offen und sind während der gesamten Larvenentwicklung erhalten. Durch diesen Kanal können Symbionten nach dem Schlüpfen aus dem Ei aufgenommen werden, was einen vertikalen Übertragungsweg gewährleistet. Über diesen Weg können sie auch an die äußere Oberfläche der Larven gelangen. Bei Puppen unterscheiden sich diese Organe in Weibchen und Männchen. Eine Verkleinerung der Organe bei Männchen führt zu einem Rückgang der Symbionten während der Verpuppung und schließlich zum vollständigen Verlust im männlichen Erwachsenen. Bei weiblichen Puppen ermöglicht die Erhaltung dieser Organe eine erfolgreiche vertikale Symbiontenübertragung sogar über den Reorganisationsprozess des inneren Gewebes während der Metamorphose. *In-vitro*- und *in-vivo*-Assays, FISH und MALDI-Bildgebung zeigten, dass die Symbionten und Lagriamid nicht nur innerhalb der symbiotischen Organe vorkommen, sondern auch auf der Oberfläche, wo sie junge Larven vor pathogenen Pilzen schützen. An der Oberfläche hemmen sie den Pilzbefall auf Puppen und Larven, was zu einer höheren Überlebenswahrscheinlichkeit der Larven führt. Ektosymbionten an der Oberfläche zu binden, veranschaulicht eine effektive Verteidigungsstrategie der Käfer gegen Infektionen durch Pilzfeinde. Darüber hinaus zeigten HPLC-MS, *in-vivo*-Assays und Genomanalysen, dass neben seiner Bedeutung und beständigen Produktion in *L. villosa* ein naher Verwandter von Lagriamid, nämlich Lagriamid II, in ähnlicher Weise für den Eischutz in *L. hirta* verantwortlich zu sein scheint. Lagriamid und ihre Produzenten sind jedoch nicht die einzigen Abwehrmechanismen, denn auch andere *Burkholderia*-Stämme und deren Schutzstoffe schützen das Larven- (LvStA) oder Eistadium (LvStA, LhStG) gegen Pilzbefall. Darüber hinaus zeigten mindestens drei andere bakterielle Symbionten (*Acinetobacter*-Lv1, *Luteibacter*-Lv2, *Variovorax*-Lv3) Abwehrfähigkeiten gegenüber den Eiern und durch das Vorhandensein von potenziellen biosynthetischen Genclustern in ihren Genomen auch ihr Verteidigungspotenzial.
Mit dieser Arbeit erforschte ich fehlende Bindeglieder in den Entwicklungsstadien der Wollkäfer und trug durch mehrere Aspekte zum allgemeinen Wissen über Verteidigungssymbiosen bei: Indem ich die Struktur, Entwicklung und Funktion der symbiotischen Organe beleuchtete, deckte ich eine einzigartige morphologische Anpassung für Insekten auf, um Symbionten während der gesamten Entwicklung und Wirtsgenerationen zu binden und zu erhalten. Die offene Struktur der kutikulären Einstülpungen, die antibiotikaproduzierende Ektosymbionten beherbergen, veranschaulicht eine effektive Verteidigungsstrategie für Insekten gegen Pilzinfektionen, insbesondere während anfälliger Phasen der Häutung und Metamorphose. Darüber hinaus lässt ihre offene Struktur einen Spielraum für den potenziellen horizontalen Erwerb anderer mutmaßlicher Symbionten, wodurch die Symbiose flexibel bleibt, um potenziell schnell auf Umweltveränderungen reagieren zu können. Diese Flexibilität spiegelt sich außerdem in der Anwesenheit und Koexistenz mehrerer schützender Symbionten neben dominanten Stämmen mit unterschiedlichen Stoffwechselfähigkeiten wider. Ob eine Vielzahl an koexistierenden Symbionten für die Symbiose vorteilhaft ist und wie diese in einer langfristigen Beziehung bestehen können, sind evolutionär interessante Fragen, die durch das *Lagria*-System weiter untersucht werden können.

LIST OF PUBLICATIONS

Chapter I is published in The ISME Journal

Janke R. S., Kaftan F., Niehs S. P., Scherlach K., Rodrigues A., Svatoš A., Hertweck C., Kaltenpoth M., Flórez L. V.
Bacterial ectosymbionts in cuticular organs chemically protect a beetle during molting stages

Chapter II is published in Frontiers in Physiology

Janke R. S., Moog S., Weiss B., Kaltenpoth M., Flórez L. V.
Morphological adaptation for ectosymbionts maintenance and transmission during metamorphosis in *Lagria* beetles

Chapter III is in preparation

Ganesan R., **Janke R. S.**, Grabe V., Kaltenpoth M., Flórez L. V.
Severe bottleneck during post-hatch vertical transmission of a defensive beetle symbiont

Chapter IV is in preparation

Janke R. S., Basic S., Ackel J., Reinecke S., Gohlke D., Köhler I., Weiss B., Vogel H., Kaltenpoth M., Flórez L. V.
Localization, function, and interaction of low-abundance symbionts in *Lagria villosa*

Chapter V is in preparation

Janke R. S., Basic S., Scherlach K., Hertweck C., Kaltenpoth M., Flórez L. V.
Coexistence and functional differentiation of culturable and non-culturable defensive symbionts in *Lagria* beetles

TABLE OF CONTENTS

GENERAL INTRODUCTION

1. Symbiosis

The phenomenon that was described as the "living-together of two different species" was first presented by Albert Bernhard Frank in 1877 as the term "symbiotism" and later referred to as "symbiosis" by Heinrich Anton de Bary in 1879 [1,2]. Since this very early research on the discovery of lichens as an exceptional association between fungi and algae and/or cyanobacteria, symbiosis is today known as a ubiquitous and central phenomenon in nature. In fact, the endosymbiotic theory is one of the leading evolutionary theories describing the origin of eukaryotic life. Now, almost 150 years later, symbiosis is acknowledged as an important driver of the biodiversity of various organisms across the planet, and research on symbiosis grows rapidly [3]. The possibilities are almost endless in terms of symbiotic partners since the relationships can involve eukaryotic, prokaryotic, and archaeal organisms living together in a dynamic continuum from positive to negative interactions, as commensals, parasites, or mutualists [4].

Since virtually all eukaryotic organisms live in an ecosystem colonized by microbes, it is not surprising that many animals are associated with microbial symbionts that impact evolutionary innovation in their hosts [4]. One of the pioneers in animal-microbe symbiosis research was Paul Buchner, whose compiled and detailed work "Endosymbiose der Tiere mit pflanzlichen Mikroorganismen" still serves as an important piece of literature illustrating the diversity of microbial symbioses [5,6]. The innovation and variety among symbioses is prominently visible in insects, which are the most diverse animal class and have adapted to many different environments from aquatic to terrestrial [7]. This success to thrive in many different habitats and utilize various food sources is also possible due to their interaction with microorganisms [8]. The different benefits that insects can gain from their microbial partners and their ecological relevance shall be described in more detail in the following section.

1.1. Ecological relevance of microbial symbionts in insects

With around one million described species, representing over half of the animal diversity, insect morphological and ecological variety has helped them to become the most diverse and successful organisms on Earth [9,10]. They thrive not only in various ecosystems, but each insect embodies an ecosystem on its own, interacting with many different organisms, including microorganisms. The estimated number of microorganisms on Earth is thought to be around one trillion [11], therefore it is not surprising that also microbes exhibit a diversity of lifestyles, such as symbionts of insects. In fact, microbial cells represent up to 1 – 10 % of a healthy insect's biomass, as residents of the exoskeleton, gut, hemocoel, specialized organs, or within cells [8].

Through their extensive metabolic potential, symbiotic microorganisms allow insects to adapt to new niches by providing versatile functional traits [12] (Figure 1). Some microbial symbionts help their hosts to cope with abiotic stressors [13], such as heat or cold [14,15], desiccation [16], or inorganic toxicants [17]. However, the majority of characterized symbioses support their hosts when challenged by biotic factors. Thereby, nutritional supplementation, such as vitamin or amino acid provisioning [18–22] is as important as help in digestion [23–25] and detoxification [26,27] for opening up new food sources and habitats or ensuring host development [18,28,29]. Another important quality of microbial symbionts is the ability to increase their host's survival and fitness when confronted with natural enemies [30–34].

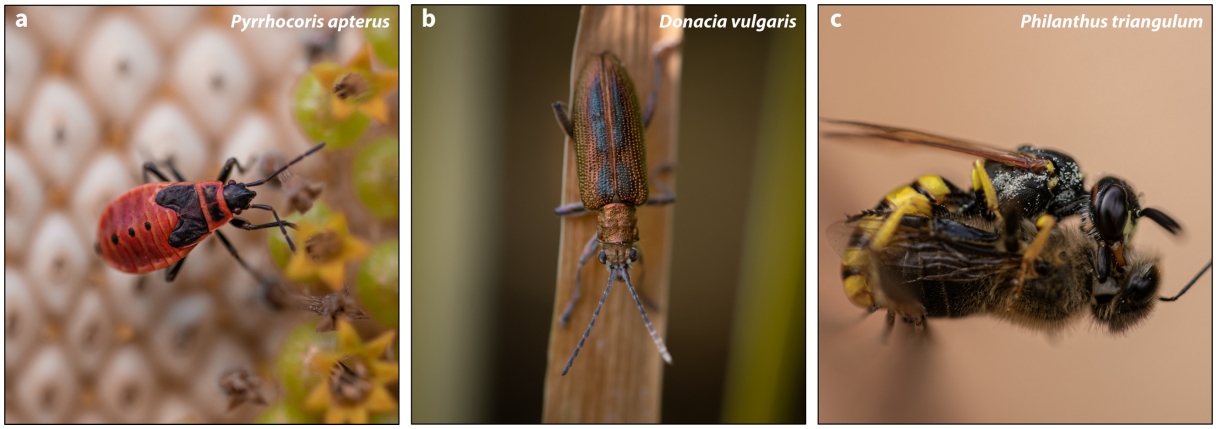

Figure 1: Variety of insects with beneficial symbionts. a Nymph of the European firebug *Pyrrhocoris apterus*. These bugs receive their vitamin supplies by gut symbionts. **b** Adult reed beetle *Donacia vulgaris*. These beetles carry intracellular and extracellular symbionts throughout their life cycle which help in digestion of plant biomass and supplement nutrients. **c** Female beewolf *Philanthus triangulum* (top) paralyzing a honeybee. These wasps harbor defensive symbionts which protect their brood against mold fungi.

1.1.1. Defensive symbionts in insects

Like any other organism, also insects face the challenge to withstand life-threatening natural antagonists in the environment to survive. Predators, parasitoids, parasites and pathogens are common antagonists, exerting selective pressures to evolve effective defense mechanisms [33,35]. Insects have found multiple ways to protect themselves, since various strategies were described so far [36]. They span a wide range including (i) chemicals, such as deterrents or toxins, that can be contained e.g. in glandular secretions or anal and oral discharges[37–39], (ii) morphological adaptations including color, warning coloration, crypsis, or mimicry [35,40,41], (iii) physiological modifications like a strong cuticle, hairs, spines or body size [42–45], (iv) various behavioral strategies including autotomy, avoidance, biting, molting, thanatosis, flight, dropping, hiding, sheltering, removal of nest-mates, sound production, stinging or heat production [46–49] and (v) responses of the immune system such as phagocytosis, melanization, encapsulation or coagulation [50–52]. With this arsenal of different strategies, insects seem to be well-equipped against different kinds of danger. But coevolving antagonists can adapt fast to cope with such mechanisms, leading to rapidly evolving traits on both sides and high selective pressures on evolving new traits or

modifying existing ones. Microorganisms can usually adapt fast, and their defensive capabilities might additionally come in handy, when host mechanisms are not sufficient or temporally unavailable. An association with microbial symbionts might therefore be an effective way of dealing with antagonists.

Microbial symbionts can protect their hosts through different ways [33]. Although primarily providing nutritional benefits for host survival and fecundity as described above, many nutritional symbionts also indirectly protect their host by improving their health [33]. Resources can be used by the host for defense, e.g. by building up a strong cuticle, which is protecting against external forces. This was recently described in the sawtoothed grain beetle *Oryzaephilus surinamensis*, in which the tyrosin-supplementing symbiont Candidatus *Shikimatogenerans silvanidophilus* is not only important for cuticle biosynthesis ensuring complete development [16,53,54], but also enhances cuticle defenses against fungal pathogen infection and predation by a spider [55].

Another symbiont-mediated defense strategy is outcompeting pathogens, which might often happen in the insect gut environment [33,56]. Concordantly, when the squash bug *Anasa tristis* is colonized by *Caballeronia* symbionts, infection by the opportunistic pathogen *Serratia marcescens* is lowered by competitive exclusion [57]. Similarly, *S. marcescens* is excluded by the gut community of the bee *Apis mellifera* [58]. Also, gut symbionts of the burying beetle *Nicrophorus vespilloides* hinder colonization of bacterial pathogens, thereby increasing the survival of larvae [59].

Resistance against different antagonists can also be achieved by stimulating the host's immune system [33]. This indirect assistance can be observed in the mosquito *Aedes aegypti*, which symbiont *Wolbachia* increases resistance to the dengue virus by inducing immune pathways that activate antimicrobial peptides effective against the virus [60]. Also, gut symbionts of the red palm weevil *Rhynchophorus ferrugineus* improve the systemic immunocompetence of their host aiding in protection against pathogens [61].

The beforementioned traits influence the host’s health rather indirectly, but some symbionts contribute to their host's safety in a more direct way by the production of chemical compounds [33,62]. One advantage of compounds that are constantly produced and present on the surface, is the potential to act ahead of encounters with antagonists, thereby preventing detrimental infections [63]. Those compounds can be harmful to enemies in various ways. They can irritate antagonists [46], indirectly protect as repellents [36], or directly affect the physiology of organisms [64]. Although the basis of chemical protection is sparsely understood [32,33], there is expanding evidence from several symbionts with bioactive potential aiding in host defense in different groups of insects. Some of the most prominent examples are within the Hymenoptera: The presence and effect of antibiotic-producing symbionts were described in beewolf digger wasps of the tribe Philanthini, which are associated with strains of *Streptomyces* bacteria that produce a mix of antibiotic compounds, thereby protecting the offspring of the wasps against environmental pathogens [65–70]. Similarly, fungus-farming leaf-cutter ants protect their fungal gardens and themselves against the fungal parasite *Escovopsis* through antimicrobial compounds that are produced by different vertically and

horizontally transmitted Actinobacteria and *Burkholderia* symbionts [71–76]. Being common in many insects including Diptera, the bacterium *Spiroplasma* is also a defensive symbiont of several *Drosophila* species, through the production of toxic ribosome-inactivating proteins that act against nematodes and wasps [77–79]. Within Hemiptera, the defensive symbiont *Hamiltonella* is best studied in several aphids, including *Acyrthosiphon pisum* for defense against parasitic wasps by bacteriophage-produced toxins [80–84]. Also, the Asian citrus psyllid *Diaphorina citri* is associated with *Profftella* symbionts, whose genome encodes for the toxic polyketide diaphorin, although its effect against natural enemies was not demonstrated yet [85,86]. This compound is related to the polyketide pederin, which was early described as an antipredatory compound produced by *Pseudomonas* bacteria in *Paederus* rove beetles [37,87,88]. *Paederus* beetles are thereby protected against spiders, while eggs and young larvae have the highest concentration of pederin [89]. Other Coleoptera species, with several antibiotic-producing strains that aid in host protection are *Lagria* beetles [68], whose symbiosis with *Burkholderia* bacteria shall be described in later sections.

1.1.2. Localizations of defensive symbionts in insects

Insects have evolved diverse specialized structures to accommodate symbionts, from very intimate places inside the insect body to areas covering the outer surface. Symbionts localized within the insect's body are generally named endosymbionts, including extracellular and intracellular endosymbionts. Symbionts that are on the other hand generally extracellular and associated with the outer cuticle including gut-associated bacteria or the close environment are termed ectosymbionts (or: exosymbionts). Few described defensive symbionts are harbored intracellularly in specialized host cells called bacteriocytes, which cluster together in a structure named bacteriome, such as Candidatus *Profftella armatura* in *D. citri* [85]. Also, several of the defensive facultative symbionts (*Hamiltonella defensa,* Candidatus *Regiella insecticola, Serratia symbiotica, Rickettsia*, *Rickettsiella*, *Spiroplasma*) of the pea aphid *A. pisum* reside in bacteriocytes and the cytoplasm of surrounding sheath cells, but also in the hemocoel, where they can protect against attacking parasitoids or pathogens [80,83,90–93]. Other bacteria are internally localized in the hemolymph protecting against invaders, such as *Sodalis glossinidius* in *Glossina* tsetse flies [94,95] or *Spiroplasma* in *Drosophila melanogaster* [96,97]. Many of these defensive endosymbionts protect against other eukaryotes, like different parasitic wasps, predators, nematodes, or protozoan parasites, while many protective symbionts that are located externally are often involved in defense against pathogenic microorganisms [33].

The localization on the cuticle might provide suitable conditions to house ectosymbionts without running into the risk of being harmed by potentially detrimental side effects of antibiotic compounds [33]. Having the protective partner located on the outer surface also favors defense against external invaders and can ward off antagonists before they even infect the insect [33]. Therefore, ectosymbionts might be especially beneficial against pathogenic microbes, including fungi, which often infect insects via the cuticle [98]. An external location

might also be favorable for housing protective partners because e.g. the gut microbiota can directly act against invading or ingested pathogens, or antibiotic-producing symbionts on the outer surface can effectively expose their substances to antagonists [33,99]. Some insects outside of holometabolous insects are described to carry ectosymbionts (including gut symbionts as defined above), such as fungus-farming termites [100,101], locusts [102,103] or cotton stainer bugs [104], but many more were discovered within Holometabola (Figure 2).

In which site ectosymbionts are associated with the cuticle is different among holometabolous insects and can involve different body parts (Figure 2). Symbionts of bees [58,105–107], mosquitos [108,109], flies [110,111], moths [112,113], and beetles [59,61,114] reside in the gut and protect against invading parasites and bacterial or fungal pathogens. Symbionts protecting against fungi from the outer surface were detected on larvae of the oriental fruit moth *Grapholita molesta* [115], on the cocoon of beewolves of the genus *Philanthus* [66,69], or in several Attine ants [74,76,116]. Attine ants have separately evolved different morphological adaptations along the ant's body for their defensive symbionts at least three times, indicating repeated selection for this symbiotic adaptation [76,117]. Also, the symbioses of mycangial bark beetles have many independent origins and show different structural modifications although most of them are not defensive [118], except for some species like *Dendrocontus frontalis* that accommodate a defensive symbiont in its lateral thoracic mycangium acting against antagonistic fungi [119,120]. On the other hand, the peculiar and specialized antennal gland reservoirs of female beewolves, which house the antifungal defensive symbionts that are later incorporated into the larval cocoon, have likely evolved once in an ancestor of the tribe Philanthini [121]. Another highly specialized invagination of the ventral abdominal cuticle can be found in the adult leaf-rolling weevil *Euops chinensis,* which harbors a defensive fungal symbiont that protects its offspring against pathogenic fungi [122,123]. Most of those complex cuticular structures house symbionts acting against antagonistic fungi and have evolved in different taxa, indicating that entomopathogenic fungi are one major driver promoting such morphological adaptations for defense. Also, the antifungal ectosymbionts of *Lagria* beetles are harbored in such structures and their importance for the beetle will be described below.

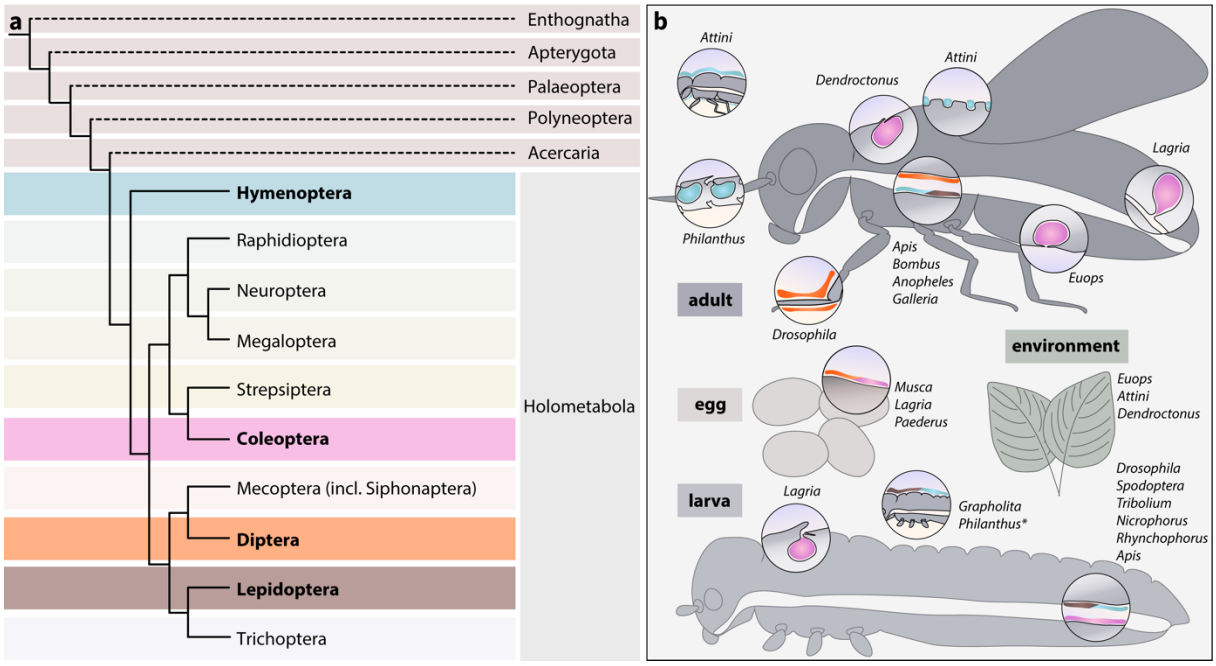

Figure 2: Defensive ectosymbionts associated with holometabolous insects. a Cladogram focusing on holometabolous insect orders associated with ectosymbionts (Hymenoptera: blue, Coleoptera: pink, Diptera: orange, Lepidoptera: brown). The phylogeny was simplified and adapted from [10]. **b** Localizations of ectosymbionts in different life stages and sites. Circles represent the localization of ectosymbionts associated with a certain body part in the insect. Different colors represent ectosymbionts according to their order in the cladogram. Silhouettes of adult, egg, larva, and environment are generic illustrations depicting no specific organism. The list may not comprehend all ectosymbionts among all holometabola and localizations but illustrates many known symbioses. *Symbionts of *Philanthus* are incorporated by the larva into the pupal cocoon.

1.2. Holometabolism-related implications for host and symbiont

Around 85 % of all insects, including bees, flies, beetles, and butterflies, belong to the superorder of Holometabola, which is defined by undergoing complete metamorphosis [124]. This characteristic is defined by four distinct life stages (egg, larva, pupa, adult), and the metamorphosis from larva to adult is associated with drastic tissue reorganization and a complete change in body plan [125]. Having different life stages unlocks several features for holometabolous insects that can be advantageous, like feeding on various food sources, colonizing different niches and evading competition among conspecifics, and specializing either on growth or reproduction [125,126]. Thereby, life stages can differ largely in their ecology, which is advantageous on the one hand, but can also lead on the other hand to adaptive conflicts of phenotypic traits. Complete metamorphosis diminishes the conflicts and breaks up trait correlations, which enables the distinct life stages to adapt independently to selective pressures, which is called adaptive decoupling [127]. However, besides the benefits of complete metamorphosis, there are also restrictions, one being associated with the insect's exoskeleton.

Despite being one of the key traits of the insects' success and diversity due to its ability to protect against natural enemies, prevention of water loss, or providing structural support, the rigidity of the cuticle leads to limitations [42,43,128]. The completely sclerotized cuticle is hard and not flexible, which restricts growth and also development [43]. Therefore, in order to develop to the adult stage, insects need to shed off their cuticle and form a new, bigger one, which is a process called molting or ecdysis. After molting, the new cuticle is not yet

sclerotized nor melanized and is relatively soft, enabling expansion. Since insects are fully covered by the cuticle with only a few membranous regions, their whole body is exposed during that time until the new cuticle is hardened again [43]. This process happens after every larval molt, but also during pupation and after adult emergence, potentially leading to increased susceptibility to predators or pathogens during these periods [55,129]. Although molting is not restricted to holometabolous insects, the implications for the pupal stage age likely much stronger, due to their lack of mobility [47].

Another trade-off associated with metamorphosis is the maintenance of symbionts during the reorganization of the host tissue. The majority of the internal organs present in the larval stage are restructured during pupation, which also includes symbiont-bearing structures. While for some symbiotic systems, this phase allows to get rid of symbionts when they are not needed [19], other systems might rely on a constant association throughout metamorphosis. Although some symbionts inside the body successfully relocate during pupation [130], endosymbionts and symbionts located in the gut might especially face the challenge of surviving this event, without being expelled or lysed by the host [131,132]. Symbionts can also be shed off with the exuvia during molting or adult emergence and might need to re-colonize the host in the adult stage [133]. In other cases, symbionts are available in the environment until the next stage picks them up [65,66]. If symbionts persist with the insect throughout metamorphosis might therefore depend on their location, but also on their function. Because if the trait beneficial for larvae is still needed in the following stages, it is favorable for the host to keep the symbiont close, as it is similarly important for symbiont transmission in females [134].

Some insects have developed strategies to protect themselves during these periods of reorganization and higher vulnerability [135], but the ecological role and fate of microbial symbionts throughout molting and pupation is rather understudied, despite their abundance across holometabolous insects.

2. The *Lagria-Burkholderia* symbiosis

First descriptions of the association between *Lagria* beetles and specific rod-shaped bacteria associated to female *Lagria* beetles were carried out by Hans Jürgen Stammer in 1929 [136]. Almost a century later, these bacteria were characterized belonging to the species *Burkholderia gladioli* and were shown to engage in a mutualistic relationship with the beetles by protecting their eggs against fungal pathogens from the environment [68,137].

2.1. Ecology and description of *Lagria* beetles

Lagria beetles belong to the tribe Lagriini in the subfamily Lagriinae within the Lagrioid branch of Tenebrionidae, one of the largest families of Coleoptera comprising nearly 20,000 described species [138]. Currently 42 species are described within the genus *Lagria* and are distributed worldwide.

Among those, *Lagria villosa* is a species native to Africa, which was introduced to Brazil, where it has been described as an agricultural pest of several crops including soybean [139]. Typically, adult beetles can be found on top of the plants or the ground on the soil (Figure 3 a, b), and eggs are suggested to be laid into the soil (Figure 3 c). Larvae and pupae are usually present in high numbers foraging in the soil or under leaf litter (Figure 3 d, e). Eggs take a developmental time of five to seven days until the first instar larvae hatch, which are white-beige colored and covered with hairs. This first instar persists for around 24 hours and remains unmelanized until its first molt. From the second to the seventh and last instar in the lab, the larval cuticle is melanized after each molt to a dark brown color. Before each larval molt, the cuticle breaks at the dorsal ecdysial line until the first abdominal segment, forming the opening from which the larva or pupa emerges. Although first instar larvae from a same egg clutch hatch almost synchronized, developmental time varies during larval growth in the lab, resulting in a total larval developmental time of about 50 days (Figure 4 a). Pupal development lasts around five to six days, during which individuals are immobile. Like larval molts, the pupal exuvia breaks dorsally and the newly formed adult emerges by crawling out of the exuvia. Freshly emerged adults have white translucent elytra and a light brown thorax, both darkening within an hour in the lab. The mature elytra are mostly dark brown with occasional metallic green or purple tones. Adult beetles can survive for several weeks in the lab and females can lay up to five egg clutches during that time [137]. Eggs are yellow to orange colored and laid in clutches of around 300 individuals (Figure 3 c, Figure 4 b). In nature, *L. villosa* can have up to three generations per year, which can overlap in lab-rearing cultures.

L. hirta on the other hand is a univoltine species occurring in Eurasia and northern Africa with adult beetles occurring between May and September in nature. In Germany, it is one of two *Lagria* species besides *L. atripes*, which resembles *L. hirta* in its habitus, but occurs earlier in the year starting in April. *L. hirta* adults can be often found in groups on the edge of forests but also in urban gardens on several plants including trees, shrubs, and grasses

(Figure 3 fh). Adults are usually found sitting and feeding on top of leaves or feeding on dry flowers or stems, while larvae and pupae can be found in the soil or leaf litter. The beetles overwinter as larvae and can be found in soil or under leaf litter but in their later stages also crawl up small plants (Figure 3 i, j). Eggs of *L. hirta* are also laid in clutches of around 50 eggs, which are white to yellow colored (Figure 4 b). Egg development can take six to ten days, and larval developmental time is dependent on the temperature lasting around 160 days under constant laboratory conditions including a diapause of three months [140,141] (Figure 4 c). Like *L. villosa*, first instar larvae remain unmelanized and take around 24 hours to develop, while the later stages melanize to a brown color. All larval stages are motile, while pupae cannot move. Throughout this thesis, mostly field-collected individuals and their direct offspring were used, due to their comparatively more difficult rearing conditions in relation to *L. villosa*.

A common feature between *Lagria* species is the association with symbiotic bacteria, which inhabit cuticular structures or organs in both beetles [68,136,142] and are involved in the protection of the egg stage, providing defense against pathogenic fungi [68,137].

Figure 3: Life stages of *Lagria* beetles. Photographs of *L. villosa* (a-e) and *L. hirta* (f-j) found in nature. **a** Close-up of an *L. villosa* adult beetle feeding on a drying soybean leaf. **b** Two mating adult beetles on top of a soybean pod. **c** Eggs (top left) and first instar larvae photographed on filter paper in the lab. **d** Big larva foraging through leaf litter on a soybean plantation. **e** Multiple pupae found under leaf litter on a soybean plantation. **f** Multiple *L. hirta* adults sitting and feeding on blackberry leaves. **g** Male (top) and female (bottom) adults feeding on dry *Impatients* sp. flowers. **h** Female adult feeding on flowering grass. **ij** Older larvae found in early spring on top of leaves.

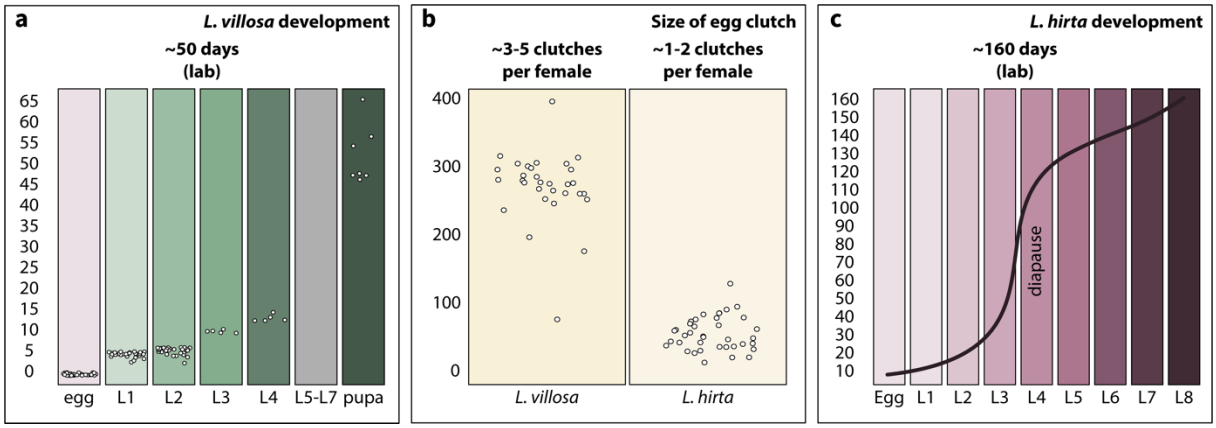

Figure 4: Life history traits of *Lagria* beetles. a Developmental time in days of *L. villosa* under laboratory conditions until pupation. Data was collected for individuals of the first laboratory generation. **b** Size and number of egg clutches per female *Lagria* beetle. Data of *L. hirta* and *L. villosa* was collected for individuals of the first laboratory generation and from estimates [137]. **c** Developmental time in days of *L. hirta* under laboratory conditions until the eighth larval stage. Data is simplified illustrated from [140].

2.2. The defensive symbiosis between *Lagria* and *Burkholderia*

Since Stammer's morphological description of the symbiotic organs in *Lagria* beetles [136], further studies have been carried out to investigate the evolution and ecology of the *Lagria-Burkholderia* symbiosis on a molecular level focusing on *L. villosa* [68,137,143–146] and *L. hirta* [142]. Female *Lagria* beetles carry most of their symbionts in two accessory glands associated with the ovipositor, which differ morphologically between species [136]. *L. hirta* has two bigger sac-like structures adjacent to the ovipositor and two smaller structures within the ovipositor, filled with a symbiont-containing secretion (Figure 5 a-c) [136]. Contrary, the two bigger structures of *L. villosa* consist of several tubules, each of them containing symbionts, while the ovipositor-associated structures are absent (Figure 5 d-f) [68]. During oviposition, the symbiont-containing secretion is smeared over the eggs, covering the egg surface [68,136]. Later, the bacteria are incorporated into three cuticular structures in the larval stage (Figure 6 a) [136]. These larval organs represent a peculiarity among insect larvae since they are invaginations of the dorsal cuticle and are filled with bacterial symbionts, which were so far not described in any other species [6].

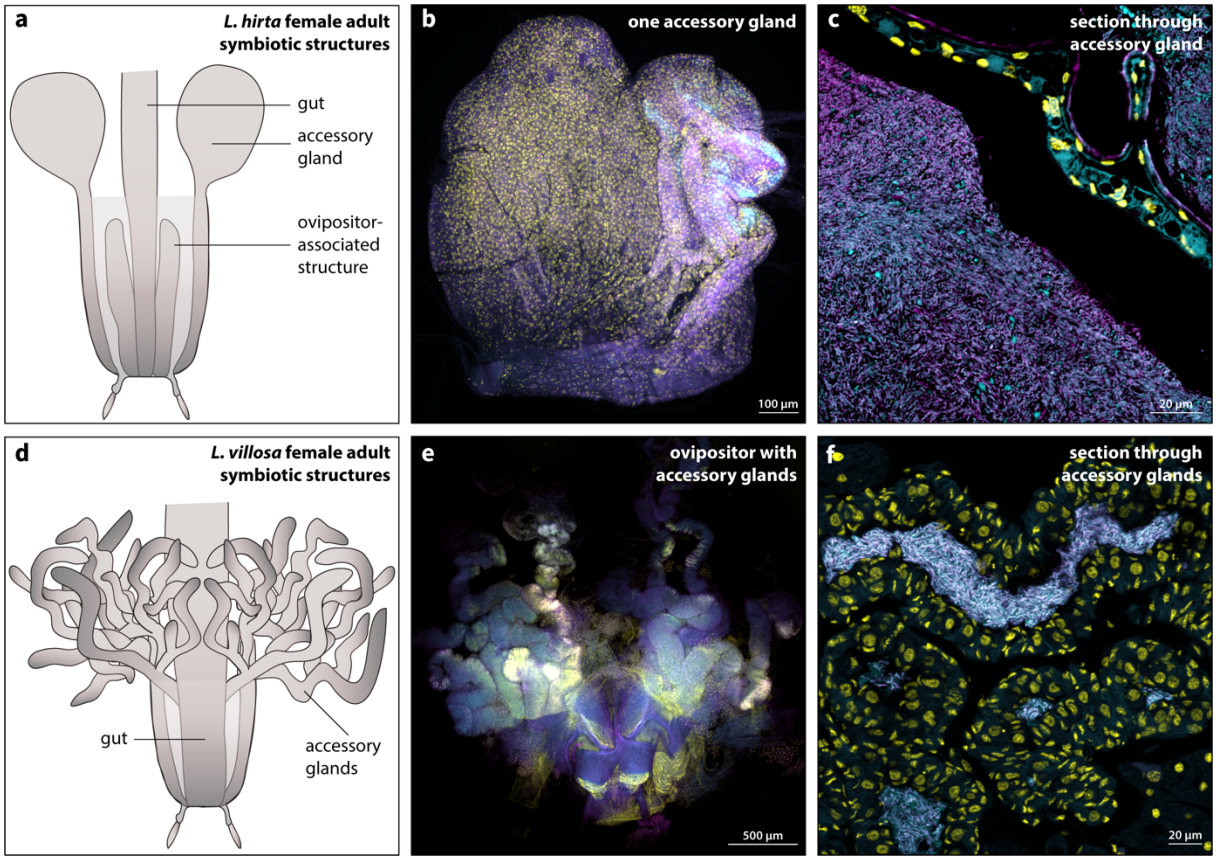

Figure 5: Symbiotic structures of *Lagria* females. *L. hirta* (a-c) and *L. villosa* (d-f). **a** Schematic representation of the symbiotic organs associated with the reproductive system in *L. hirta.* **b** Fluorescence *in situ* hybridization (FISH) on one dissected accessory gland. **c** FISH on a transversal section through a female abdomen showing symbiont cells within the accessory gland. **d** Schematic representation of the symbiotic organs associated with the reproductive system in *L. villosa*. **e** FISH on a whole ovipositor including tubular accessory glands. **f** FISH on a transversal section through a female abdomen showing symbiont cells within the accessory glands. Illustrations adapted from [136]. In FISH images, host nuclei are shown in yellow, *Burkholderia* symbionts in magenta, general Eubacteria in cyan, and overlap in purple-white.

In *L. hirta*, Stammer already observed that rod-shaped microbes dominate within the symbiotic organs (Figure 6 a) [136], which were later identified as bacteria belonging to the species *Burkholderia gladioli* [142]. *B. gladioli* was found to be present within the accessory glands of female adults and in larvae of both sexes, while male adults lack the symbionts (Figure 6 b) [142]. During larval development, *B. gladioli* titer increases until the fourth larval instar, where it reaches a plateau at around 10^6 symbiont cells in single individuals (Figure 6 c) [142].

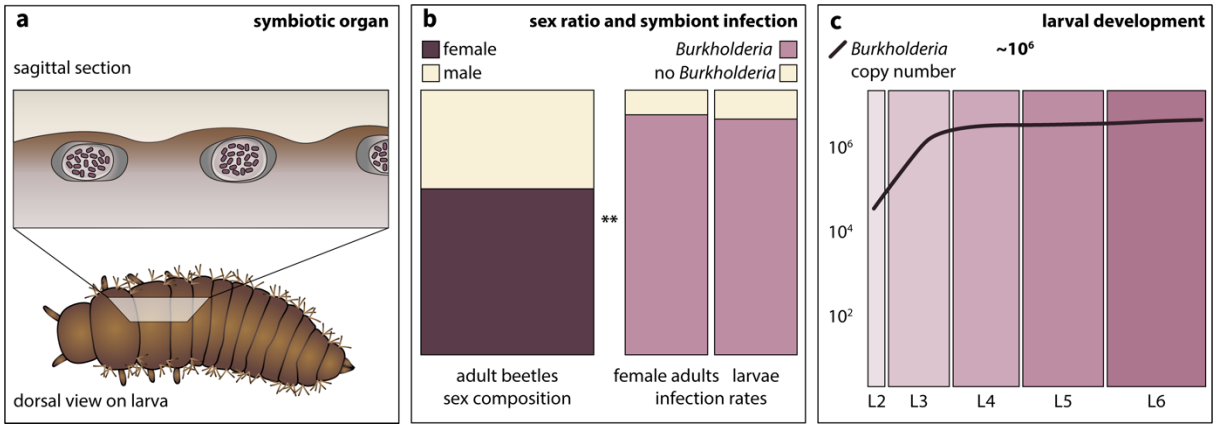

Figure 6: The symbionts of *L. hirta* beetles. a Localization of the symbiotic organs: Larvae accommodate symbionts in three dorsal compartments between the thoracic and first abdominal segments (illustration adapted from [136,142]). **b** Symbiont presence in females and males: *Burkholderia* symbionts are absent in male adults, but according to the sex ratio in adult beetles, present in both sexes in larvae (illustration simplified and adapted from [142]). **c** Symbiont abundance during larval development: *Burkholderia* titers reach a plateau around the L4 stage (illustration simplified and adapted from [142]).

Despite *B. gladioli* being highly abundant in *Lagria* beetles, other bacteria are also present in the symbiotic organs [68,142]. In *L. villosa*, *B. gladioli* represents 65-86 % of the community in females and 30 – 71 % on the egg surface (Figure 7 a, b) [68]. Interestingly, within *B. gladioli*, different putative strains can be distinguished by their 16S rRNA [68,137,142]. In *L. villosa*, the genome-eroded and unculturable strain *Burkholderia* Lv-StB (henceforth "Lv-StB") dominates on the eggs of field-collected female beetles, representing 50 – 95% of the *Burkholderia* community (Figure 7 c), while other strains are occasionally present in the community [137]. From these other strains in *L. villosa*, *B. gladioli* Lv-StA (henceforth "Lv-StA") is the only one isolated and culturable *in vitro* [68]. Similarly, multiple *B. gladioli* strains are present in the females of *L. hirta*, from which *B. gladioli* Lh-StH and *B. gladioli* Lh-StG (henceforth "Lh-StH" and "Lh-StG") were identified [147], and the latter is isolated and culturable *in vitro*. Notably, this distribution of strains can only be observed in field-collected individuals because some strains, including the genome-eroded LvStB and Lh-StH, are lost under laboratory conditions [142]. The specific factors driving this loss of certain symbionts are yet unknown, although they have been previously discussed [144].

Since the symbionts are smeared onto the egg surface during oviposition and are later incorporated into the larval organs, where they are present in both sexes, *B. gladioli* symbionts are mainly transmitted vertically from mother to offspring [68,136]. However, they can also be horizontally acquired from plants or leaf litter in the environment (Figure 7 d), representing a link to their evolution from plant associated ancestors [68,144]. On the eggs of *L. villosa*, *B. gladioli* strains and the whole symbiont community inhibit fungal infestation and increase the survival of hatching larvae in comparison to eggs without any symbionts (Figure 7 e) [68,137]. This protective effect is largely driven by the production of several antimicrobial compounds, which were identified from Lv-StA and Lv-StB (Figure 7 f) [68,137]. Lv-StB is dominant under natural conditions on the eggs and is thereby one of the main candidates responsible for defense by producing the antifungal polyketide lagriamide, whose corresponding biosynthesis gene cluster was horizontally acquired by LvStB [137]. Notably, LvStA can also produce various antibacterial and antifungal compounds, including sinapigladioside [68,148], toxoflavin [68], caroynencin [68], lagriene [68], gladiofungin [145], icosalide [146],

haerreogladin [146], burriogladin [146] and gladiobactin [149], aiding in protection against different fungal antagonists (Figure 7 f).

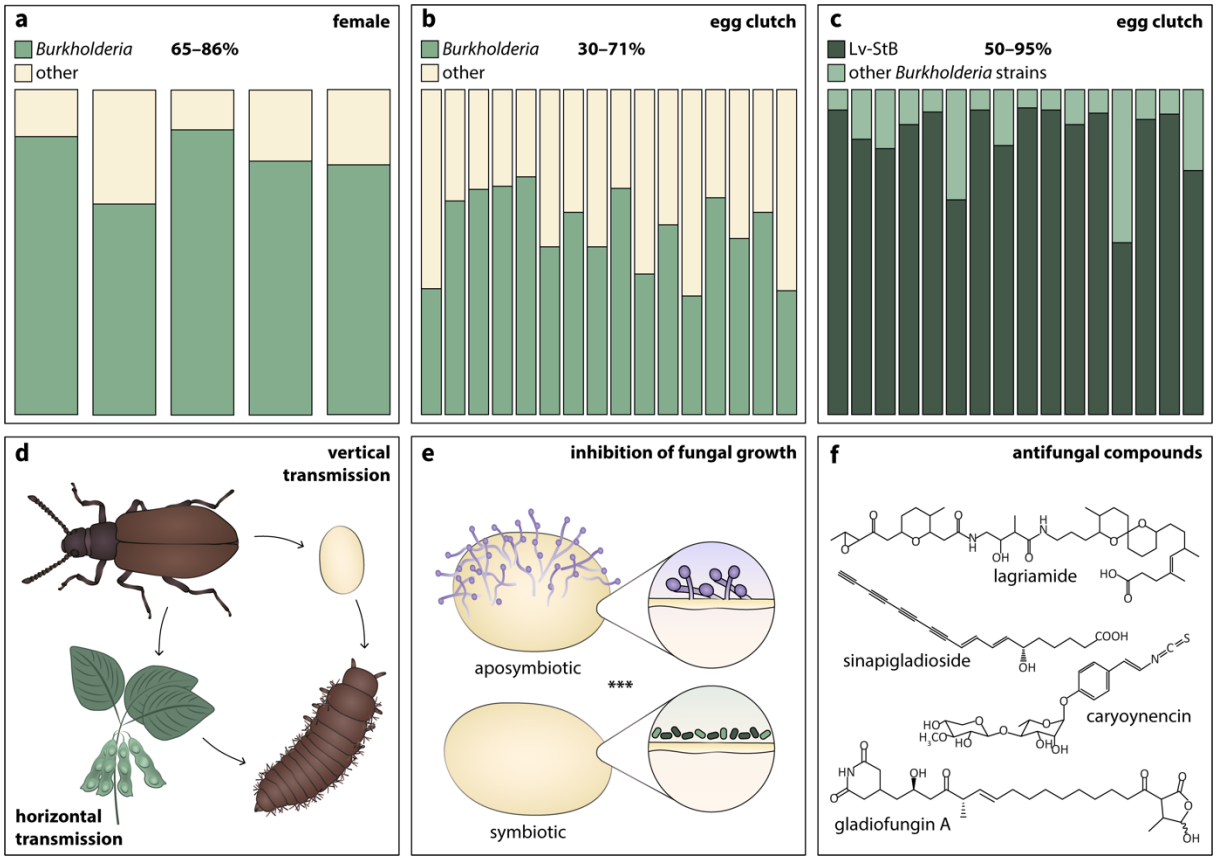

Figure 7: The defensive symbiosis between *L. villosa* beetles and *Burkholderia* bacteria. a Relative abundance of *Burkholderia* symbionts within accessory glands of field-collected females. "Other" includes multiple bacterial taxa (illustration simplified and adapted from [68]) **b** Relative abundance of *Burkholderia* symbionts on the surface of eggs from field-collected females. "Other" includes multiple bacterial taxa (illustration simplified and adapted [68]). **c** Relative abundance of the dominant *Burkholderia* strain Lv-StB within all *Burkholderia* strains. "Other" includes multiple *Burkholderia* strains (illustration simplified and adapted from [137]). **d** Mixed-mode symbiont transmission in the *Lagria-Burkholderia* symbiosis: Symbionts are mainly transmitted vertically from mother to offspring during oviposition, but horizontal acquisition from the beetle's host plant is also possible [68,144]. **e** Protective symbionts in the egg stage: The symbiont community inhibits infestation from multiple pathogenic fungi on the egg surface [68,137]. **f** Defensive compounds: *Burkholderia* strains produce different antimicrobial compounds that inhibit fungal growth (not all bioactive compounds are shown) [68,137,145].

3. Thesis Outline

Lagria beetles accommodate antimicrobial-producing *Burkholderia* symbionts in accessory glands of the adult's female reproductive systems, and in peculiar dorsal invaginations in larvae, but not in male adults. Since they defend eggs of *L. villosa* against fungal pathogens and are present in both sexes in larvae, the aim of this thesis was to explore the symbiosis between *Lagria* beetles and their defensive symbionts, focusing on the post-hatch life stages of the host before reaching adulthood. Research on the protection of immature insect stages of Holometabola and the role and fate of microbial symbionts throughout metamorphosis is scarce, especially during molting. Therefore, I aimed to (i) investigate symbiont-mediated protection against fungal pathogens of immature and molting stages by *Burkholderia* and non-*Burkholderia* members of the symbiont community, (ii) examine the abundance, localization, and potential function of several *Burkholderia* strains of two *Lagria* species, and (iii) shed light on the morphological adaptation of the host allowing for successful symbiont maintenance and transmission throughout host development and complete metamorphosis.

In **Chapter I**, we investigate the defensive potential of *L. villosa* symbionts beyond the egg stage by using a comprehensive experimental toolbox of microbial community sequencing, qPCR, fluorescence *in situ* hybridization (FISH), *in vivo* and *in vitro* assays, liquid chromatography mass spectrometry (LC-MS), and mass-spectrometry imaging (MSI). Thereby, we unravel a special symbiont-mediated defense mechanism of molting stages, which is facilitated by morphological adaptations of the host. We show that the unique symbiotic organs of larvae and pupae enable symbiont maintenance and release during vulnerable phases of molting and metamorphosis, thereby protecting the larvae against fungal infestation. Furthermore, we identified the dominant symbiont strain throughout host development and provided evidence for its crucial role in the symbiosis, by consistently producing the antifungal compound lagriamide. Importantly, we could localize the symbiont strain as well as the compound *in situ* in tissues relevant for defense, showed its production in every life stage, and tested its defensive potential *in vitro*, providing a comprehensive overview of its importance for the symbiosis beyond the already described egg stage.

To further reveal the fate of the symbionts across the beetle's life cycle, we examined in **Chapter II** the differences between female and male *Lagria* pupae. By using qPCR, FISH, and micro-computed tomography (µCT), we show that morphological differences between the sexes lead to the loss of symbionts in male adults and symbiont maintenance despite metamorphosis-driven tissue reorganization in females. We show that a drop in symbiont titers during pupation is likely caused by morphological changes of the symbiotic organs in pupae, which partially remain vestigial as cuticular linings between the segments lacking accumulations of symbionts. Furthermore, we demonstrate that female pupae retain one big symbiotic organ to facilitate symbiont transmission to the next stage, while this organ is strongly reduced in males. Lastly, we propose an external transmission route, which enables successful symbiont translocation towards the symbiotic organs in early female adults.

In **Chapter III**, we address the question of when and how different *Burkholderia* strains colonize the symbiotic organs in the first larval instar of *L. villosa* from the egg surface, using reinfection experiments, colony-forming-unit (CFU) counts, light-sheet-microcopy with green-fluorescent-protein (GFP) labeled symbionts, and FISH. We provide evidence that the symbionts infect the dorsal organs during or shortly after hatching from the eggshell, contradicting the described colonization mechanisms of the embryo through the eggshell, which was proposed by Stammer in 1929 for *L. hirta*. Furthermore, we show that symbiont acquisition from the egg stage is more successful than during larval development, although symbionts can also be taken up later from the environment.

The potential for horizontal symbiont acquisition might have also initially led to the association with other bacteria in the community besides *Burkholderia*, which association is described in **Chapter IV**. There, we reveal the presence, potential role, and interaction between different members of the microbial community in *L. villosa* with a combination of microbial community sequencing, FISH, *in vivo* and *in vitro* assays, as well as genome analysis. We identified at least three additional bacteria (*Acinetobacter*, *Luteibacter*, and *Variovorax*) that are consistently associated with the beetle. They inhabit the same habitat as *Burkholderia* within the symbiotic organs of all life stages and are likely coexisting by positively influencing each other's growth. Preliminary experiments also showed their potential for egg defense when exposed to a known pathogen of *L. villosa*, probably complementing the defensive traits of the *Burkholderia* symbionts.

Since not only multiple bacterial families are present in the symbiont community of *Lagria* beetles, but also strain-level diversity was found among the *Burkholderia* symbionts, , we investigated in **Chapter V**, the coexistence and functional differentiation of two *Burkholderia* strains in *L. hirta*. By using microbial community sequencing, qPCR, FISH, *in vivo* assays, as well as genome analysis of strains from two *Lagria* species we discuss the dynamics and implications of harboring multiple strains in two *Lagria-Burkholderia* symbioses. We show that *L. hirta* eggs are defended by their symbionts, likely through different chemical mediators as demonstrated for *L. villosa*. In addition, we demonstrate the presence of two distinct *Burkholderia* strains in *L. hirta*, which fluctuate in abundance across host development and show genomic and metabolic analogies to strains of *L. villosa*, despite differing in their prevalence within natural populations.

In a general discussion, the above-mentioned results are brought together and discussed considering symbiont and host characteristics, which might be essential for a long-term persistence of a defensive symbiosis. Moreover, I aim to discuss future perspectives of the *Lagria* system addressing questions that are also relevant to host-microbe interactions at large.

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CHAPTER I

Bacterial ectosymbionts in cuticular organs chemically protect a beetle during molting stages

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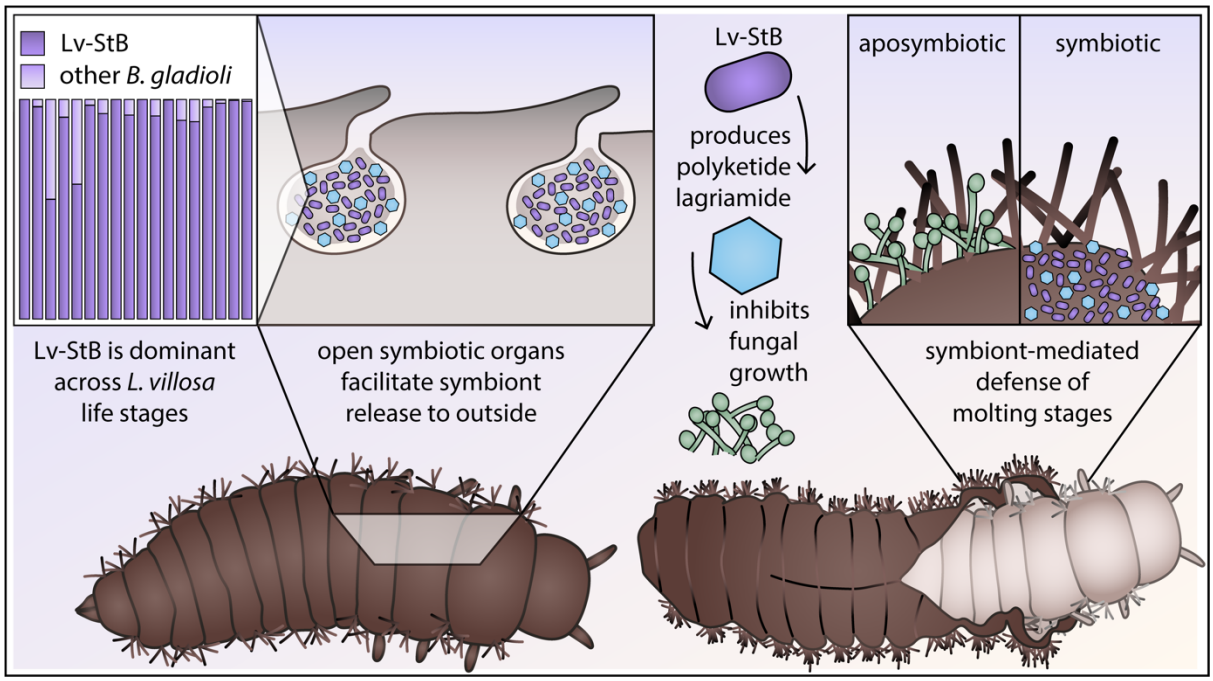

R.S.J., M.K. and L.V.F. conceived the study and designed the experiments. **R.S.J.** carried out all manipulative assays, qPCR, FISH experiments, and illustrations. L.V.F. was responsible for high throughput amplicon sequencing and the corresponding analysis. F.K. carried out MS imaging, and S.P.N. and K.S. were in charge of lagriamide qualitative quantification via LC-MS. Samples were collected by **R.S.J.**, L.V.F., and A.R. Experiments and data analysis were done with input from A.R., A.S., C.H., M.K., and L.V.F. **R.S.J.**, L.V.F. and M.K. wrote the manuscript and all authors commented on the final draft.

1. Abstract

In invertebrates, the cuticle is the first and major protective barrier against predators and pathogen infections. While immune responses and behavioral defenses are also known to be important for insect protection, the potential of cuticle-associated microbial symbionts to aid in preventing pathogen entry during molting and throughout larval development remains unexplored. Here, we show that bacterial symbionts of the beetle *Lagria villosa* inhabit unusual dorsal invaginations of the insect cuticle, which remain open to the outer surface and persist throughout larval development. This specialized location enables the release of several symbiont cells and the associated protective compounds during molting. This facilitates ectosymbiont maintenance and extended defense during larval development against antagonistic fungi. One *Burkholderia* strain, which produces the antifungal compound lagriamide, dominates the community across all life stages, and removal of the community significantly impairs the survival probability of young larvae when exposed to different pathogenic fungi. We localize both the dominant bacterial strain and lagriamide on the surface of eggs, larvae, pupae, and on the inner surface of the molted cuticle (exuvia), supporting extended protection. These results highlight adaptations for effective defense of immature insects by cuticle-associated ectosymbionts, a potentially key advantage for a ground-dwelling insect when confronting pathogenic microbes.

2. Introduction

Fungal pathogens are important natural enemies of insects and exert strong selective pressures on populations in both natural and agricultural settings [1,2]. By adhesion and germination across the surface followed by direct penetration of the cuticle and secretion of toxins, entomopathogens attack a major protection barrier of insects [3]. In response, insects have evolved diverse defense mechanisms including elaborate immune responses, as well as behavioral, mechanical, and chemical defenses [4,5]. While animals can often produce defensive compounds themselves, there is growing evidence that associated microorganisms can support protection against fungi and other enemies [6,7]. Symbiotic microbes inhibit fungal infections in aphids [8,9], eggs of *Lagria* beetles [10,11], immature life stages of solitary wasps [12–14], fruit moths [15], and leaf-rolling weevils [16], as well as the nutritional resources of ants [17–19], termites [20] and ambrosia-beetles [21]. Although numerous compounds produced by defensive symbionts have been identified [7,22–24], there are only a few systems in which these have been spatially tracked [25–28], since their quantification and detection *in situ* remains challenging.

Symbiont-mediated defense might be especially important for animals with temporarily limited protection mechanisms, like insects with complete metamorphosis, in which the distinct life stages —adult, egg, larva, pupa— face different challenges and demand specific defense strategies. Eggs are particularly susceptible to predation, parasitism, and pathogen infection since behavioral defenses are limited by the lack of mobility [29]. In *Lagria villosa*

beetles, fungal infections are prevented by *Burkholderia* symbionts on the eggs, which produce different antimicrobial compounds [10,11]. A similar strategy has been observed in squids [30], hoopoe birds [31], and lizards [32], indicating that egg protection through beneficial microorganisms has evolved in different environments for invertebrate and vertebrate hosts [33].

Insect larvae and pupae also face high infection risks, as they often inhabit pathogen-rich environments like soil or phyllosphere. The leaf-rolling weevil *Euops chinensis* increases the survival of its larval offspring in leaf cradles by depositing the symbiotic fungus *Penicillium herquei* with the eggs [16]. Also in solitary beewolf wasps, antibiotic-producing *Streptomyces* bacteria are incorporated into the cocoon silk protecting the pre-pupa from mold fungi [12,13]. Given its occurrence across animals and life stages, symbiont-mediated defense might be a common defense strategy for immature stages. The localization of microbial symbionts on the host surface or in connection to the external environment could be especially convenient to prevent infections. As such, cuticle-associated host defenses, including melanization [34,35] and immune factors in the molting fluid [36,37] might be complemented by ectosymbionts. However, immature insects recurrently shed their cuticle during development, which has both advantages and potential risks. Molting can clear out detrimental but also beneficial microbes from the surface, and it might lead to temporal vulnerability to antagonists until a fully sclerotized and melanized cuticle is reestablished [38,39]. While these tradeoffs are likely common across many invertebrates that shed their cuticle, research on defense strategies during molting phases is lacking.

In *L. villosa* beetles (Coleoptera: Tenebrionidae), ectosymbionts with high bioactive potential are associated with the insect throughout immature life stages [10,11,40](Figure 1a). This polyphagous beetle originates from sub-Saharan Africa and was introduced to South America, where it is found on several crop plants. Adults feed on leaves and flowers, while the larvae are mostly detritivorous [41]. Hence, *L. villosa* beetles occur in soil and around decaying plant material, where they deposit eggs, and the larvae feed, molt (Figure 1b, c), pupate (Figure 1d), and emerge as adults, demanding effective defense mechanisms against a variety of potential pathogens throughout their lifetime. Concordantly, the beetles harbor a community of bacterial symbionts including several strains of *Burkholderia gladioli* (Gamma-Proteobacteria, previously Beta-Proteobacteria), which are vertically transmitted from female accessory glands onto the egg surface during oviposition [10,40]. Multiple strains can infect the beetles, and at least two, *B. gladioli* Lv-StA and *B. gladioli* Lv-StB (henceforth “Lv-StA” and “Lv-StB”, respectively), are capable of producing antimicrobial compounds that inhibit fungal growth on the beetle eggs [10,11]. Under field conditions, Lv-StB is consistently present in high abundance within female accessory glands and on the egg surface, where it is important for defense presumably by producing the antifungal polyketide lagriamide [11]. The symbionts are absent in male adults but are present in both female and male larvae in the related species *L. hirta* [40], where they are housed as ectosymbionts in peculiar dorsal cuticular invaginations formed during embryonic development [42].

Given the localization of the ectosymbionts in larvae in cuticle-lined organs of both sexes, we reasoned that, in addition to egg defense and transmission to the next generation, symbiont presence during larval development may be associated with the protection of molting stages. To test this, we investigated the functional role of antibiotic-producing *B. gladioli* symbionts in larvae and pupae of *L. villosa* beetles by (i) evaluating symbiont function in larval and pupal stages via bioassays against antagonistic fungi, (ii) characterizing and localizing the symbiont community in all life stages through 16S rRNA gene amplicon sequencing, qPCR and FISH, and (iii) quantifying and localizing the bioactive secondary metabolite lagriamide *in situ* and monitoring its biosynthesis during development using high-performance liquid chromatography mass spectrometry (HPLC-MS) and mass-spectrometry imaging with a high-resolution atmospheric pressure scanning microprobe (APSMALDIHR MSI). Thereby, we show prolonged antibiotic-mediated defense provided by a *Burkholderia* bacterial symbiont during the immature life stages of the beetles and highlight the symbiotic organs as unique host morphological adaptations that support the protective association.

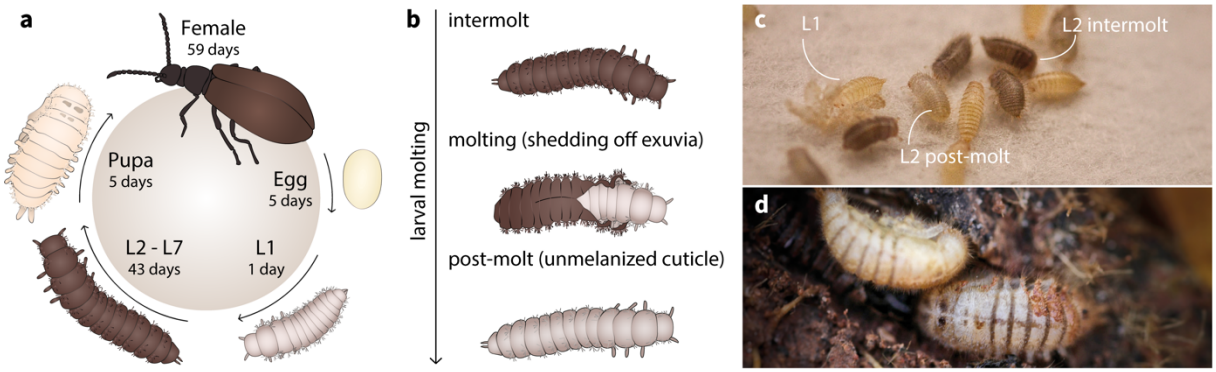

Figure 1. Life cycle of *L. villosa* beetles. a Schematic overview of the beetle's life stages including average duration of every stage in days. The seven larval instars are abbreviated as L1 – L7. **b** Illustration of the larval molting phase. c Photograph of young larvae reared in the laboratory. L1 larvae remain unmelanized, while the cuticle of L2 larvae melanizes after the post-molt phase. d Photograph of pupae in the field, found under decaying leaves in the soil of a soybean plantation.

3. Results

3.1. Symbiont-mediated defense against entomopathogens in early larval and pupal stages

To assess the potential protective function of the symbionts after egg hatching, we carried out bioassays exposing larvae to different pathogenic fungi and monitored survival and fungal growth (Table S1). We evaluated the first days of larval development covering multiple molting events since freshly molted larvae are likely more susceptible to infections [43]. First, we exposed young larvae to a fungal pathogen previously isolated from *L. villosa* beetles, *Purpureocillium lilacinum* [44]. In these conditions, aposymbiotic larvae were less likely to survive compared to symbiotic individuals including those that were untreated, reinfected with the natural symbiont community recovered from eggs, or with the culturable symbiont strain Lv-StA (Figure 2 a, Table S2, Cox mixed effects model, *p* values: untreated: 0.0017, reinfected-egg wash: <0.001, reinfected-StA 0.023), while there was no significant difference between untreated and reinfected larvae (Table S2, *p* value: 0.2). In a second assay, we focused on the natural symbiont community dominated by LvStB and tested its protective effect against two generalist entomopathogens – *Beauveria bassiana* and *Metarhizium anisopliae*– applying fungal titers comparable to amounts of fungal conidia in other studies and agricultural applications of entomopathogens [45–47] or natural soil [48,49]. Because there was no difference between untreated larvae and larvae reinfected with the natural community in the previous assay, we compared symbiont-free larvae to reinfected larvae in the following assays, controlling for the surface-sterilization procedure [10]. To rule out the possibility that symbiotic larvae survived better because of a nutritional benefit by the symbionts, we added a control group that was not exposed to fungi. Again, when exposed to *B. bassiana* or *M. anisopliae,* symbiotic larvae were more likely to survive compared to aposymbiotic larvae (Figure 2 b, Table S3, Cox mixed effects model, *p* values: *B. bassiana*: 0.0028, *M. anisopliae*: 0.026). While the tested *M. anisopliae* strain was overall more virulent than that of *B. bassiana* and symbiont-conferred protection was weaker against *M. anisopliae* (Fig. 2b), the probability of fungal growth was significantly lower in symbiotic individuals for both fungi (Figure 2 c, Table S4, Cox mixed effects model, *p* values: *B. bassiana*: <0.001, *M. anisopliae*: <0.001), in line with a broad-spectrum defense. There were no differences in survival between symbiotic and aposymbiotic larvae in the absence of pathogenic fungi.

To test if symbionts can also protect pupae, we exposed aposymbiotic and symbiotic individuals directly after pupation to fungi and used two set-ups (Table S1). One set-up used a combination of different fungi (fungus-mix: *P. lilacinum, B. bassiana, M. anisopliae*) which was applied to the environment and the other set-up used a topical application of *M. anisopliae* conidia onto the dorsal thorax of pupae. When we exposed pupae to the fungus-mix or only to *M. anisopliae*, there was no difference in emergence rate (Figure S1 a) or survival of adults after emergence between the treatments (Figure S1 b, c). However, we observed melanization spots on the pupal surface, likely as a reaction to fungi, which was

significantly higher in aposymbiotic individuals infected with *M. anisopliae* (Figure S1 d, Cox proportional hazards regression model, p value = 0.037). Additionally, we observed a higher probability of visible fungal growth on pupae infected with *M. anisopliae* (Figure S1 e, Cox proportional hazards regression model, p value = 0.012). Although adult emergence rate and survival after emergence were not different between both groups infected with fungi (aposymbiotic and untreated), all emerged adults died within eight days (Figure S1 c) which is not expected given the usual longevity in absence of fungal infections (Figure 1). This might be due to the high exposure to the pathogen, and/or the gradual decline in Lv-StB titers under laboratory conditions, which has been previously documented [40,50]. Foreseeing this problem, we maintained the symbiotic individuals on soil and live plants, which decreases the risk of symbiont loss [50]. However, only three out of five female pupae tested before the experiment still carried *Burkholderia* spp., and none of the adults that emerged (Figure S1 f). In summary, the natural symbiont community present in the dorsal organs and on the insect's surface inhibited fungal infections in young larvae and pupae and increased the chances of larval survival in the presence of fungal pathogens.

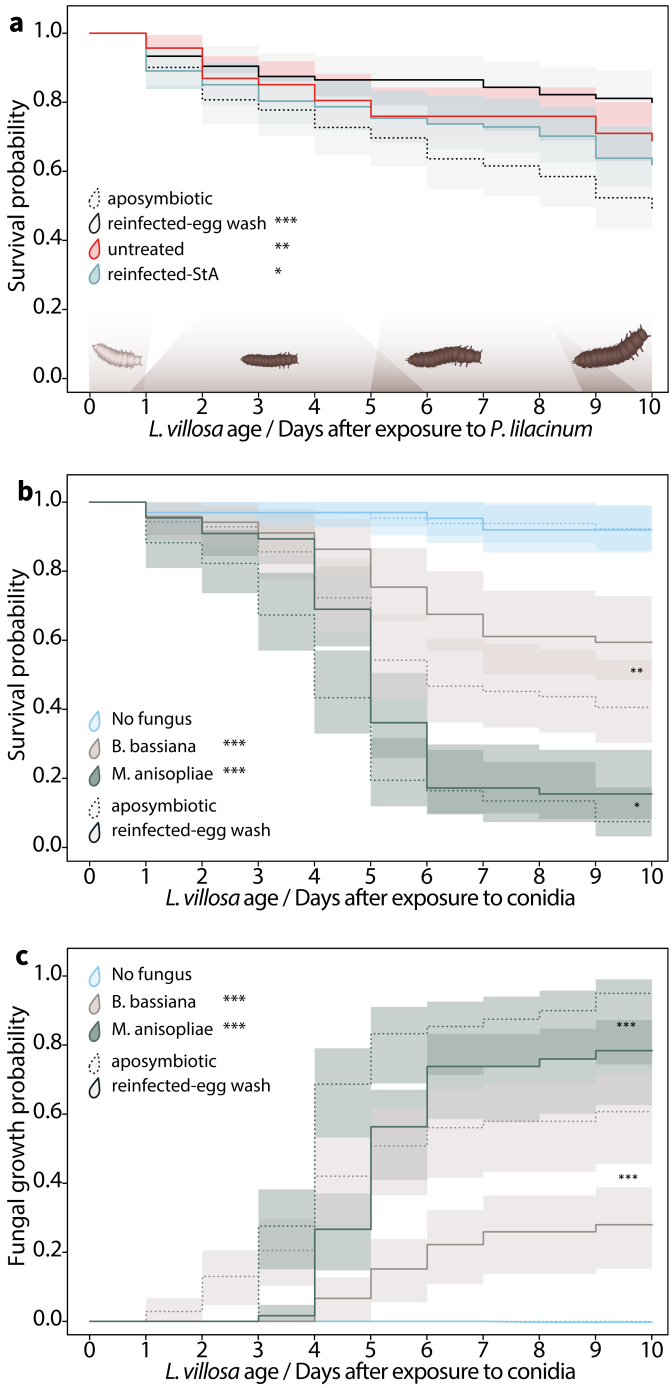

Figure 2: Bacterial symbionts reduce growth of pathogenic fungi and enhance survival of *L. villosa* larvae. 1st instar larvae with (solid lines) or without symbionts (dotted lines) were exposed to different fungal pathogens in small groups and were single-blind monitored for 10 days. **a** Survival was assessed for an environment with 7.5×10^3 *P. lilacinum* conidia in 460 larvae from 8 clutches. **b** Survival and **c** visible fungal infection was assessed for an environment with either 10^6 conidia of *B. bassiana* (beige lines), *M. anisopliae* (dark green lines) or a no-fungus-environment (light blue lines) (in total 404 larvae from 4 clutches). Statistically significant differences in relation to aposymbiotic controls or no fungus controls (treatment legends in b and c) based on Cox mixed effects model: $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. Estimated survival curves (Kaplan–Meier) and the corresponding 95% confidence intervals are shown.

3.2. Symbiont strain Lv-StB is maintained in special dorsal cuticle invaginations and becomes dominant during *L. villosa* development

To evaluate if protection in larval and pupal stages is mediated by the same symbionts as previously described in eggs, we investigated the strain composition of field-collected *L. villosa* individuals and tested whether Lv-StB dominates the community beyond the egg stage (Figure 1 a). As in female glands and on the egg surface [10], Burkholderiaceae were consistently present in larvae and pupae (Figure S2 a). While Enterobacteriaceae and Rhizobiaceae were also predominant in larvae, the profiles correspond to the full body, and these are therefore most likely associated with the gut. As observed directly by in-situ localizations using microscopy, *Burkholderia* is present and becomes dominant in the dorsal structures of older larvae (Figure S3). LvStB is particularly predominant in the community, being present in 95% of the samples and showing the highest relative abundance among *Burkholderia* strains in 82% of the 58 samples (Figure 3 a). The three individuals in which Lv-StB was not detected showed high relative titers of the culturable strain *B. gladioli* Lv-StA, or a closely related strain (Figure 3 a, Figure S2 b). In early larvae, Lv-StB was present in very low abundance, judging from the absolute read counts in these samples (Figure S2 b) and FISH (Figure S3 a-c). While this might result from a bottleneck during vertical transmission or natural variation among individuals, we cannot rule out that the low titers are associated with laboratory conditions, since *Burkholderia* symbionts are often lost during controlled rearing [40,50].

To identify potential differences in absolute symbiont abundance at a fine temporal scale during development, we quantified Lv-StB symbiont titer using qPCR across larval and pupal stages. Additionally, we considered both field-collected and laboratory-raised individuals for the life stages in which this was feasible (Figure 3 b). The mean abundances of Lv-StB copy numbers were the highest in females and tended to decrease in laboratory-raised individuals as host development advanced. As expected [40], Lv-StB titers of later timepoints were higher in field-collected individuals than in laboratory-raised individuals. Taken together, the results suggest that Lv-StB is the dominant *Burkholderia* strain across larval development and is consistently present throughout the life cycle (Figure 3 a). Additional involvement of other bacteria in protection is also possible, given the consistent presence of specific groups, albeit in lower relative abundance in symbiotic organs.

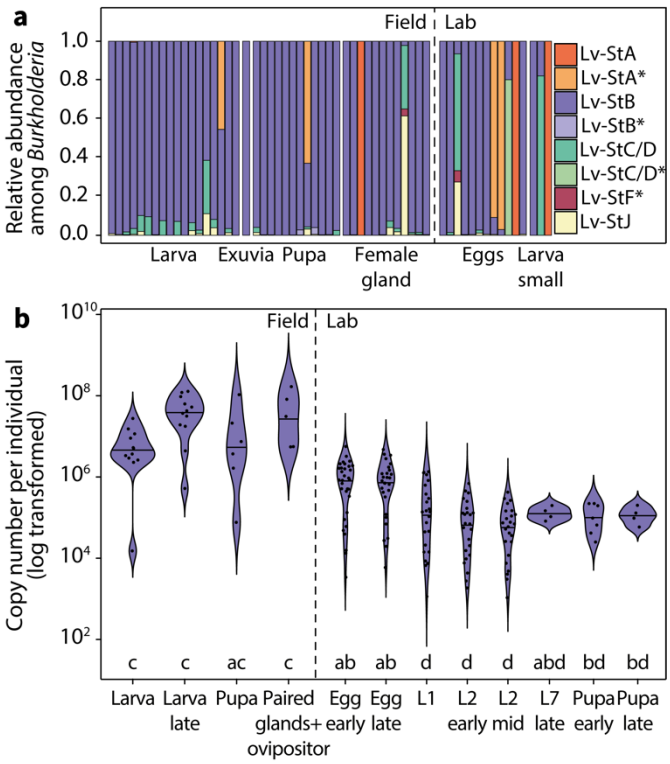

Figure 3: *Burkholderia* Lv-StB abundance throughout host development. a Relative abundance of *Burkholderia* strains across different life stages based on Illumina sequencing of the 16S rRNA gene V4 region compared to whole genome or MAG references of previously described strains. Each bar corresponds to a single individual, dissected organ of an adult female ("female gland") or egg clutch. An asterisk (*) in the legend denotes pairwise identity above 98% but below 100%. **b** Lv-StB titers across host development in field and laboratory-raised individuals (Table S6) measured by qPCR with primers targeting the single-copy trans-AT PKS/NRPS lgaG gene involved in the biosynthesis of lagriamide by Lv-StB. Different letters indicate significant differences between life stages (Kruskal-Wallis $\chi 2 = 97.712$, df = 11, p value = 5.1×10^{-16}, post hoc Dunn's Test, $\alpha \leq 0.05$).

To localize Lv-StB in each life stage and further assess its dominance within the symbiotic organs, we carried out fluorescence *in situ* hybridization (FISH) with specific probes targeting this strain (Figure S4, Figure S5) on histological sections throughout host development (Figure 4). After symbionts are transmitted from female accessory glands (Figure 4 a) to the egg surface (Figure 4 b), embryos develop three equally sized organs located dorsally, which likely originate as invaginations of the cuticle. From the first larval instar on (L1, Figure 4 c), the pouches are filled with symbionts. These peculiar organs remain open to the outside through a small cuticle-lined canal and are maintained throughout larval development, increasing in size as the insect grows [42] (Figure 4 d & e). In pupae, symbionts can be found in the first dorsal pouch (Figure 4 f) and on the surface of the head region between bristles (Figure 4 g). To evaluate if ectosymbionts are released from the pouches, we carried out FISH on whole exuviae of the first (L1) and last larval instar (larva-to-pupa molt), as well as exuviae from pupae (pupa-to-adult molt). We detected *Burkholderia* symbionts on the external surface of L1 exuviae (Figure 4 h), which might be explained by contact with the egg chorion during hatching. We also found high amounts of *Burkholderia* on the internal surface of larva-to-pupa exuviae (Figure 4 i) and the outside of those from pupa-to-adult (Figure 4 g). These findings indicate that the symbionts not only colonize the dorsal organs but are also present on the surface of early larvae and pupae. The probe used to target Lv-StB cells showed low-intensity unspecific labeling on a pure Lv-StA culture (Figure S4 a-b), but a direct comparison on an egg wash (Figure S4 c, d) and tissue sections (Figure S5) confirmed much more prominent labeling of Lv-StB cells, supporting the results observed by sequencing. In summary, Lv-StB is the most prevalent bacterial strain in symbiotic pouches during the life cycle of *L. villosa*, and symbionts are also released to the surface of larvae and pupae, as indicated by their presence on exuviae and pupal bristles.

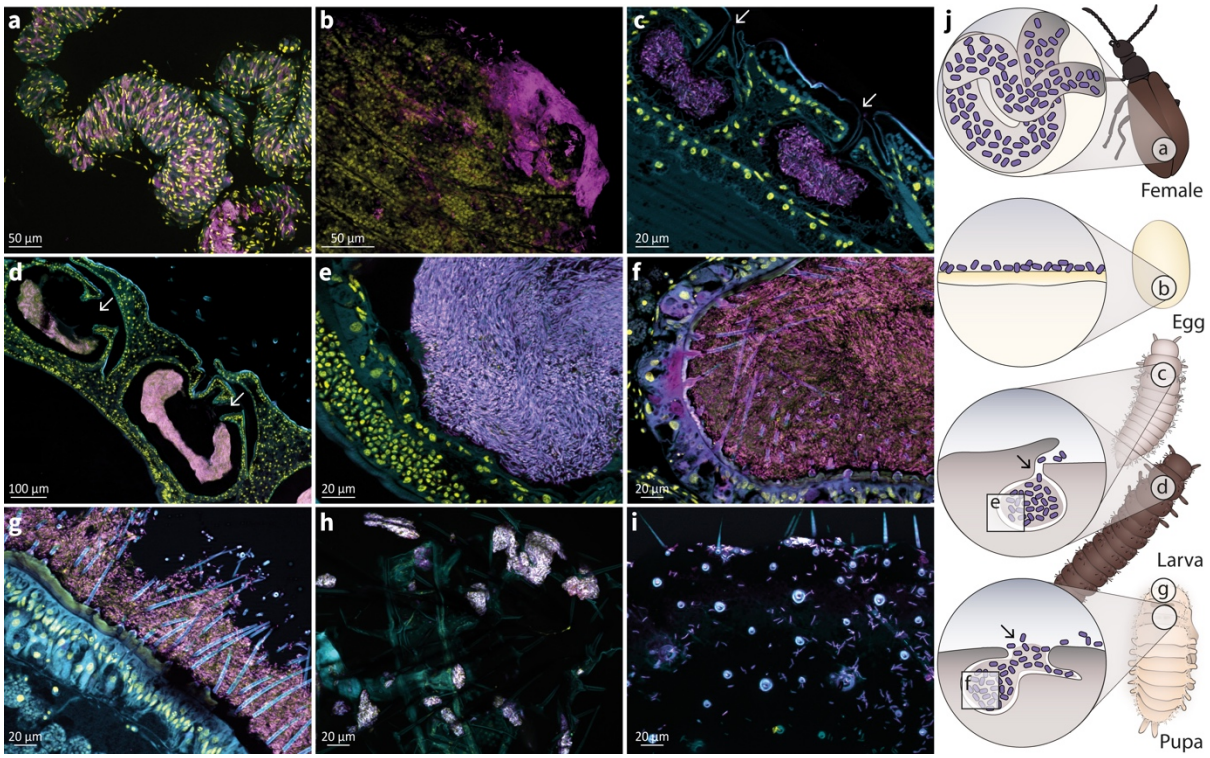

Figure 4: *Burkholderia* Lv-StB symbiont localization and prevalence throughout the different life stages of *L. villosa* using fluorescence *in situ* hybridization. Symbiont cells are generally depicted in magenta, and host cell nuclei in yellow. **a** Whole mount of a female abdomen showing a dense population of Lv-StB in tubes of the accessory glands. **b** Whole-mount of an egg revealing Lv-StB dominance. **c** Sagittal section of a 1st instar larva revealing a dense culture of Lv-StB in the dorsal symbiotic structures as well as an opening to the external environment (white arrows) **d** Sagittal sections through the three pouches of a later larval stage showing the same morphology and **e** the dominance of Lv-StB inside the pouch. **f** 1st dorsal symbiont compartment and **g** outer surface of a pupa showing dominance of Lv-StB. In a-g *B. gladioli* specific staining is shown in cyan (Burk16S_Cy3), Lv-StB-specific staining in magenta (Burk16S_StB_2_Cy5), and host cell nuclei in yellow (DAPI). **h** First larval exuvia covered with *B. gladioli*. **i** Inner side of a larva-pupa exuvia with visible symbionts cells. FISH on exuviae (h, i) show general eubacterial staining in cyan (EUB338_Cy3) and *B. gladioli* specific staining in magenta (Burk16S_Cy5). **j** Schematic guide illustrating symbiont localization throughout *L. villosa* development. 1st instar larva individuals are from the 1st lab generation, and all other individuals are field collected. In every image autofluorescence of the host tissue is shown in cyan and overlap of all three channels is shown in purple-white. Arrows indicate the opening of the symbiotic structures in larvae and pupae. Scale bars correspond to 20 µm, except for panels a (50 µm) and d (100 µm).

3.3. Presence of lagriamide throughout the host life cycle and bioactive potential

As shown in Figure 4, Lv-StB is highly abundant in larvae and pupae, the symbiotic pouches are connected to the outer surface and the symbionts are present on the outer and inner surface of exuviae. To gain further insight into the chemical basis and dynamics of defense observed in early larval and pupal stages, we investigated whether the Lv-StB bioactive secondary metabolite lagriamide (Figure 5 a) is also produced during these time points and where it is located in the host. We first analyzed crude extracts of field-collected individuals and offspring of field-collected females by HPLC-HRESI-MS and evaluated the presence of lagriamide by comparing retention time, UV absorbance, and high-resolution mass spectra to an authentic reference. We detected lagriamide in every life stage except in adult males (which lack symbionts), showing that lagriamide is consistently produced across symbiont-bearing life stages of *L. villosa* and is also present on shed exuviae (Figure 5 b).

Using APSMALDI-HR MSI, we localized lagriamide on the egg surface, in larval and pupal symbiotic organs as well as distributed across the surface of exuviae from larval molts (Figure 5 c-h, Figure S6), whereas we could not detect it on individuals without the natural symbiont community (Figure S7). Thus, lagriamide co-localizes with Lv-StB throughout host development, i.e., in female accessory glands, on the egg surface, in symbiotic organs of larvae and pupae, and on exuviae. Although we did not detect lagriamide on the surface of larval sections via APSMALDI-profiling, possibly because the abundance resulting after histological sectioning is below the detection limit, HPLC-MS, and APSMALDI-profiling confirmed its presence on intact molted exuviae, and it spatially coincided with Lv-StB presence. Additionally, *in vitro* activity profiling of pure lagriamide revealed that it hinders the growth of *B. bassiana* and *M. anisopliae* on plate, showing partial inhibition (Figure S8 a-b). This observation is in line with the previously reported antifungal activity against *P. lilacinum* and *Aspergillus niger* [11], and the larval antifungal assays (Figure 2). However, we did not observe antibacterial effects against the entomopathogen *Bacillus thuringensis* under these conditions (Figure S8 c). Taken together, these findings indicate that the dominant symbiont strain Lv-StB produces the antifungal polyketide lagriamide throughout the developmental stages of *L. villosa* from eggs to pupae.

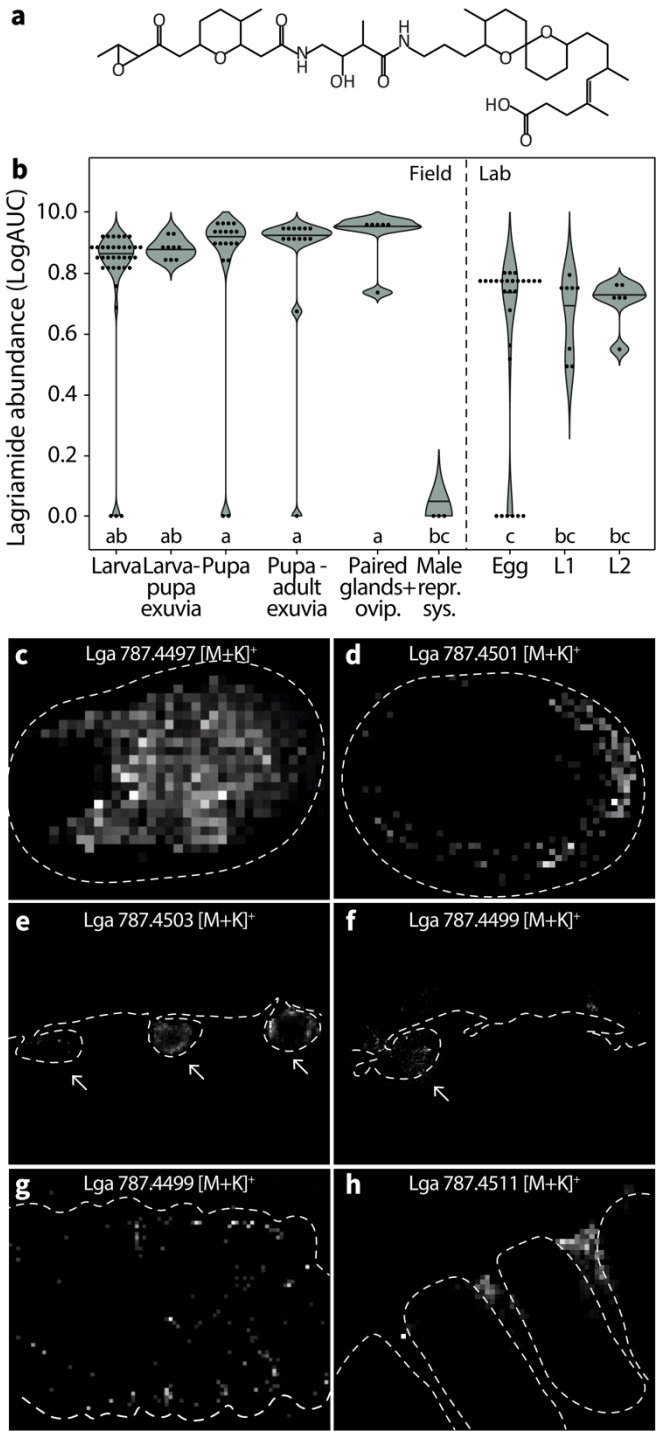

Figure 5: Lagriamide is present across *L. villosa* life stages and co-localizes with Lv-StB in regions exposed to the external environment. a Chemical structure of lagriamide 11. **b** Area under curve (AUC) of the extracted ion chromatogram (EIC) of lagriamide (m/z = 747.4769 – 747.4843 [M-H]-) representing abundance across host development (Table S7), as quantified from crude methanol extracts. Eggs, as well as first (L1) and second (L2) instar larvae correspond to offspring from field-collected females. All others correspond to field specimens. Different letters indicate significant differences between life stages (Kruskal-Wallis $\chi 2$= 66.988, df = 8, p value = 1.95e-11, post hoc Dunn's Test, $\alpha \leq 0.05$). c-h 2D ion maps obtained by AP-SMALDI-MSI representing the potassium adducts of lagriamide [M+K] + across *L. villosa* life stages. **c** Surface analysis of an intact egg and **d** an egg cryosection showing lagriamide presence on the surface. **e** In larval sections, lagriamide is present inside the symbiotic organs (arrows). **f** In

pupal sections, lagriamide is mainly detected in the first symbiotic organ (arrow). **g** On the inner surface of a larva-to-larva exuvia, lagriamide is either scattered over the thoracal segments or **h** distinctly located between the thoracic segments and first abdominal segment, which corresponds to the location of the symbiotic organs. Dotted lines were manually added based on corresponding light microscopy pictures (Figure S7) to indicate specimen profiles.

4. Discussion

L. villosa beetles are associated with a bacterial community dominated by *Burkholderia* [10] harbored in specialized symbiotic structures only absent in male adults. The symbionts are vertically transmitted, although sporadic acquisition from the environment likely occurs [9, 42]. Multiple *Burkholderia* strains can infect the beetles, occasionally simultaneously [40], and at least two —Lv-StB and Lv-StA— are known to produce antimicrobial compounds inhibiting fungal growth on the insect's eggs and thereby enhance survival [10,11]. Here, we show consistent presence of the antimicrobial lagriamide and its producer Lv-StB throughout the host life cycle. *In situ* localization of both Lv-StB and lagriamide confirms their presence within open symbiotic organs and their distribution across the surface of shed exuviae, demonstrating release of symbionts and the protective compound during larval molting. Reduced fungal growth and enhanced survival of young larvae in presence of Lv-StB, when confronted with different fungi and *in vitro* antifungal activity of pure lagriamide against fungi, demonstrate the protective role. These findings reveal a case of host adaptation to house ectosymbionts in specialized cuticular organs, which facilitate external release of bacteria and the associated compounds that aid in host protection. At the same time, these organs offer a suitable environment for growth and maintenance of antibiotic-producing ectosymbionts despite recurrent molting.

Like other arthropods living in the soil, *L. villosa* larvae and pupae face the challenge of fungal pathogens in the environment, a risk that is particularly high in phases with reduced structural protection by the incompletely sclerotized cuticle after molting [38,39]. The cuticle is a key protective barrier against pathogenic fungi in almost all arthropods because of its rigidity [3], but also through the presence of antifungal proteins (e.g. chitinase-inhibitors or glucanases), antimicrobial peptides, or small molecules with inhibitory activity, e.g. cuticular fatty acids or benzoquinones [43,51,52]. For example, *Galleria mellonella* morphs with higher amounts of melanin, increased dihydroxyphenylalanine (DOPA) decarboxylase activity but lower amounts of hydrocarbons showed reduced fungal attachment and germination, resulting in increased host survival in comparison to weakly melanized larvae [53]. Yet, relying on the cuticle as a primary armor can involve important tradeoffs. While the phenomenon of molting practiced by ecdysozoans, including arthropods and nematodes, enables growth or reaching the next life stage, it also entails the cost of a temporarily softer and thinner integument. Right after molting, individuals might therefore be more susceptible to natural enemies, as described for some crustaceans [54,55]. However, molting itself can shed off epibionts [56] or pathogens that were not able to penetrate the cuticle. In the crustacean *Daphnia magna*, for example, the bacterial pathogen *Pasteuria ramosa* has lower chances of a successful infection when the host molts soon after exposure to the pathogen [57]. Also, in the diamondback moth *Plutella xylostella*, larvae inoculated with fungi early during an instar are more likely to die than if inoculated later during the same instar [58]. It is also possible that fungi can breach the cuticle during vulnerable periods and proceed with infection within the insect host even after several molting events, as occurs in larvae of Colorado potato

beetles [59]. Taken together, such findings in different arthropods indicate that while the cuticle itself is the first major barrier against pathogens and molting can remove both beneficial and harmful microbes from the surface, the early period after ecdysis is particularly vulnerable due to a softer cuticle and/or the longer period available for pathogens to colonize and establish before the next molt.

Despite a long history of research on defense mechanisms of arthropods against fungal pathogens [3], the vulnerability of molting stages and adaptations for protection in these phases have rarely been studied, particularly in insects. However, in *Bombyx mori* and *Tribolium castaneum* the molting fluid that is secreted and accumulates during molting between the old and new cuticle [60] not only enables successful ecdysis but could also protect vulnerable post-molt insects against microbial pathogens [37]. In *L. villosa*, critical phases where structural protection is limited are the first larval instar, which remains unmelanized for around 24 hours, and the phases after each of the seven larval molts and during pupation, where individuals also lack mobility. By exposing symbiotic and aposymbiotic individuals to pathogens over the first 10 days after hatching and during pupation, respectively, we covered multiple molting events as well as metamorphosis and showed that cuticle-associated symbionts can persist despite recurrent molting and provide an effective means for protecting these life stages against fungal pathogens. Our setup does not directly demonstrate that fungal entry is occurring immediately after ecdysis in aposymbionts, yet the presence of both the symbionts and the antifungal lagriamide over extended areas of the inner surface of shed exuviae are in line with their role as a protective barrier to infections upon molting. In pupae, our results suggest a moderate inhibition of fungal growth by the symbionts, although we did not find a strong advantage in pupal emergence or survival shortly after emergence. This might be associated to the potential loss of symbionts after some time under laboratory conditions in untreated individuals, as previously reported for this insect [40]. Aposymbiotic beetles that are not challenged with fungal infections can fully develop and lay eggs in the laboratory [10]. It is thus challenging to manipulate and individually track the symbiotic status of *Lagria* beetles up to the adult stage. However, the inhibition of fungal growth on pupae, the high abundance of Lv-StB symbionts on the pupal surface, and lagriamide detection inside the enlarged dorsal organ are in line with symbiont-mediated protection during pupation. Altogether, our results reveal symbiont-provided protection against fungal pathogens as an adaptive defense strategy of immature insect life stages, which likely supplements existing immune responses and structural defenses.

The evolution of the protective symbiosis in *Lagria* beetles likely relied on the dorsal cuticular invaginations as a morphological adaptation facilitating accommodation and release of ectosymbionts. The external location of symbionts makes defensive metabolites readily available for deterring antagonists that attack insects from the exterior [61], while the crypt-like structures grant shelter to symbionts and likely provide nourishment from surrounding glandular cells [42]. Similarly, attine ants carry defensive *Pseudonocardia* bacteria in cuticular crypts on their surface, which protect both ants and their fungal gardens against pathogens [6264]. Another structurally and functionally similar organ is found in solitary

beewolf wasps, which harbor *Streptomyces* bacteria in cuticle-lined reservoirs of their antennae that protect the developing larva by producing an antibiotic cocktail [12,13,65,66]. Also, ectosymbionts of marine ciliates defend their host against predators, and bacterial symbionts on developing embryos of certain shrimps and lobsters can inhibit infections by pathogenic fungi [67,68]. Cuticular organs that foster symbiont colonization are also described in several other insects, and fungus-bearing mycangia of wood-boring and bark beetles, as well as leaf-rolling weevils, resemble pouches of *L. villosa* larvae [16]. Interestingly, although the pouches of *L. villosa* larvae are invaginations of the cuticle and are connected to glandular cells, they are not shed off with the exuvia as usually seen in exocrine glands of insects [69]. This prevents a complete loss of symbionts during molting, which is also reflected by similar symbiont titers from larvae after molting and intermolt stages (Figure 3 b, comparison between early L2 and mid L2). Similarly, other molting arthropods like nymphs of the phasmid *Oreophoetes peruana* do not discard the cuticular lining of their defensive glands during molting, conserving the costly secretion and keeping it available as a chemical defense directly after molting [70]. The external location of the symbionts might be additionally favorable for the host to avoid contact with potentially harmful bioactive secondary metabolites produced by the bacteria [7].

Although it was previously demonstrated that multiple *Burkholderia* strains (including LvStA) can coinfect single *L. villosa* beetles [10] and FISH revealed occasional presence of other bacteria and strains in the organs of younger larvae (Figure S3 a-c), LvStB is generally predominant and shows signs of a tight association with its host. Possibly, host provisions support its proliferation and enable it to remain in the organs despite its reduced genome [71]. The dominance of this strain also underlines the putative importance of the bioactive polyketide lagriamide for host protection or competitive exclusion of coinfecting microorganisms. However, other *Burkholderia* strains and community members might also contribute to host protection, as they were either shown to be capable of producing bioactive metabolites [10,72,73] or to carry gene clusters for putative defensive molecules [71]. Such coinfections by closely related symbiont strains in a single individual as observed in *L. villosa* are rare in insects, or so far understudied. However, defensive symbioses are often described as dynamic and can be shaped by multiple coinfections throughout a population or by symbiont replacements [7], offering an opportunity to acquire multiple defensive partners.

Like other insects harboring defensive symbionts with a broad bioactive potential [12,13], *Lagria* beetles are not challenged by a single antagonist, but rather by a variety of pathogens in the soil. Having the possibility for symbiont uptake via a mixed-mode symbiont transmission and simultaneously a tight association to one specific defensive symbiont strain, the beetle might have evolved a flexible strategy to adapt to various antagonists. Thus, the protective effect of the dominant symbiont LvStB through the production of lagriamide may be complemented by other compounds of associated microorganisms.

In conclusion, we show that in *L. villosa*, the adaptations to house antifungal-producing *Burkholderia* bacteria in cuticular invaginations in larvae and pupae enable defense against entomopathogens throughout development including multiple molting phases. These structures foster a specialized association with a specific defensive symbiont strain while maintaining flexibility to acquire additional protective strains from the environment. In contrast to highly intimate nutritional symbioses in which morphological adaptations such as bacteriomes have evolved, defensive symbioses usually have intrinsically different constraints regarding where and how microorganisms are maintained for effective protection, especially if deploying bioactive compounds. Cuticle-associated ectosymbionts can therefore be an effective and dynamic defense strategy influencing antagonistic interactions between arthropods and pathogenic microbes.

5. Methods

5.1. Insect collecting and rearing

L. villosa individuals were collected in plantations of various crops in the states of São Paulo and Paraná, Brazil, from 2017 to 2020 (Table S7). Individuals were reared in plastic containers in a climate chamber (16:8 L:D light regime with 0.5 h dusk, 26 °C, and 60% humidity). Adult beetles were fed with iceberg lettuce, soybean, and kidney bean leaves, and larvae were fed with dry soybean and kidney bean leaves. Centrifuge tubes with autoclaved tap water and cotton on top and centrifuge tube lids with moist cotton were provided for humidity control and as egg-laying substrate.

5.2. Bacterial community profiling via 16S rRNA gene amplicon sequencing

L. villosa specimens were collected directly from the field or recovered in the lab as offspring of field-collected females in case of eggs and small larvae and preserved in ethanol or methanol. Accessory glands of female specimens were dissected before DNA extraction. Eggs were extracted as full clutches or a fraction of a single clutch (min. 18 eggs per clutch), while small larvae were extracted individually and later pooled by clutch combining DNA from either three (10 days old) or two (15 days old) individuals. All other samples were extracted individually. The Epicentre MasterPure Complete DNA and RNA Purification Kit was used for DNA extraction of a total of 58 samples following the manufacturer's instructions and including lysozyme treatment before protein digestion. The V4 region of the 16S rRNA gene was sequenced by a commercial provider (StarSeq, Mainz, Germany) on a MiSeq platform (Illumina), using primers 515F [74] and 806bR [75,76], double indexing, and a paired end approach with a read length of 300 nt. Amplicon sequence variants (ASVs) were inferred using the R package DADA2 [77] with default parameters including dereplication, chimera removal, and trimming lengths of 250 and 140 nt for forward and reverse reads, respectively. Taxonomy was assigned using the pre-trained classifier SILVA 132 [78,79] with subsequent removal of reads classified as chloroplast or mitochondria. For graphical representation, only ASVs were shown that reached 1% or more of the reads per sample, in at least one sample. ASVs within Burkholderiaceae classified as "*Burkholderia-Caballeronia-Paraburkholderia*" or not assigned at the genus level were evaluated manually to identify previously described *Burkholderia* symbiont strains [11,71], for which whole-genome or Metagenome-assembled Genome (MAG) references are available. The representative sequences were blasted against a local database containing Lagriinae-associated *Burkholderia* strain sequences. Four of these ASV representative sequences which had a pairwise identity of 100% to a sequence in the local database and another four ASVs that were between 98–99.6% in similarity were assigned accordingly. All others were below 93% in similarity.

5.3. Quantification of Lv-StB throughout beetle life stages

Different developmental stages (Table S5) of *L. villosa* individuals from the field and the 1st lab generation were collected in replicates and frozen at −80 °C before nucleic acid extraction. Females were dissected beforehand to obtain only the symbiont-bearing structures. Samples were homogenized with liquid nitrogen and nucleic acids were extracted using the innuPREP DNA/RNA Mini Kit (Analytik Jena) following the manufacturer's guidelines. qPCRs were carried out with the DNA targeting the trans-AT PKS/NRPS lgaG region of the lagriamide gene cluster with the specific primers LgaG_3_fwd (CGCCGTATCGAGCAGTTTC) and LgaG_3_rev (CAACTGGTCGAGCGTATCAA) under the following conditions: Initial activation at 95 °C for 15 min, denaturation at 95 °C for 15 s, annealing at 65 °C for 15 s and elongation at 72 °C for 15 s. qPCRs were carried out using the 5x HOT FIREPol EvaGreen HRM Mix EvaGreen (Solis BioDyne) on a RotorGene-Q cycler (Qiagen) in 10 µL reactions including 0.5 µL of each primer and 1 µL template DNA. Standard curves were generated by amplifying the fragment, followed by purification and determination of the DNA concentration using a Qubit fluorometer (Thermo Fisher). Afterwards, a standard containing 1 ng/µL was generated and 1:10 serial dilutions to 10^{-8} ng/µL were prepared. All standards were included in the qPCR run for absolute quantification. qPCR copy numbers were corrected for the number of individuals extracted in each sample (Table S5). Influence of the different life stages on the symbiont titer was analyzed by a Kruskal-Wallis and a post hoc Dunn's test using the PMCMRplus package (Version 1.9.0) in RStudio (Version 1.2.5042). Plots were created using the ggplot2 package (Version 3.3.0) and Adobe Illustrator (Adobe, Version 14.1, CC 2020).

5.4. Symbiont localization by fluorescence *in situ* hybridization (FISH)

FISH was either performed on entire tissue (whole-mount), on semithin sections of *L. villosa* individuals, on an egg wash, or pure symbiont culture (Lv-StA). Either field-collected individuals (female, bigger larvae, pupae, larva-pupa-exuvia) or offspring of field-collected females (egg, L1 larva, L1 exuvia) were used. Before FISH, *L. villosa* individuals and exuviae were fixed in 4% formaldehyde for at least 3 days, while the egg wash and symbiont culture were fixed on glass slides with ethanol. Embedding, semithin sectioning, and FISH were performed as described previously [80] using a hybridization temperature of 50 °C. The Cy3- or Cy5-labeled Burk16S probe (5'TGCGGTTAGACTAGCCACT'3) was used to mark all *Burkholderia* strains, and the Cy5-labeled Burk16S_StB_2 probe (5'GGCAACCCTTTGTTTTGACC'3) was used for the symbiont strain Lv-StB, Cy5-labeled Burk16S_StA probe (5'GCACCCTCAGATCTCTCCAAGG3) was used for symbiont strain Lv-StA and Cy3-labeled EUB338 probe [81] (5'GCTGCCTCCCGTAGGAGT'3) was used for general eubacteria. DAPI (40,6-diamidino-2-phenylindole) was used to label the host cell nuclei and as counterstaining. Images were taken on an AxioImager.Z2 fluorescence microscope (Zeiss, Jena, Germany).

5.5. *In vivo* bioassays with *L. villosa* larvae

Larval bioassays (Table S1) were carried out at 26 °C in small Petri dishes prepared as follows: Sterile vermiculite (1-2 mm) was distributed over the bottom of the dish and humidified with sterile water. Sterile filter paper inoculated either with 100 µL sterile water (no-fungus-treatment) or 100 µL of a fungal conidial suspension in sterile water (75 conidia/µL of *P. lilacinum* (LV1, Accession number: KY630747, KY630748, KY630749), or 10^4 conidia/µL of *B. bassiana* (LESF477, Database Identifier: CRM 1216, CRM - UNESP) or *M. anisopliae* (LESF 206, Database Identifier: CRM 530, CRM - UNESP), respectively) was added. In each dish, maximum five 1st instar larvae were placed according to their treatment and single-blind monitored for 10 days. UV-sterilized dried soybean and kidney bean leaves were provided as food sources every other day. To obtain aposymbiotic and differently reinfected 1st instar *L. villosa* larvae, 1–2 day(s) old egg clutches of field-collected females were divided into different groups. One part of the clutch was used as a control with the natural microbial community (untreated). Remaining eggs were first washed with sterile PBS, slightly shaken for 5 min in 70% ethanol, washed 2 times with sterile water, immersed for 30 s in 12% NaClO, and washed three times with sterile water. Eggs were then inoculated with PBS to obtain symbiont-free individuals (aposymbiotic), with the previously obtained egg-wash that contains the natural microbial community (reinfected-egg wash, previously named "reinfected-natural" [10]), or with a PBS suspension of a cultured symbiotic strain of *L. villosa* beetles (10^6 cells/µL) — *B. gladioli* Lv-StA (reinfected-LvStA). While this strain is only occasionally found in the beetles in natural conditions, we included it in this first assay to evaluate its protective potential, since it has been previously shown to chemically inhibit fungal growth on *L. villosa* eggs [10]. The *P. lilacinum* assay was carried out with larvae from eight clutches laid by different *L. villosa* field-collected females in two consecutive years. Survival of hatching larvae was observed for 10 days in a single-blind assay. B. gladioli and Lv-StB presence on the egg clutches was verified via qPCR using *B. gladioli* specific primers Burk16S_1_F (GTTGGCCGATGGCTGATT) and Burk16S_1_R (AAGTGCTTTACAACCCGAAGG) and Lv-StB specific primers LgaG_3_fwd and LgaG_3_rev on the five clutches from the 2020 population qPCR was carried out under the following conditions: Initial activation at 95 °C for 15 min, denaturation at 95 °C for 15 s, annealing at 65 °C for 15 s and elongation at 72 °C for 15 s.

The assay including *B. bassiana*, *M. anisopliae*, and the no-fungus-control was carried out with four clutches laid by different *L. villosa* field-collected females comparing aposymbiotic and reinfected-egg wash individuals. Given the inconsistent occurrence of Lv-StA in natural conditions and giving priority to the naturally dominant strain Lv-StB, a Lv-StA reinfection treatment was not included in this assay to guarantee a robust sample size per group. After hatching, larvae were observed for 10 days monitoring survival, developmental time, and fungal infection in a single-blind assay. To assess fungal growth, larvae were monitored under a stereoscope for visible fungal hyphae and sporulating fungus on the larval surface. Fungal and bacterial strains were grown on King B Agar plates or King B liquid medium.

Symbiont presence of Lv-StB on the original eggs and larvae was verified via qPCRs of DNA extracts from a subset of untreated eggs and surviving larvae from each treatment using Lv-StB specific primers LgaG_3_fwd and LgaG_3_rev.
To determine the effect of different treatments on survival and fungal growth probability on *L. villosa* larvae, a Cox mixed effects model with treatment and fungus as fixed effect and a random intercept per clutch and per year was fitted using the coxme package (Version 2.2-16) in RStudio (Version 1.2.5042) (Table S2-S4). Plots were obtained with the rms package (Version 5.1-4), making use of the Kaplan-Meier-estimator, and Adobe Illustrator (Adobe, Version 14.1, CC 2020).

5.6. *In vivo* bioassays with *L. villosa* pupae

The pupal bioassays (Table S1) were carried out at 26 °C with no light in 24 well-plates. Each well was prepared with soil that was inoculated with 100 µL of fungus suspension 2 - 4 days before the experiment. The fungus mix for the first assay contained 10^6 conidia of each of the three fungi *B. bassiana*, *M. anisopliae*, and *P. lilacinum*. For the second assay, 10^6 conidia of *M. anisopliae* were inoculated into the soil and additional 10^6 conidia were inoculated onto the dorsal thorax of the pupae directly after pupation. Aposymbiotic and untreated pupae from the first lab generation were randomly placed in each well as soon as they emerged. Melanization and fungal growth were observed single-blind until emergence. Aposymbiotic individuals were obtained through the egg sterilization procedure described above and kept in a semi-sterile box until pupal emergence (around seven weeks). Symbiotic individuals were reared in a small terrarium on natural soil with growing soybean plants until the last larval stage. To determine the effect of the different treatments on survival, visible fungal infection, and melanization of *L. villosa* pupae, a Cox model was fitted to the data using the survival package (Version 2.2-16) in RStudio (Version 1.2.5042). Plots were obtained with the rms package (Version 5.1-4), making use of the Kaplan-Meier-estimator, and Adobe Illustrator (Adobe, Version 14.1, CC 2020).

5.7. Chemical extraction and quantification of lagriamide

L. villosa specimens (Table S6) were collected in the field or recovered in the lab as offspring of field-collected females in the case of eggs and larvae of the two first instars. Eggs were extracted as a fraction of the clutch including up to 60 eggs. First and second instar larvae were combined in multiple pools of up to 22 individuals for extraction. Field-collected larvae, pupae, and exuviae were extracted individually. For female adult specimens, the ovipositor and both accessory glands were dissected. For adult males, the full reproductive system was dissected. Replicate numbers for each sample type are indicated in Table S3. Each sample was extracted in a known amount of methanol (200–600 µL) for at least 24 h. Extracts were kept at 10 °C until further processing. Crude extracts were analyzed by LC-MS on an Exactive Orbitrap High Performance Benchtop LC-MS (Thermo Fisher Scientific)

with an electron spray ion source and an Accela HPLC System, C18 column (Betasil C18 5 µm, 150 × 2.1 mm, Thermo Fisher Scientific), solvents: acetonitrile and water (both supplemented with 0.1% formic acid), flow rate: 0.2 mL min^{-1}; program: hold 1 min at 5 % acetonitrile, 1–16 min 5–98% acetonitrile, hold 3 min 98% acetonitrile. Lagriamide amounts were measured by integration of the peak areas of the extracted mass traces (m/z = 747.4769 – 747.4843 $[M-H]^-$) (ICIS integration algorithm in XCalibur, Thermo Fisher Scientific).

5.8. Lagriamide localization by AP-SMALDI-HR MSI experiments

L. villosa specimens were collected in the field or recovered in the lab as offspring of field-collected females in the case of eggs and exuviae. Aposymbiotic individuals were collected from a culture of the 6th lab generation. All samples were analyzed using an atmospheric pressure - scanning microprobe matrix-assisted laser desorption ionization (AP-SMALDI10, TransMIT, Gießen, Germany) ion source equipped with a UV (337 nm) nitrogen laser (LTB MNL-106, LTB, Germany). AP-SMALDI10 was coupled to a mass spectrometer Q-Exactive Plus (Thermo Fisher Scientific, Bremen, Germany) providing high-resolution mass spectra. Raw MS data were collected via Xcalibur software v.2.8 Build 2806 (Thermo Fisher Scientific), while the acquisition of spatial scans, predefined by the user in x- and y-directions as a rectangular sample region, was controlled by the Master Control Program (TransMIT [82]).
Sample heterogeneity required minor changes in specific parameters of the AP-SMALDI-HR MSI method. Therefore, egg samples (cryosections and intact eggs) were imaged with a step size of 10 - 15 µm. Exuviae samples were analyzed based on their size using 25 µm - 45 µm. Sagittal cryosections of pupae and larvae sections were analyzed with a step size of 30 µm. The lagriamide standard (30% DMSO at a concentration of 168 ng/µL) was pipetted on hexane extracted fine paper tissue in a volume of 1 µL/mm^2. All samples were imaged with the number of laser shots per spot set to 30 (approximately 1.1 µJ × $shot^{-1}$ to analyze the egg samples; 1.0 µJ × $shot^{-1}$ for exuviae samples and 1.2 µJ × $shot^{-1}$ for the pupae and larvae sections) within a laser frequency of 60 Hz. A mass range was set from m/z 100 to m/z 1,000 with a resolving power of 140 000. AP-SMALDI-HR MS profiling of lagriamide standard was performed in positive ion mode with several laser shots per spot set to 30 (approximately 13 µJ × $shot^{-1}$) within a laser frequency of 60 Hz, a mass range was set from m/z 700 to m/z 850 with a resolving power of 280,000. Ion intensity maps of selected m/z values were generated using a Mirion 3D V3.3.64.13 software package [82] with an m/z width of 0.01 u. Ion maps were normalized separately to the highest intensity for each ion species. All acquisitions were performed in positive polarity and in laser desorption ionization mode (LDI) without applying the MALDI matrix to the sample.
Samples were prepared as follows: exuviae samples were cut open along the dorsal posterior region to flatten them on a double-sided adhesive tape (3M, Maplewood, MN, USA) using a fine tweezer (Figure S9 a, b). This was attached to a microscope glass slide (25 mm × 15 mm, Thermo Scientific, Menzel-Gläser, VWR, Darmstadt, Germany) and finally fixed on the AP-

SMALDI metal target. Intact eggs were carefully picked up from a clutch using a clean tweezer and fixed on a double-sided tape attached to a microscope glass slide (25 mm × 15 mm). Glass slides with egg samples were mounted on an AP-SMALDI metal target and promptly placed into the AP-SMALDI ion chamber to be analyzed. Because of the conditions in the ion chamber and the nature of the egg samples, a negative effect of drying and shape distortion was observed (Figure S9 c, d). For sections, samples were embedded in O.C.T. medium (Tissue-Tek O.C.T. Compound; Sakura Finetek, Torrance, CA, USA), frozen in liquid nitrogen, and cut into 30 µm sections using a Leica cryomicrotome (Leica CM1850, Leica Mikrosysteme Vertrieb GmbH, Germany). Fresh sections were transferred from a cryomicrotome chamber (–20 °C) into a desiccator (filled with potassium hydroxide as a drying agent) to dry without a vacuum for 20 min.

The identity of acquired lagriamide-related ions from *L. villosa* samples, which was based on exact mass, was supported by best matching chemical formulas and isotope patterns generated via Qual Browser/Xcalibur software 3.0.63. This enabled to putatively assign the peaks to lagriamide ($C_{41}H_{68}N_2O_{10}$) adducts (Δ error <1 ppm), which was confirmed with AP-SMALDI-HR MS profiling of a lagriamide standard (Figure S9 e). 3 µL of the lagriamide standard (30% DMSO at concentration 168 ng/µL) was pipetted in three parts onto small pieces of tissue paper glued to a clean microscope glass slide. Except for the potassium adduct, which was predominantly found in MS/MSI datasets, ions representing the $[M+H]^+$ or $[M+Na]^+$ adducts were detected. Presence of all three lagriamide adducts was demonstrated on an extract obtained from an *L. villosa* larval exuvia (Figure S9 f).

5.9. *In vitro* assays for lagriamide antimicrobial activity

Antimicrobial activity of lagriamide was studied by agar diffusion assays against *Bacillus thuringiensis* DSM2048, *Beauveria bassiana* ST000047, and *Metarhizium anisopliae* STH00420 (ATCC 24942). For the fungal assays, fifty microliters of a solution of lagriamide (168 ng/µL in 30% DMSO in methanol) were filled in agar holes of 9 mm diameter (malt extract agar, Merck, seeded with a spore suspension). After incubation at 23 °C for 4 d, the inhibition zone was evaluated. For the antibacterial assay, nutrient agar plates (Standard I nutrient agar, Merck) seeded with the bacterial culture were used and incubation took place at 30 °C. The inhibition zones were determined after 24 h.

5.10.Data availability

Raw sequence data generated in this study and used for 16S rRNA gene bacterial community profiling of *L. villosa* life stages is available in the GenBank SRA database under the BioProject accession number PRJNA790999.

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8. Significance statement

The cuticular exoskeleton of arthropods plays a vital role as a protective barrier against natural enemies. Although shedding the cuticle enables growth and superficial cleansing, it also comes with the potential cost of temporal susceptibility to antagonists. This might be a common challenge among invertebrates, however, little is known about protective mechanisms that are especially relevant during larval development including frequent molting events. Here, we demonstrate symbiont-mediated protection against fungal pathogens by antibiotic-producing ectosymbionts throughout the immature stages of the beetle *Lagria villosa*. We localize an antifungal compound relevant for protection and its bacterial producer, and demonstrate impaired host survival upon symbiont elimination and fungal exposure in larval stages. Our results reveal cuticle-associated bacterial symbionts as an effective and flexible strategy for protecting immature stages in insects.

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10. Supplement

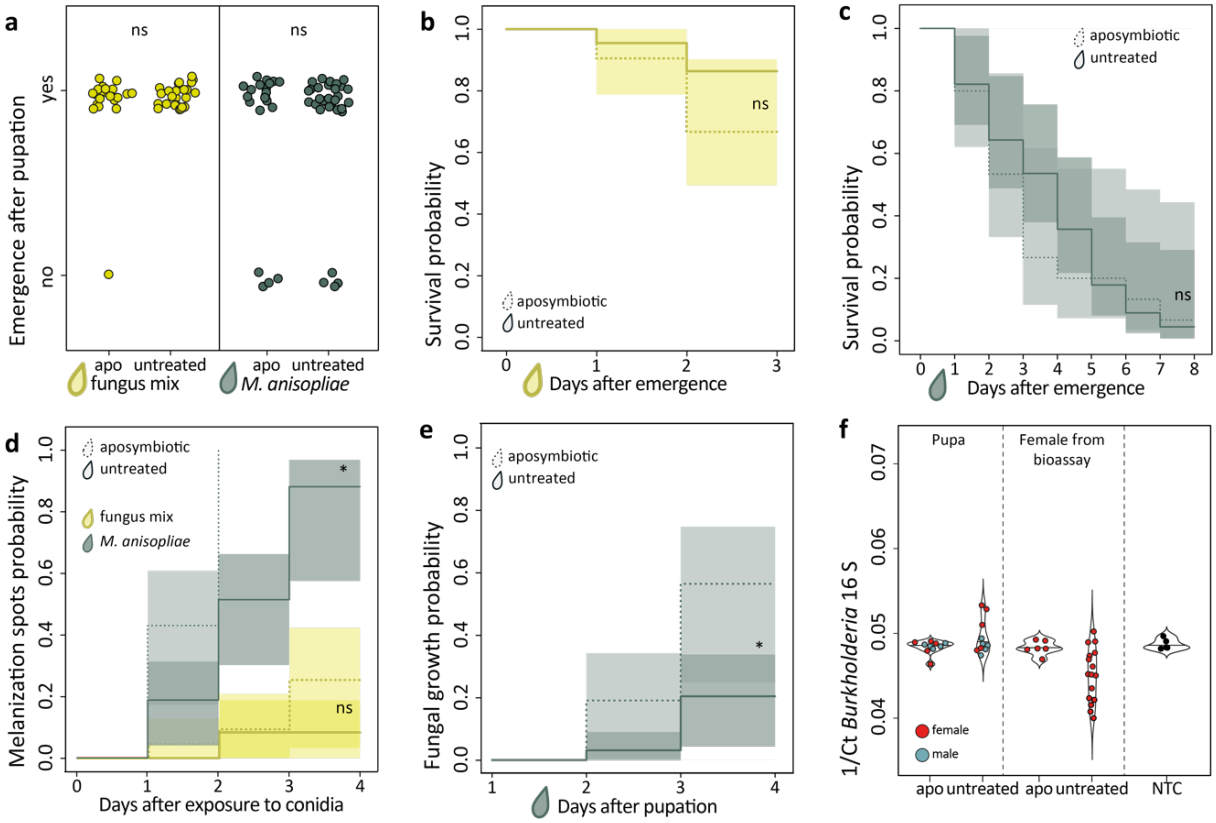

Figure S1: Bacterial symbionts reduce growth of pathogenic fungi on *L. villosa* pupae. Single pupae with (untreated, solid lines) and without symbionts (aposymbiotic, dotted lines) were exposed to different fungal pathogens (fungus mix in yellow or *M. anisopliae* in dark green) and were monitored single-blind. **a** Emergence rate after pupation according to fungal treatment and symbiont infection status. **b** Survival probability of aposymbiotic and untreated adults after infection by a fungus mix (10^6 conidia of each *B. bassiana*, *M. anisopliae* and *P. lilacinum*) during pupation. **c** Survival probability of aposymbiotic and untreated adults after an infection of 10^6 conidia of *M. anisopliae* and additional topical application of 10^6 conidia on the dorsal thorax. **d** Melanization probability of aposymbiotic and untreated pupae in response to different fungus treatments. Black lines indicate presence of a fungus mix, green lines indicate presence of *M. anisopliae*. **e** Visible fungal growth on the pupae infected with *M. anisopliae* during pupation. For b - e statistically significant differences from Cox mixed effects model are indicated as: $*p < 0.05$, ns= $p > 0.05$. Estimated survival curves (Kaplan–Meier) and the corresponding confidence intervals are shown. **f** Symbiont presence of aposymbiotic and untreated pupae and adult females was assessed using qPCR. These individuals belong to the same batch as pupae used in the bioassays, but were not exposed to fungus. Females correspond to individuals from the bioassay. The results show *Burkholderia* presence in 3 out of 10 untreated pupae, and symbiont absence in all females after the bioassay.

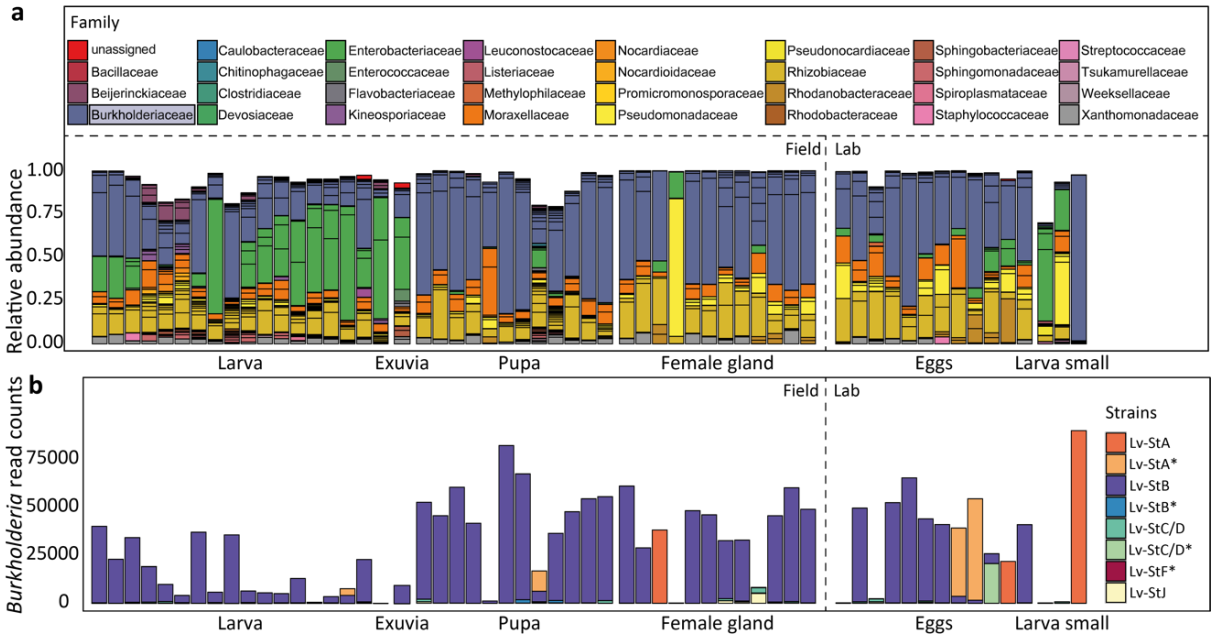

Figure S2: Relative abundance of bacterial families and read counts of *Burkholderia* strains across *L. villosa* life stages. **a** *Lagria villosa* bacterial community composition of different life stages shown at bacterial family level. Burkholderiaceae was abundant in most individuals of all life stages. In larvae, Enterobacteriaceae and Rhizobiaceae were also predominant and are most likely associated to the gut. **b** Read counts per sample of *Burkholderia* strains across different life stages. (*) in legend denotes pairwise identity above 98% but below 100%.

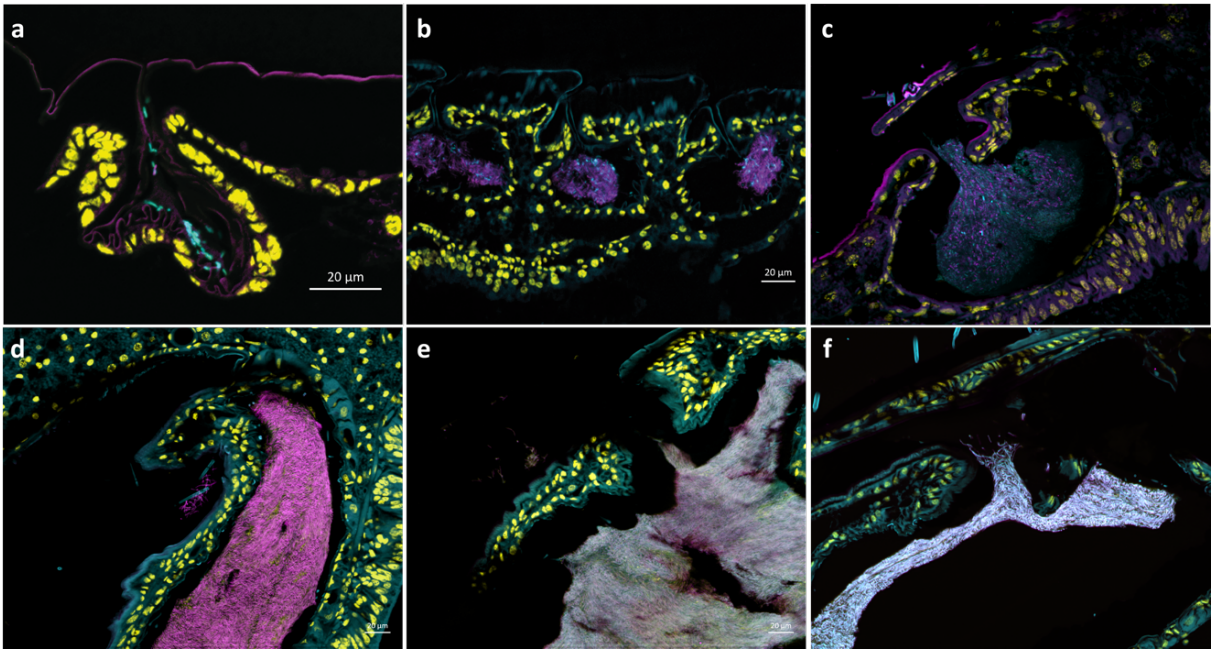

Figure S3: Presence of other bacterial symbionts or other *Burkholderia* strains in the symbiotic organs of *L. villosa* larvae. **a** Sagittal section of an early L1 larva showing very few *Burkholderia* cells (magenta) among other bacteria (cyan). **b** Sagittal section of an L1 larva showing few other *Burkholderia* strains (cyan) in between Lv-StB (magenta). **c** Sagittal section of a medium-sized larva showing *Burkholderia* cells (magenta) along with other bacteria (cyan) being released through the opening to the outer surface. **d-f** Sagittal sections through organs of older larvae showing higher abundance of *Burkholderia* (magenta) among all bacteria (cyan), while most of the cells are labeled with both probes (violet-white).

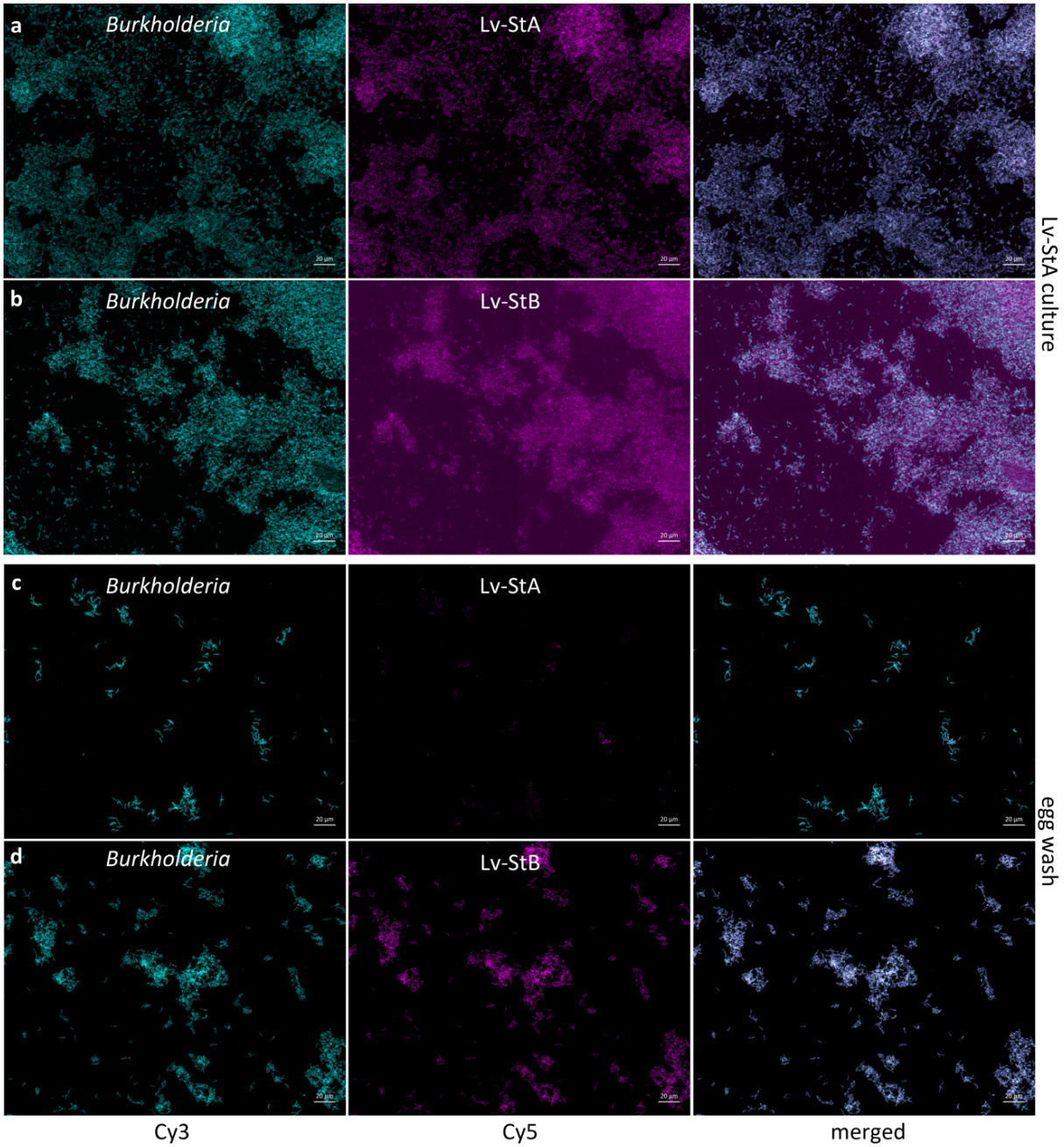

Figure S4: Specificity of FISH probes used for Lv-StB and Lv-StA staining. *Burkholderia*-specific staining in Cy3 (left panels) is depicted in cyan while Lv-StB and Lv-StA-specific staining in Cy5 (middle panels) is shown in magenta and a merged staining is shown in violet-white (right panel). **a** Culture of Lv-StA showing a clear signal for the general *Burkholderia* and Lv-StA-specific probe in all the cells. **b** Culture of Lv-StA showing clear signal for the *Burkholderia*-specific probe, and low signal to noise ratio with the Lv-StB probe. **c** Egg wash of *L. villosa* eggs showing labeling for single cells with the *Burkholderia* probe and only a few single cells with the Lv-StA probe. **d** Egg wash of *L. villosa* eggs stained with the Lv-StB-specific probe showing labeling of all cells. Images were taken with the same exposure times. The white labels on the upper region of each panel refer to the FISH-probe used, while the y-axis labeling shows the sample and the x-axis label the fluorescence channel.

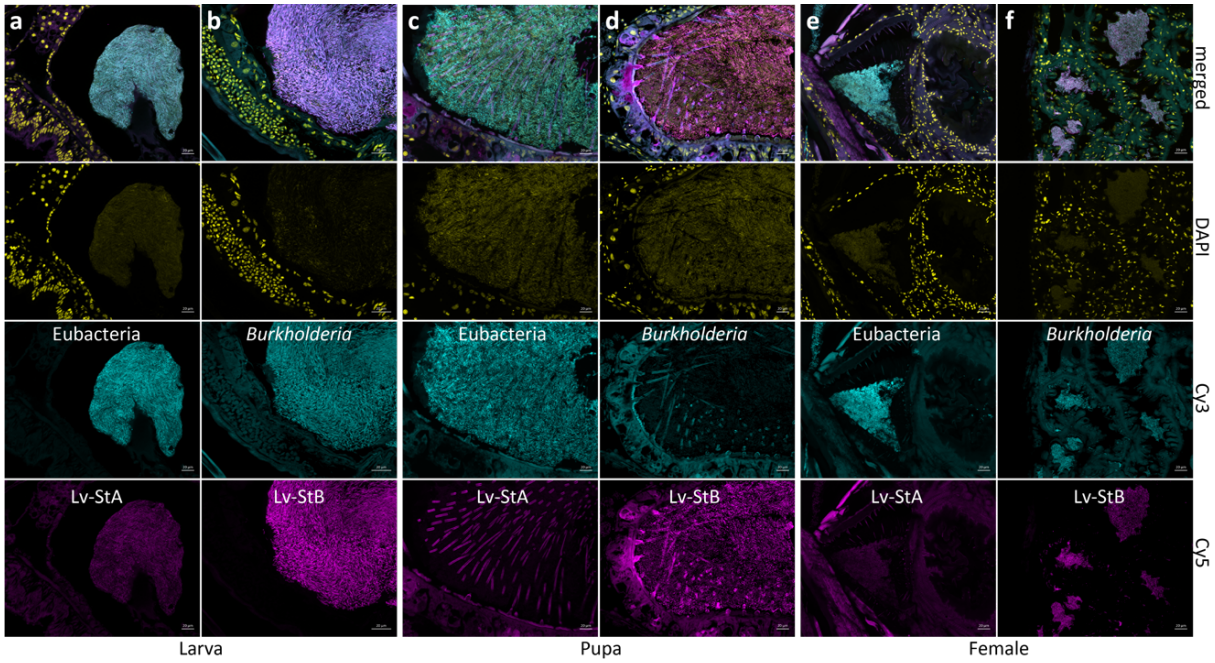

Figure S5: Lv-StA and Lv-StB presence on *L. villosa* sections. Sagittal sections of field individuals (same as in Figure 4) were used to compare the FISH signal of the strains-specific probes. The first row shows the merged image of the channels. The following rows show the individual channels corresponding to DAPI (yellow), probes for Eubacteria (a,c,e) or *Burkholderia* (b,d,f) labeled with Cy3 (cyan) and specific probes designed for Lv-StA (a,c,e) or Lv-StB (b,d,f) labeled with Cy5 (magenta). **a,b** Larval organ (Figure 4 e) showing intense signal for Eubacteria, *Burkholderia* and Lv-StB, in contrast to the Lv-StA probe. **c,d** Pupal organ (Figure 4 f) showing clear signal for the Eubacteria and Lv-StB probes, moderate signal for *Burkholderia* and no signal for Lv-StA. **e,f** Female reproductive system showing strong labeling for Eubacteria, *Burkholderia* and Lv-StB, as opposed to the Lv-StA probe. In all *L. villosa* sections, there was usually minor autofluorescence for the host tissue in the Cy3 channel, except for strong cuticular structures, which is usual for this wavelength range. The Cy5 channel usually showed no autofluorescence of the host tissue when specific labeling is visible for the symbionts. The white labels on the upper region of each panel refer to the FISH-probe used, while the y-axis labeling shows the fluorescence channel and the x-axis label shows the sample.

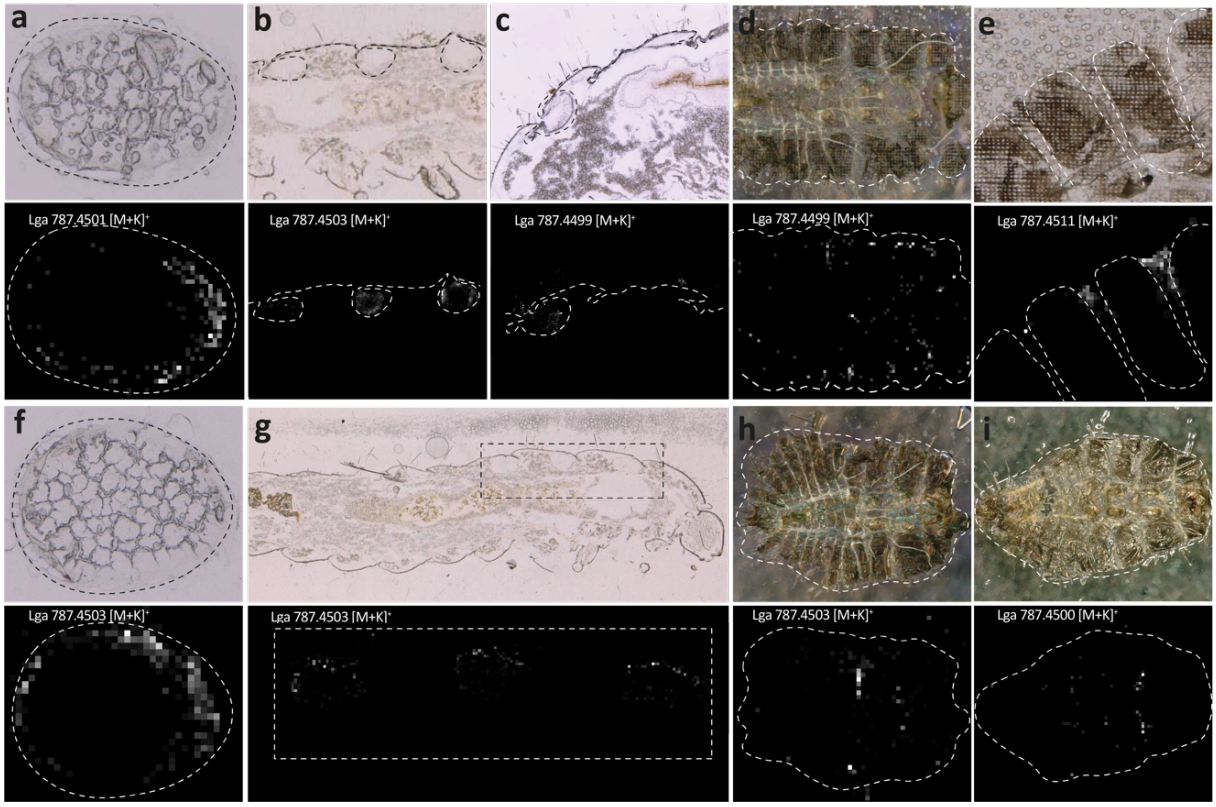

Figure S6: Lagriamide detection on *L. villosa* tissue using AP-SMALDI-HR MSI. Micrographs and the corresponding MSI images from Figure 5 c-h of sections through **a** an egg, **b** a larva, **c** a pupa, and on **d** a whole exuvia, and **e** parts of an exuvia. Additional replicates of **f** an egg section, **g** a larval section and **h** and **i** whole larval exuviae.

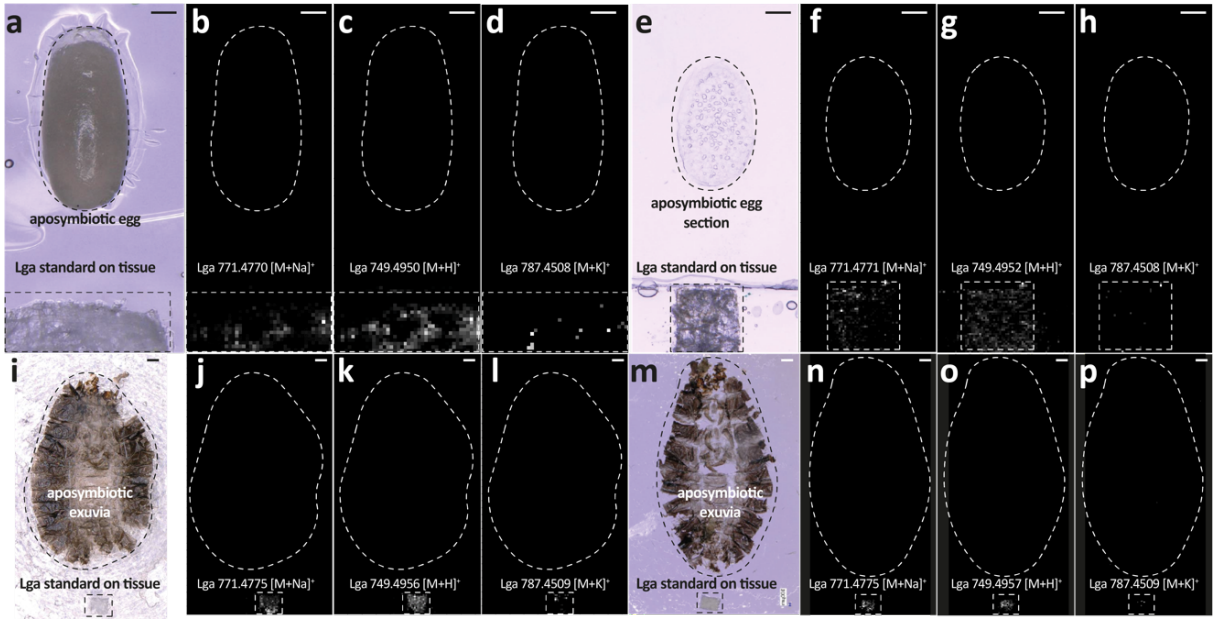

Figure S7: Positive (lagriamide standard) and negative controls (aposymbiotic *L. villosa* samples) for lagriamide detection using AP-SMALDI-HR MSI. Micrographs and the corresponding MSI images showing [M+Na]+, [M+H]+ and [M+K]+ adducts of lagriamide on negative controls of *L. villosa* samples without Lv-StB (outlined ovals) and positive controls of pure lagriamide on tissue (outlined rectangles). **a-d** Intact aposymbiotic egg. **e-h** Aposymbiotic egg section. **i-p** Two flattened aposymbiotic exuviae from larvae. Scale bars 100 µm for the top row and 500 µm for the bottom row.

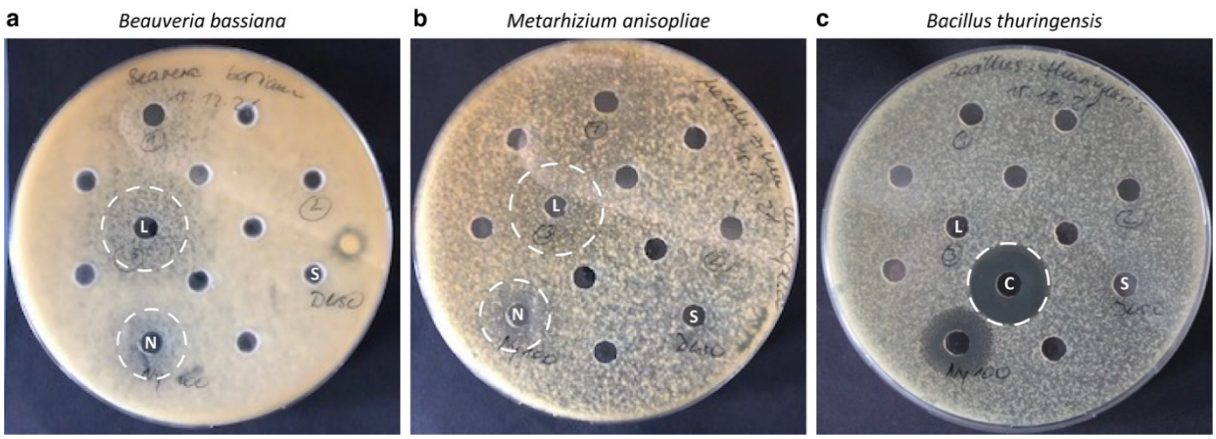

Figure S8: *In vitro* activity profiling of lagriamide. a Inhibition zone of 24 mm by lagriamide (L) against *B. bassiana*. **b** Inhibition zone of 18 mm by lagriamide (L) against *M. anisopliae*. **c** No inhibition by lagriamide (L) against *B. thuringensis*. L=lagriamide, N=nystatin, C= ciprofloxacin, S=solvent (DMSO).

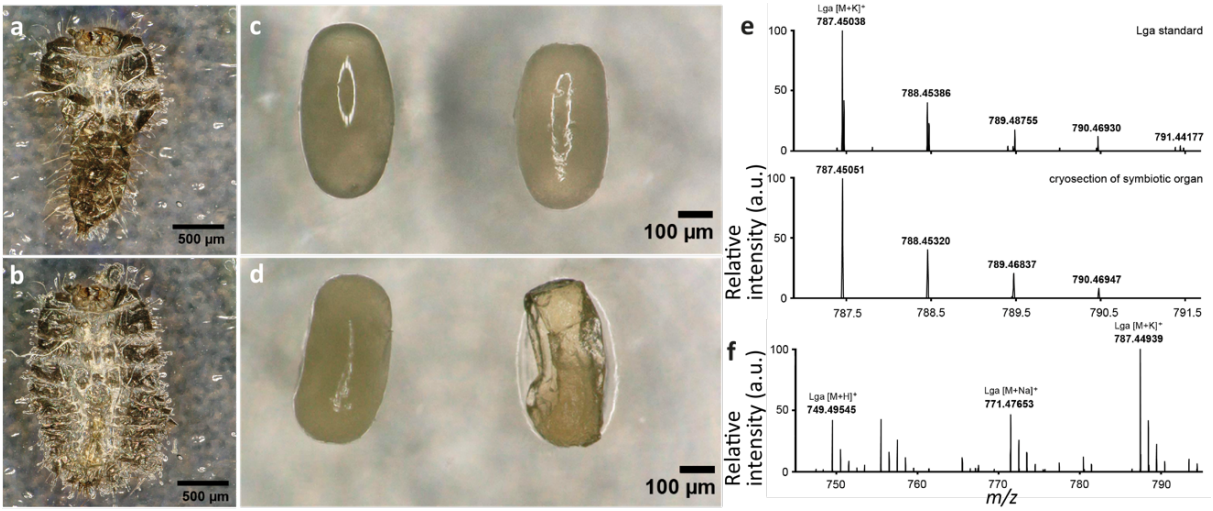

Figure S9: AP-SMALDI-HR MSI experimental procedure. a-d) Fixing process of *L. villosa* samples on double sided tape: **a** Exuvia before flattening and **b** after flattening. **c** Intact eggs before and **d** after 30 min inside AP-SMALDI ion chamber (+30 °C), egg at the right side was analyzed via AP-SMALDI-HR MSI. **e** AP-SMALDI-HR MS profiling of lagriamide standard (upper MS spectrum) in comparison with a cryosection of a symbiotic organ of a *L. villosa* larva (lower MS spectrum). Lagriamide was detected mainly as a potassium adduct at m/z 787.450 [M+K]+. **f** AP-SMALDI-HR MS profiling of *L. villosa* larva exuviae crude methanolic extract. Typical lagriamide adducts formed during AP-SMALDI-HR imaging and profiling experiments.

Table S1: Experimental set-up and replicate numbers of bioassays including differently treated *L. villosa* life stages and different fungi

Life stage	Experiment	Fungus	Number of applied conidia	Number of clutches	Treatment	Number of individuals
Larvae	Survival	*P. lilacinum*	7.5 x 10^3	8	aposymbiotic	111
					untreated	111
					reinfected-egg wash	103
					reinfected-LvStA	127
	Survival & Fungal growth	*B. bassiana*	10^6	4	aposymbiotic	70
					reinfected-egg wash	69
		M. anisopliae	10^6	4	aposymbiotic	68
					reinfected-egg wash	66
		No-fungus-control	-	4	aposymbiotic	65
					reinfected-egg wash	66
Pupae	Melanization & fungal growth	fungus mix	10^6	6	aposymbiotic	22
					untreated	24
		M. anisopliae	10^6 & topically applied 10^6	6	aposymbiotic	21
					untreated	32
Adults	Survival	*M. anisopliae*	10^6	6	aposymbiotic	17
					untreated	28

Table S2: Statistical analysis of the larval bioassay against *P. lilacinum* using a Cox mixed-effects model fit by maximum likelihood with survival as output, treatment as fixed effect and random intercepts per clutch and per year.

Cox mixed-effects model fit by maximum likelihood		NULL	Integrated	Fitted		
	Log-likelihood	-789.3066	-775.1302	-768.8992		
		Chisq	df	p	AIC	BIC
	Integrated loglik	28.35	5.00	3.1051e-05	18.35	3.86
	Penalized loglik	40.81	7.64	1.6216e-06	25.53	3.39
Random effects		Std Dev	Variance			
	Clutch	0.3590747895	0.1289347044			
	Year	0.0199984204	0.0003999368			
Fixed coefficients		coef	coef(exp)	se(coef)	z	p
aposymbiotic	untreated	0.7642137	0.4656999	0.2436558	3.14	1.7e-03
aposymbiotic	reinfected-egg wash	1.1607199	0.3132606	0.2791207	4.16	3.2e-05
aposymbiotic	reinfected-StA	0.4840728	0.6162683	0.2129933	2.27	2.3e-02
untreated	reinfected-egg wash	0.3965062	0.6726661	0.3111214	1.27	0.2000
untreated	reinfected-StA	0.2801409	1.3233163	0.2520390	1.11	0.2700
reinfected-egg wash	reinfected-StA	0.6766471	1.967271	0.2852862	2.37	1.8e-02

Table S3: Statistical analysis of the larval bioassay including no-fungus-control, *B. bassiana* and *M. anisopliae* using Cox mixed-effects models fit by maximum likelihood with survival as output, treatment and fungus as fixed effect and a random intercept per clutch. In addition, Cox mixed-effects models within the single fungal treatments were carried out with treatment as fixed effect and a random intercept per clutch.

Cox mixed-effects model fit by maximum likelihood		NULL	Integrated	Fitted		
	Log-likelihood	-1076.26	-966.8294	-962.2197		
		Chisq	df	p	AIC	BIC
	Integrated loglik	218.86	4.00	0	210.86	197.87
	Penalized loglik	228.08	5.66	0	216.76	198.38
Random effects		Std Dev	Variance			
	Clutch	0.4110391	0.1689531			
Fixed coefficients		coef	coef(exp)	se(coef)	z	p
aposymbiotic	reinfected-egg wash	-0.4653203	0.6279339	0.1467154	-3.17	1.5e-03
B. bassiana	*M. anisopliae*	1.0749012	2.9297034	0.1576536	6.82	9.2e-12
B. bassiana	no-fungus-control	-2.2907314	0.1011924	0.3411659	-6.71	1.9e-11

M. anisopliae	no-fungus-control	3.3656326	28.9518052	0.3347164	10.06	0.0e+00
Cox mixed-effects model fit by maximum likelihood		NULL	Integrated	Fitted		
	Log-likelihood	-48.11166	48.10818	-48.0339		
		Chisq	df	p	AIC	BIC
	Integrated loglik	0.01	2.00	0.99652	-3.99	-4.60
No-fungus-control	Penalized loglik	0.16	1.07	0.72143	-1.99	-2.31
Random effects		Std Dev	Variance			
	Clutch	0.10004711	0.01000942			
Fixed coefficients		coef	coef(exp)	se(coef)	z	p
aposymbiotic	reinfected-egg wash	0.03887024	1.039636	0.6326311	0.06	0.95
Cox mixed-effects model fit by maximum likelihood		NULL	Integrated	Fitted		
	Log-likelihood	-302.1836	-290.7721	-286.0536		
		Chisq	df	p	AIC	BIC
	Integrated loglik	22.82	2.00	1.1068e-05	18.82	14.44
B. bassiana	Penalized loglik	32.26	3.68	1.1417e-06	24.90	16.84
Random effects		Std Dev	Variance			
	Clutch	0.7435752	0.5529041			
Fixed coefficients		coef	coef(exp)	se(coef)	z	p
aposymbiotic	reinfected-egg wash	-0.7759086	0.4602854	0.2600352	-2.98	0.0028
Cox mixed-effects model fit by maximum likelihood		NULL	Integrated	Fitted		
	Log-likelihood	-474.072	-470.218	-467.7515		
		Chisq	df	p	AIC	BIC
	Integrated loglik	7.71	2.0	0.0211960	3.71	-1.76
M. anisopliae	Penalized loglik	12.64	2.9	0.0049451	6.85	-1.07
Random effects		Std Dev	Variance			
	Clutch	0.25010300	0.06255151			
Fixed coefficints		coef	coef(exp)	se(coef)	z	p
aposymbiotic	reinfected-egg wash	-0.4217744	0.655882	0.1892313	-2.23	0.026

Table S4: Statistical analysis of the larval bioassay including no-fungus-control, *B. bassiana* and *M. anisopliae* using Cox mixed-effects models fit by maximum likelihood with fungal infestation as output, treatment and fungus as fixed effect and a random intercept per clutch. In addition, Cox mixed-effects models within the single fungal treatments were carried out with treatment as fixed effect and a random intercept per clutch. Having only ceros in individual groups impedes running this kind of model. Therefore, given that in the no-fungus-control treatment none of the aposymbiotic and natural-reinfected individuals originally showed signs of fungal growth, a single fungus-positive sample was artificially added to each of these two groups.

Cox mixed-effects model fit by maximum likelihood		NULL	Integrated	Fitted		
	Log-likelihood	-822.0822	-675.8562	-669.1933		
		Chisq	df	p	AIC	BIC
	Integrated loglik	292.45	4.00	0	284.45	272.52
	Penalized loglik	305.78	5.9	0	293.98	276.37
Random effects		Std Dev	Variance			
	Clutch	0.9624150	0.9262427			
Fixed coefficients		coef	coef(exp)	se(coef)	z	p
aposymbiotic	reinfected-egg wash	-1.393525	0.248198776	0.1868122	-7.46	8.7e-14
B. bassiana	M. anisopliae	-1.263624	0.282627988	0.1823727	-6.93	4.2e-12
B. bassiana	no-fungus-control	4.336962	76.4748646	0.7272239	5.96	2.5e-09
M. anisopliae	no-fungus-control	-5.600586	270.5848957	0.7298289	-7.67	1.7e-14
Cox mixed-effects model fit by maximum likelihood		NULL	Integrated	Fitted		
	Log-likelihood	0	0	0		
		Chisq	df	p	AIC	BIC
	Integrated loglik	0	2	1	-4	Inf
No-fungus-control	Penalized loglik	0	1	1	-2	Inf
Random effects		Std Dev	Variance			
	Clutch	2e-02	4e-04			
Fixed coefficients		coef	coef(exp)	se(coef)	z	p
aposymbiotic	reinfected-egg wash	0	1	0	NaN	NaN
Cox mixed-effects model fit by maximum likelihood		NULL	Integrated	Fitted		
	Log-likelihood	-240.7052	-209.7258	-203.8468		
		Chisq	df	p	AIC	BIC
	Integrated loglik	61.96	2.00	3.5194e-14	57.96	54.02
B. bassiana	Penalized loglik	73.72	3.84	2.8866e-15	66.04	58.48
Random effects		Std Dev	Variance			
	Clutch	1.304286	1.701162			

Fixed coefficients		coef	coef(exp)	se(coef)	z	p
aposymbiotic	reinfected-egg wash	-1.812083	0.1633136	0.3258503	-5.56	2.7e-08
Cox mixed-effects model fit by maximum likelihood		NULL	Integrated	Fitted		
	Log-likelihood	-370.1209	-349.1938	-343.726		
		Chisq	df	p	AIC	BIC
M. anisopliae	Integrated loglik	41.85	2.00	8.1554e-10	37.85	32.83
	Penalized loglik	52.79	3.79	6.8880e-11	45.22	35.72
Random effects		Std Dev	Variance			
	Clutch	0.8185416	0.6700103			
Fixed coefficients		coef	coef(exp)	se(coef)	z	p
aposymbiotic	reinfected-egg wash	-1.234547	0.2909667	0.2361363	-5.23	1.7e-07

Table S5: Characteristics and replicate numbers for specimens used to quantify *B. gladioli* Lv-StB across *L. villosa* life stages.

Sample type	Replicates	Source	Content per replicate
Larva	12	Field	Single individual
Larva late	12	Field	Single individual
Pupa	6	Field	Single individual
Female ovipositor + paired accessory glands	5	Field	Single individual, dissected organs
Fraction of early egg clutch	32	1st lab generation	Pools of 10-15 individuals from one clutch
Fraction of late egg clutch	28	1st lab generation	Pools of 6-15 individuals from one clutch
Larva L1	24	1st lab generation	Pools of 9-15 individuals from one clutch
Larva early L2	25	1st lab generation	Pools of 2-10 individuals from one clutch
Larva mid L2	24	1st lab generation	Pools of 7-10 individuals from one clutch
Larva late L7	4	1st lab generation	Single individual
Pupa early	7	1st lab generation	Single individual
Pupa late	4	1st lab generation	Single individual

Table S6: *L. villosa* specimens used for quantification of lagriamide throughout host development including replicate numbers and source environment.

Sample type	Replicates	Source	Content per replicate
Fraction of egg clutch	27	1st lab generation	13–60 eggs, titer normalized per individual egg
Larva L1	8	1st lab generation	1st instar, 20–22 individuals, titer normalized per individual
Larva L2	6	1st lab generation	2nd instar, 6–20 individuals, titer normalized per individual
Larva (unknown instar)	35	Field	Single individual, unknown instar
Pupa	18	Field	Single individual
Pupa-adult exuvia	14	Field	Single individual
Female ovipositor + paired accessory glands	6	Field	Single individual, dissected organs
Male reproductive system	3	Field	Single individual, dissected organs

Table S7: Original localities of collected beetles and their use for this study.

Year	State	Locality	Latitude	Longitude	Use
2020-2	São Paulo	Perdobas, Cordeirópolis	22°29'27.1"S	47°26'04.7"W	Bioassay pupae
2020	São Paulo	Limeira	22°38'20.4"S	47°19'38.0"W	Bioassay larvae, MALDI
2020	São Paulo	Cordeirópolis	22°29'22.6"S	47°23'45.6"W	
2020	São Paulo	Santa Gertrudes	22°27'56.6"S	47°31'56.8"W	
2020	São Paulo	Cordeirópolis	22°29'14.6"S	47°27'52.0"W	
2020	São Paulo	Jaú	22°15'53.6"S	48°31'08.2"W	
2019-2	São Paulo	Jundiaí	S23° 8' 3.732"	W46° 58' 47.352"	Bioassay larvae, FISH, MALDI
2019-2	São Paulo	Jundiaí	S23° 7' 42.06"	W46° 59' 25.296"	
2019-2	São Paulo	Cordeirópolis	S22° 30' 11.376"	W47° 25' 40.08"	
2019-2	São Paulo	Cordeirópolis	S22° 30' 13.32"	W47° 25' 28.092"	
2019-2	São Paulo	Itirapina	S22° 15' 15.84"	W47° 50' 43.728"	
2019-2	São Paulo	Brotas	S22° 16' 18.516"	W47° 56' 4.452"	
2019-2	São Paulo	Brotas	S22° 17' 25.98"	W48° 3' 12.276"	
2019	São Paulo	Cordeirópolis	S22° 29' 26.88"	W47° 25' 58.476"	Quantification of Lv-StB, FISH, MALDI
2019	São Paulo	Brotas	S22° 17' 25.98"	W48° 3' 12.276"	
2019	São Paulo	Jaú	S22° 15' 49.896"	W48° 31' 12.396"	
2019	São Paulo	Cordeirópolis	S22° 29' 40.776"	W47° 23' 48.192"	
2019	São Paulo	Santa Gertrudes	S22° 27' 56.196"	W47° 31' 55.488"	
2019	São Paulo	Cordeirópolis	S22° 30' 11.376"	W47° 25' 40.08"	
2018	São Paulo	Jundiaí	-23°08'04.7040"	-046°58'53.5800"	
2018	São Paulo	Cordeirópolis	-22°30'13.3200"	-047°25'28.0920"	

2018	São Paulo	Itirapina	-22°15'18.9000"	-047°51'00.1440"	Bacterial community profiling, FISH, MALDI
2018	São Paulo	Guarapuã	-22°14'49.5960"	-048°17'47.9400"	
2018	São Paulo	Jaú	-22°15'20.7720"	-048°33'27.2520"	
2018	São Paulo	Santa Cruz da Conceição	-22°05'08.3760"	-047°25'17.2920"	
2018	São Paulo	Santa Cruz da Conceição	-22°05'50.9280"	-047°25'16.1400"	
2018	São Paulo	Pirassununga	-22°03'12.7440"	-047°32'05.3520"	
2018	São Paulo	Brotas	-22°16'18.5160"	-047°56'04.4520"	
2017	São Paulo	Itajú	-21°56'30.1920"	-048°51'23.5080"	Bacterial community profiling
2017	São Paulo	Jaú	-22°15'49.7880"	-048°31'10.2360"	
2017	São Paulo	Jaú	-22°15'14.4000"	-048°33'49.4640"	
2017	São Paulo	Jaú	-22°12'32.4720"	-048°36'35.4960"	
2017	São Paulo	Itirapina	-22°16'03.3600"	-047°55'48.2880"	
2017	São Paulo	Pirassununga	-22°03'12.7440"	-047°32'05.3520"	
2017	Paraná	Ponta Grossa	-25°05'46.9644"	-050°02'55.6008"	
2017	Paraná	Ponta Grossa	-25°05'35.2824"	-050°02'56.8392"	

CHAPTER II

Morphological adaptation for ectosymbiont maintenance and transmission during metamorphosis in *Lagria* beetles

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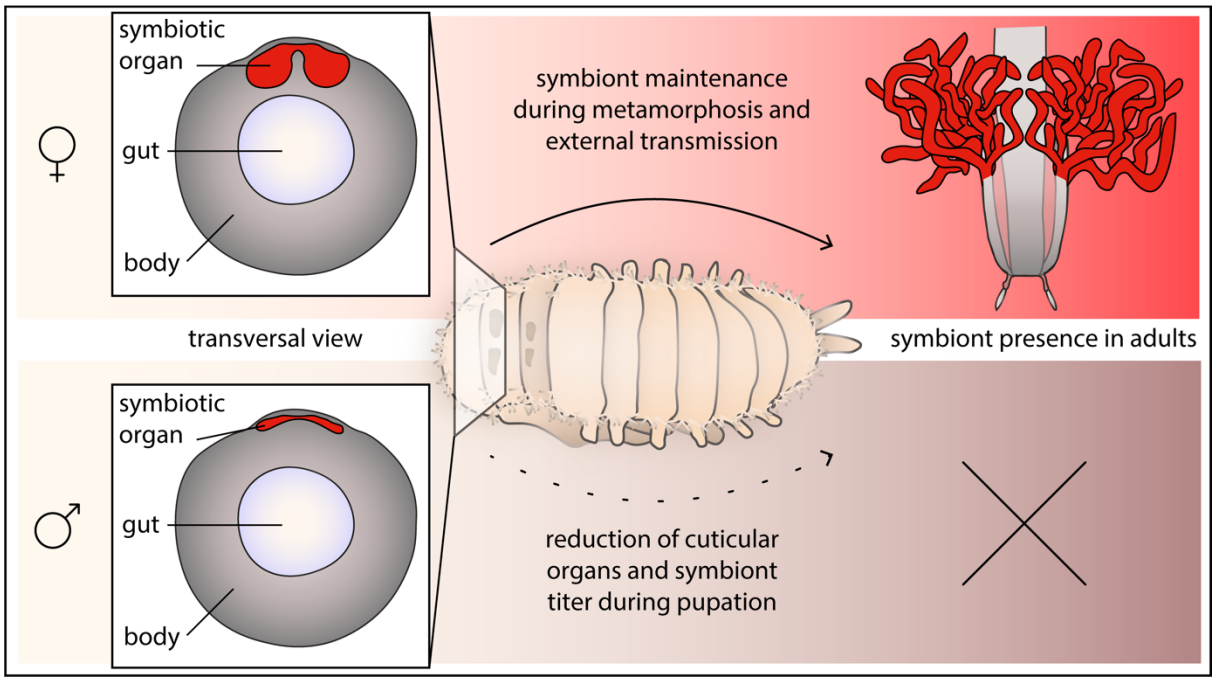

R.S.J., M.K., and L.V.F. conceived the study and designed the experiments. **R.S.J.** and S.M. carried out DNA extraction, qPCR, and FISH experiments. B.W. advised histological sectioning and carried out µCT scans. **R.S.J.** carried out other microscopy experiments and analyzed µCT scans. **R.S.J.**, L.V.F, and M.K. wrote the manuscript and all authors reviewed and commented on the final manuscript.

1. Abstract

The diversity and success of holometabolous insects is partly driven by metamorphosis, which allows for the exploitation of different niches and decouples growth and tissue differentiation from reproduction. Despite its benefits, metamorphosis comes with the cost of temporal vulnerability during pupation and challenges associated with tissue reorganizations. These rearrangements can also affect the presence, abundance, and localization of beneficial microbes in the host. However, how symbionts are maintained or translocated during metamorphosis and which adaptations are necessary from each partner during this process remains unknown for the vast majority of symbiotic systems. Here, we show that *Lagria* beetles circumvent the constraints of metamorphosis by maintaining defensive symbionts on the surface in specialized cuticular structures. The symbionts are present in both sexes throughout larval development and during the pupal phase, in line with a protective role during the beetle's immature stages. By comparing symbiont titer and morphology of the cuticular structures between sexes using qPCR, fluorescence *in situ* hybridization, and micro-computed tomography, we found that the organs likely play an important role as a symbiont reservoir for transmission to female adults, since symbiont titers and structures are reduced in male pupae. Using symbiont-sized fluorescent beads, we demonstrate transfer from the region of the dorsal symbiont-housing organs to the opening of the reproductive tract of adult females, suggesting that symbiont relocation on the outer surface is possible, even without specialized symbiont adaptations or motility. Our results illustrate a strategy for holometabolous insects to cope with the challenge of symbiont maintenance during metamorphosis via an external route, circumventing problems associated with internal tissue reorganization. Thereby, *Lagria* beetles keep a tight relationship with their beneficial partners during growth and metamorphosis.

2. Introduction

Microbial symbionts are widespread in nature and can be found in various body parts of animals, sometimes forming tight associations with their hosts [1]. Often, host morphological modifications for housing and guiding symbionts ensure the establishment and maintenance of mutualistic interactions [2,3]. In some animal-microbe symbioses, particular tissues, structures, or organs are likely adapted to favor specific microbial inhabitants [4] like bacteriocytes in aphids [5] and other sap-sucking insects [6], or grain beetles [7,8], light organs in squids [9], trophosomes in tubeworms [10,11], antennal reservoirs of solitary digger wasps [12–14], or midgut ceca in bean bugs [15].

In insects, symbiont locations are as diverse as their functional contributions to their hosts. Generally, localization correlates with – and likely constrains – symbiont function. For example, defensive microbes often occur as ectosymbionts or in proximity to the outer surface [16]. In contrast, symbionts involved in digestion or detoxification are commonly found within or around the gut [17], while nutrient-supplementing symbionts are either localized in

gut-associated organs or inhabit bacteriomes [18]. Symbiont localization and/or function can however change under different circumstances. In reed beetles, symbiont translocations from intracellular to extracellular sites in the host occur along with changes in symbiont function [19]. Alternatively, symbionts can be lost when the contributions are no longer needed, as observed for the tyrosine-supplementing symbionts in the cereal weevil and the sawtoothed grain beetle [20,21]. In particular, this occurs in adult males of several insect taxa, since they are not involved in passing symbionts to the next generation [1].

To enable successful symbiont transmission across generations, different mechanisms and adaptations have evolved to relocate symbionts. Generally, symbionts can be acquired and transferred through strict vertical transmission from parents to offspring, via horizontal acquisition every new generation, or by a mixed-mode combining both [2,22]. Many obligate intracellular endosymbionts that provide essential functions for host survival are transmitted transovarially through the female germ line [1,23]. In contrast, transmission of extracellular symbionts can be more diverse and sometimes flexible, often occurring through mechanisms that are in play during or after egg-laying [2].

Guaranteeing the maintenance or re-colonization of symbionts is not only a challenge over generations but also throughout different life stages. Reorganization of tissues, including symbiotic organs, is usually drastic during complete metamorphosis, posing a challenge on symbiont transmission and maintenance [3]. A defined translocation route for symbionts when insects mature has been described in a few cases [24,25], sometimes involving shifts from intra- to extra-cellularity as internal structures reorganize [26]. While the persistence of symbionts in the host will depend on the successful translocation during metamorphosis, this issue has so far received much less attention than the transmission across generations.

Here, we focus on the impact of symbiont localization and transmission during metamorphosis on the symbiosis between two *Lagria* beetle species and their bacterial symbionts. The presence of putative symbiont-bearing structures in the adults of 83 species of this subfamily of darkling beetles (Lagriinae, Tenebrionidae) was reported based on morphological observations [27], yet the symbiotic association with bacteria and the corresponding housing structures in the larvae have been described specifically in *Lagria hirta* [28] and *Lagria villosa* [29, Chapter I]. *L. villosa* and *L. hirta* house a community of symbionts throughout their life cycle, dominated by bacteria of the genus *Burkholderia* [28,29, Chapter I]. Hereinafter, we refer to the *Burkholderia* symbionts as the symbionts or ectosymbionts, although other bacterial taxa are also present in the community [28,29]. While occasional environmental exchange occurs, the symbionts are predominantly transmitted vertically from female accessory glands onto the egg surface during oviposition, from where they colonize three unusual dorsal cuticular invaginations of the mesothorax, metathorax, and the first abdominal segment in both sexes of larvae [27–29, Chapter]. *L. villosa* pupae maintain the symbionts in similar structures on the surface, from where they presumably translocate to accessory glands of the reproductive tract in females. By contrast, male adults lack the symbionts. In addition, some strains can be transferred from females to the environment or horizontally acquired during the larval stage [30]. In *L. villosa*, the ectosymbionts protect the

eggs and larvae against fungal infestation by producing antibiotics [29,31, Chapter I]. One symbiont strain (*Burkholderia* Lv-StB, henceforth "Lv-StB") consistently produces the antifungal compound lagriamide and likely plays a pivotal role in defense [31, Chapter I]. Although this strain lacks common genes for motility [32], it successfully colonizes the larval stage, persists in all life stages in high numbers, and dominates the microbial community in the beetle [Chapter I]. It is unclear yet when symbionts are lost in male *Lagria* beetles and if they might be ecologically relevant for defense in both sexes also during pupation [Chapter I]. In *L. hirta* it was hypothesized that the symbionts are transmitted to the female accessory glands externally via the molting fluid [1], however, how symbiont transmission and maintenance is facilitated during metamorphosis, especially for the presumably immotile LvStB, is not yet known. Therefore, we explored the pupal stages of *L. villosa* and *L. hirta* to shed light on the symbiosis at this stage. We compared symbiont titers via qPCR, visualized symbiont localization and morphology of the symbiotic organs in the host using histological sections and fluorescence *in situ* hybridization (FISH) and micro-computed tomography (µCT), and simulated a potential transmission route of Lv-StB using fluorescent beads and microscopy. Thereby, we demonstrate that symbiont loss in males already begins in the pupal stage, which is accompanied by morphological changes of the symbiotic organs during metamorphosis. Furthermore, we show that symbiont transmission from female pupae to adults probably occurs externally via the host surface. These findings indicate that the ecological importance of the symbionts likely drove the evolution of specialized structures in the host to house and maintain the bacteria during metamorphosis.

3. Results

3.1. Symbiont presence in *Lagria* pupae

Since there is evidence in *L. hirta* that both male and female larvae carry symbionts, but adult males lack them in both *Lagria* species [27,28], we aimed to better understand the process and timing of symbiont loss. We initially compared symbiont titers of field-collected female adults, pupae, and larvae without separating immature stages by sex. In *L. villosa,* symbiont titers increase during larval development, but decrease in the pupal stage and finally reach the highest numbers in adult females (Figure 1 a). To evaluate whether this lower symbiont titer in pupae was specific to a certain sex and if it is similar between the two *Lagria* species, we compared the symbiont titer of female and male *L. villosa* and *L. hirta* pupae (Figures 1 b, c). Indeed, male pupae had significantly lower symbiont titers than females in both beetle species. Also, symbiont titers were generally lower in *L. hirta* than in *L. villosa*, possibly corresponding to the observed size differences between the species (Figure S1).

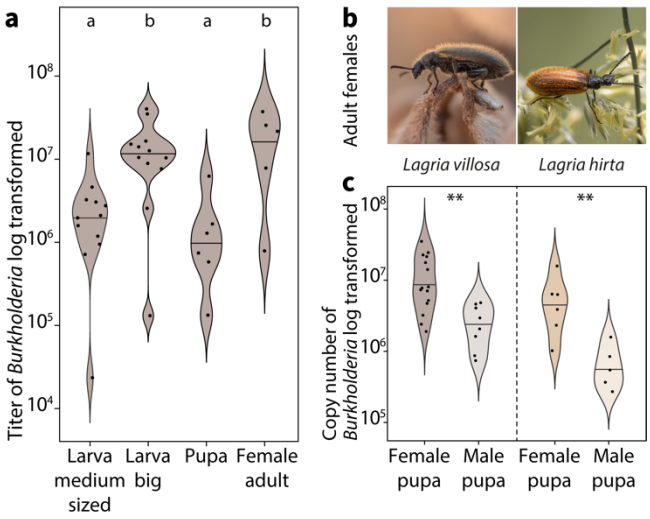

Figure 1: Symbiont abundance in male and female *Lagria* pupae. a Abundance of *B. gladioli* (gyrase B gene copies) of field-collected *L. villosa* larvae (larva medium-sized and larva big, sex unknown), pupae (sex unknown), and adult females. Different letters indicate significant differences between experimental treatments (Kruskal-Wallis test, $\chi 2 = 14.3$, df = 3, p-value = 0.0026, posthoc Dunn's Test) **b** Photographs of *Lagria* females from the two evaluated species *L. villosa* (left, Brazil) and *L. hirta* (right, Germany). **c** Abundance of *B. gladioli* copies (16S rRNA gene of *B. gladioli*) of field-collected male and female pupae of *L. villosa* (left) and *L. hirta* (right). Asterisks indicate significant differences between females and males (Two Sample t-test, ** p<0.01).

To evaluate potential differences in the pupal symbiotic organs between the two sexes, we carried out FISH experiments on one female and one male *Lagria* pupa in each of the two species (Figure 2). In *L. villosa* pupae, females (Figure 2 a) carried dense accumulations of symbionts mainly on the surface of the dorsal thorax (Figure 2 b) and in the bigger first symbiotic organ (Figure 2 c). In contrast, the first dorsal symbiotic organ is only vestigial in males (Figure 2 d) and accumulations of symbionts were not observed (Figure 2 e). In *L. hirta* pupae, the female (Figure 2 f) showed an accumulation of symbionts in a pit of the dorsal thorax between bristles (Figure 2 g), but not in the region of the first symbiont organ as observed in *L. villosa* pupae. In males, we did not detect the first organ nor any symbiont cells (Figure 2 h, i). In summary, these results indicate that the symbiotic organs of male *Lagria* pupae degenerate during pupation and symbiont reservoirs in the respective parts are missing, which relates to a decline of symbionts during metamorphosis.

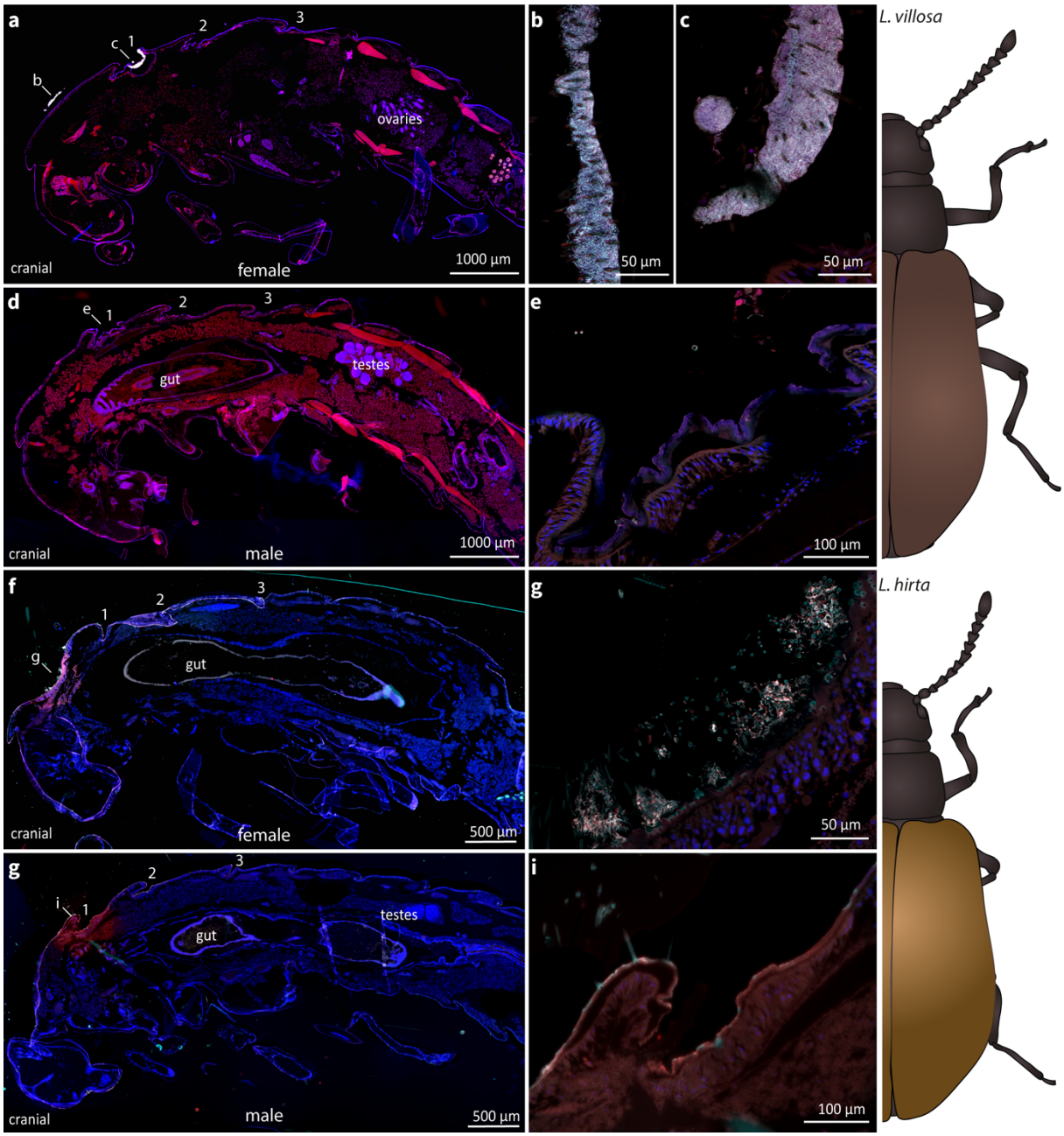

Figure 2: Sexual dimorphism of symbiotic organs and symbiont localization in *Lagria* pupae using FISH. FISH was carried out on semithin sagittal histological sections of *L. villosa* (a-e) and *L. hirta* (f-i) pupae. Symbiont cells are generally depicted in white, host cell nuclei in blue, and autofluorescence of the host tissue in red. Symbionts were present in **a** female *L. villosa* pupae, **b** on the surface of the thorax between bristles, and **c** within the first dorsal symbiont organ, while symbionts were not detectable in **d, e** male pupae. Symbionts were detected in **f** *L. hirta* females, **g** in a pit of the dorsal thorax between bristles, while they were not detectable in **h, i** males. *Burkholderia-specific* staining is shown in cyan, general eubacterial staining in red, and host cell nuclei in blue (DAPI). Overlapping signal is shown in white. Numbers indicate the location of symbiotic organs or cuticular structures in the respective position and all images show sagittal sections with the cranial end to the left.

3.2. Morphology of symbiotic organs in larvae and pupae

To identify if the observed decrease in symbiont titers, especially in male pupae, can be attributed to morphological differences in the cuticular symbiont-bearing structures, we used µCT imaging of female and male *L. villosa* pupae. We measured the total volume of female and male pupae and their cuticular structures at different time points (Figure 3 a). The results indicate that although the total volume of pupae is not different across sexes and time points (approximately 100 mm^3 for both sexes), the volume of the first structure differs between males and females. The bigger volume in females is in line with the observation of single sections via FISH (Figure 2). By looking at the whole 3D scan, we found that the first structure has a double-lobed morphology in females, while it is much less pronounced in males (Figure 3 b). The lobes correspond to the bigger part of the structure, which are oriented towards the caudal side in a sagittal orientation (Figure 3 c). A coronal view indicated that the first structure is similar across female time points but is progressively reduced during male pupal development (Figure 3 d). 3D representations of the whole pupa (Figure 3 e, Video S1 & S2) also showed that generally, the first structure looks more like a specialized organ, while the second and third structure are visually similar to the cuticular folds between the other segments.

During the identification of the sexual dimorphism in the symbiotic organs in *Lagria* pupae, we also observed that the morphology of the symbiotic organs generally changed from the larval to the pupal stage. *L. villosa* larvae have three equally sized dorsal symbiont organs (Figure 4 a, b), which increase in size while the insect grows as described for *L. hirta* [27]. However, female pupae have a bigger first organ, and the second and the third structures decrease in size (Figure 4 a). Contrastingly, male pupae have three almost equally sized cuticle-lined structures, which are smaller in comparison to those in large larvae.

To identify at which time point this change in morphology occurs, we sectioned two late final instar larvae which were about to pupate and assessed the morphology of the symbiotic organs. Unfortunately, due to the lack of sex-specific morphological characters, we were not able to identify the sex of the larvae, but we found two different morphologies of symbiotic organs in the two specimens. In one specimen, we observed three organs that decreased in size from the first to the third organ (Figure 4 c), which would be in line with an intermediate state leading to the morphology of an early female pupa (Figure 4 d). In the other specimen, we found three similarly sized organs, which looked compressed and to some extent detached from the developing new cuticle (Figure 4 e). This potentially precedes the condition in male pupae, which appear to have lost the bigger structures accommodating the symbionts (Figure 4 f). In addition, we observed that the symbiotic organs in larvae looked almost symmetrical in sagittal sections (Figure 4 b), whereas the caudal side of the organ looked more pronounced in the female pupae (Figure 4 c, d). Overall, these observations suggest that the symbiotic organs of males are possibly already detached shortly before pupation and are shed off with the last larval molt. By contrast, females retain their symbiotic organs during the last larval molt but undergo morphological changes,

leading to one enlarged first organ with the majority of the symbionts, and two remnants of the former second and third organ.

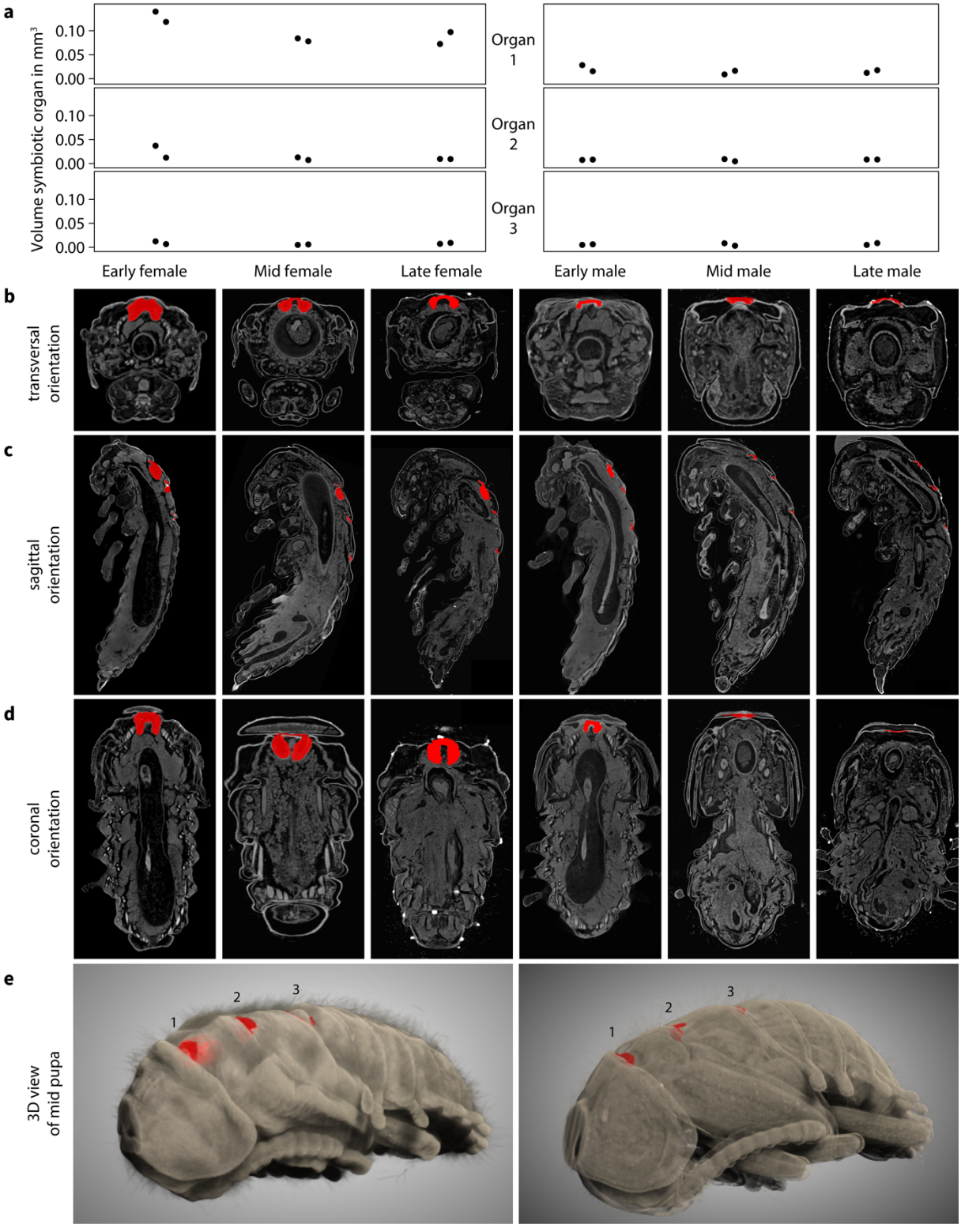

Figure 3: Symbiotic organs in female and male *L. villosa* across pupal development. a Volume of the symbiotic organs or cuticular structures measured from 3D µCT scans of *L. villosa* pupae of different sexes and stages. Female samples are shown on the left panels and males on the right. **b-d** Images show single sections in **b** transversal, **c** sagittal, and **d** coronal orientations through specimen (from left to right: early, mid and late female; early, mid, and late male pupa) corresponding to the columns in **a**. Cuticular structures are labeled in red and show the first organ in transversal and coronal sections, and all three structures in sagittal sections. **e** 3D representation of a female (left) and male (right) mid pupa showing the cuticular structures in red within the dorsal surface.

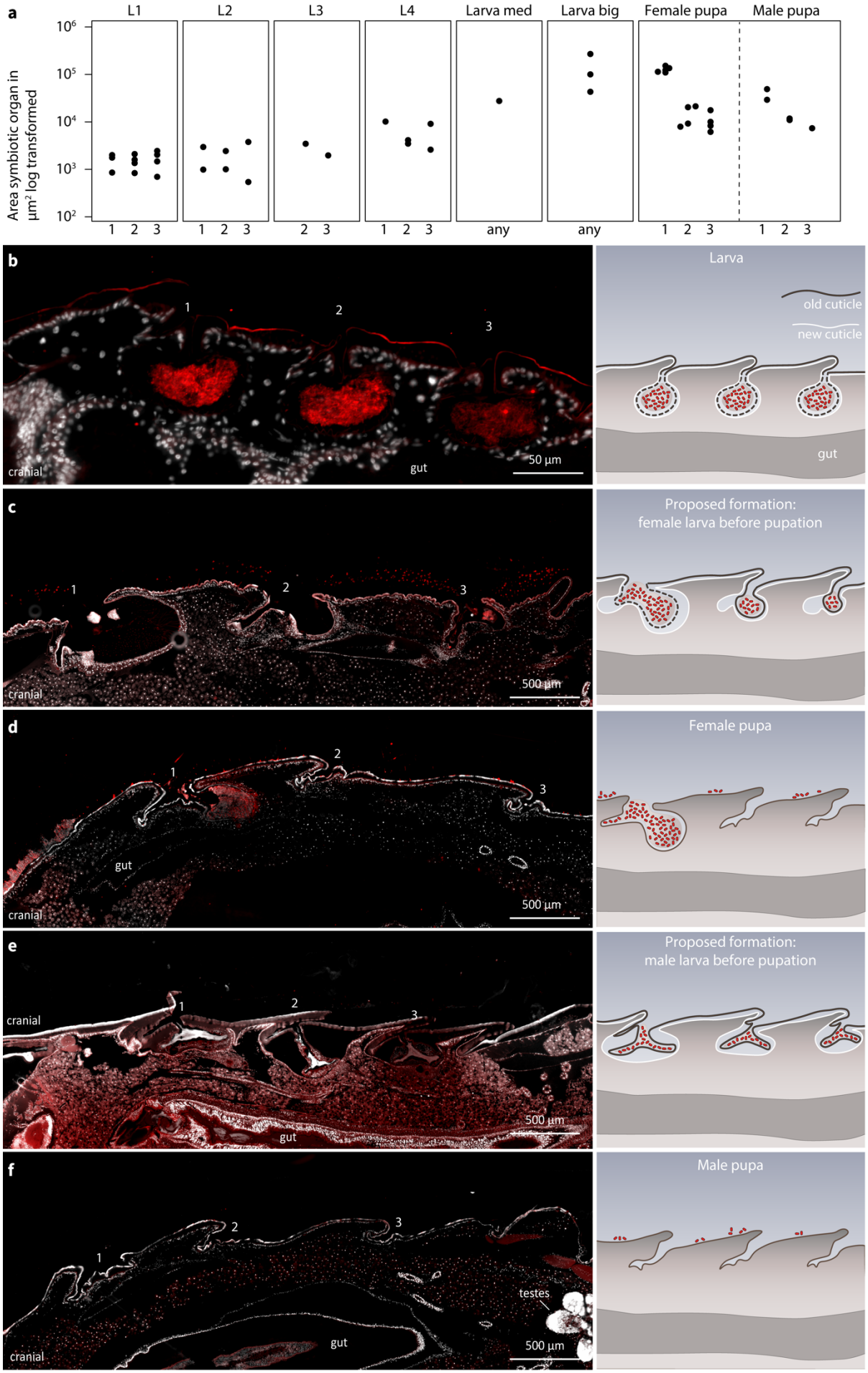
a
L1
L2
L3
L4
Larva med
Larva big
Female pupa
Male pupa
Area symbiotic organ in µm² log transformed
10⁶
10⁵
10⁴
10³
10²
1 2 3
1 2 3
2 3
1 2 3
any
any
1 2 3
1 2 3
b
1
2
3
cranial
gut
50 µm
Larva
old cuticle
new cuticle
gut
c
1
2
3
cranial
500 µm
Proposed formation:
female larva before pupation
d
1
2
3
gut
cranial
500 µm
Female pupa
e
cranial
1
2
3
500 µm
gut
Proposed formation:
male larva before pupation
f
1
2
3
testes
gut
cranial
500 µm
Male pupa

Figure 4: Development of the symbiotic organs in late *L. villosa* larvae and pupae. a Size of the symbiotic organs or cuticular structures measured in sagittal sections of *L. villosa* larvae and pupae. For standardization, sections of individuals which contained the opening canal of the symbiotic organ were chosen. However, variations in exact location, shape, and accuracy during sectioning possibly influenced the measured area. Numbers on the x-axis label refer to the first (1), second (2), third (3) symbiotic organ, or to an unknown (any) symbiotic organ or cuticular structure at the respective location. **b-f** The left panel shows relevant micrographs of histological sections covering the region of the three dorsal structures and the right panel shows illustrations representing the proposed morphological change of the organs from larvae to pupae in females and males, with the old cuticle in brown and the new cuticle in white. FISH was carried out on semithin sagittal histological sections of a **b** *L. villosa* larva, **c, e** larvae right before pupation (sex unknown but assumed), and **d** female and **f** male pupae. Numbers indicate the location of symbiotic organs or cuticular structures in the respective position and all images show cutouts of sagittal sections with the cranial end to the left.

3.3. Symbiont transmission from pupa to female adults

It is unclear how symbionts are transmitted from the dorsal structures in pupae to the accessory glands of the reproductive system in female *Lagria* beetles (Figure 5 a, b). In our FISH experiments, we only detected symbionts on the outer surface (Figure S2) and in the cuticular organs of pupae, but never inside the body. Therefore, we hypothesized a transfer of the symbionts on the body surface to the reproductive tissues during metamorphosis, as proposed for *L. hirta* and observed for the sucking louse *Haematopinus*, where symbionts are transmitted through the molting fluid towards their origin into the ovaries of females [1]. Hence, we investigated when and how the symbionts colonize the accessory glands of female *L. villosa*. We first compared accessory glands of a mature and a newly emerged female via FISH to evaluate potential differences in symbiont presence and quantity. In mature females, the accessory glands are densely packed with symbionts (Figure 5 c, d), and dense symbiont cultures were also observed in the newly emerged female (Figure 5 e, f). We estimate that this specimen had emerged within a few hours prior to fixation, as deduced from its light and incompletely melanized wings when fixated. Nonetheless, it housed a large number of symbionts, which suggests either transmission of many symbionts from pupa to adult, or a notably fast symbiont population growth rate within the accessory glands. Since the dominant symbiont strain in *L. villosa* beetles, *B. gladioli* Lv-StB does not have genes involved in chemotaxis or flagella assembly [32], we were interested in whether an immotile bacterium could be transmitted over a distance from the thorax to the abdomen. Therefore, we used fluorescent latex beads to simulate immotile symbionts of comparable size (1 µm) and tracked them during pupation via microscopy (Figure 6). We applied 10^6 latex beads to the dorsal thorax of pupae resembling natural symbiont titers (Figure 6 a) and then localized the beads in newly emerged adults. We found the beads at the caudal end of the ventral abdomen (Figure 6 b, c) as well as on the elytra (Figure 6 d) near the thorax (Figure 6 e) and on the dorsal caudal end (Figure 6 f). After removal of the elytra and integument, beads were found in the caudal region of a male beetle (Figure 6 g) and in a female beetle in the region of the reproductive system (Figure 6 h-k). These observations suggest that immotile symbionts can be externally translocated by the host from the dorsal thorax to the region around the reproductive system (Figure 6 l). From there, they likely colonize the symbiotic organs only present in females by so far unknown mechanisms.

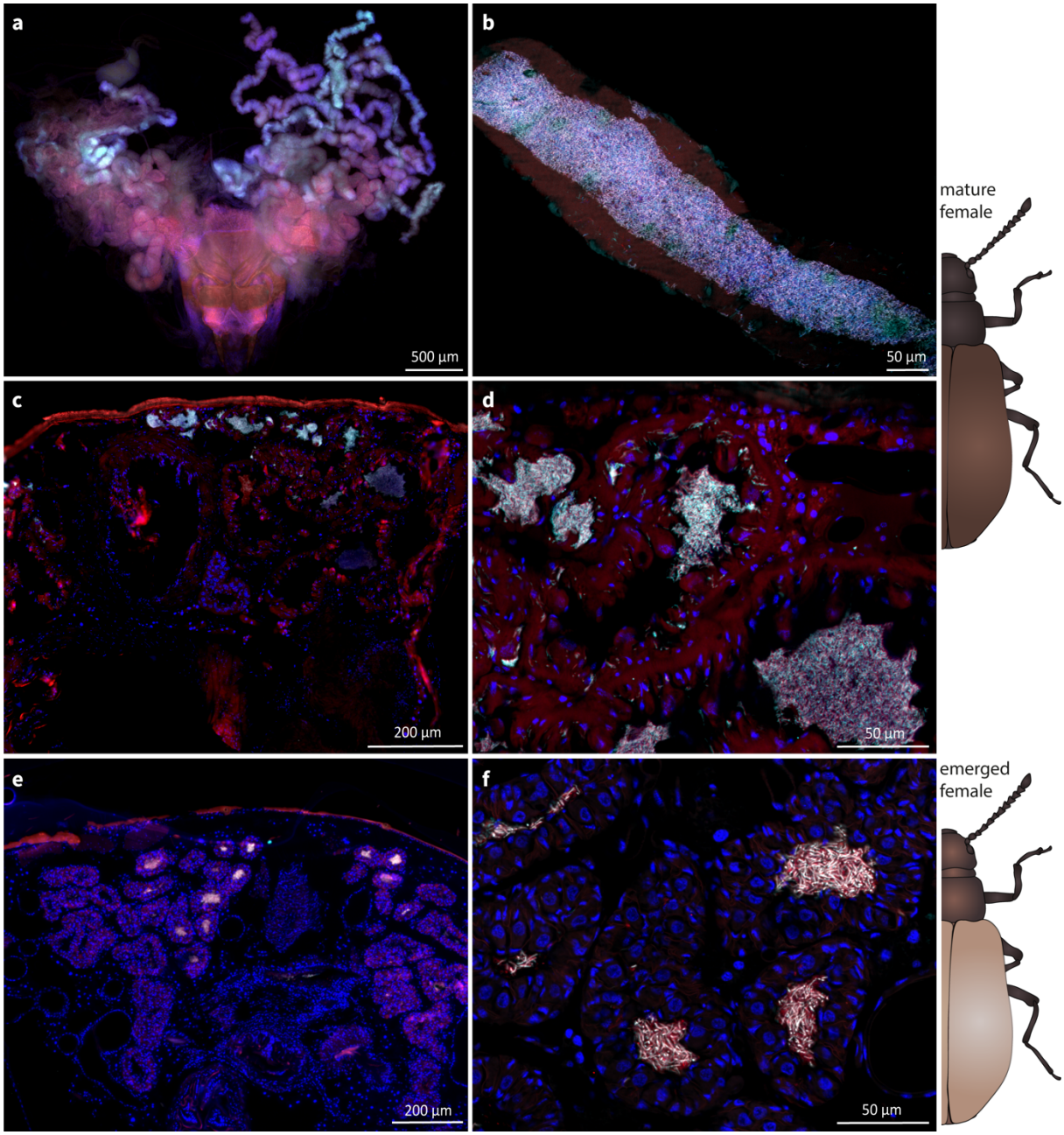

Figure 3: Symbiont presence and localization in mature and recently emerged *L. villosa* females. FISH images of the accessory glands of mature (a-d) and freshly emerged (e,f) field-collected females. Symbionts are generally depicted in white, host cuticle and tissue in red and host nuclei in blue. **a** FISH of a whole-mount dissected ovipositor and accessory glands showing the tubular structure of the symbiont-bearing organ. **b** Higher magnification of one tube which is densely filled with bacteria. **c, d** Transversal section through a mature female abdomen showing symbionts within the accessory glands. **e, f** Transversal section through a freshly emerged adult also showing dense accumulations of symbionts within the accessory glands. For (a-d) *Burkholderia*-specific staining is shown in red, Lv-StB specific staining in cyan, and host cell nuclei in blue (DAPI). For (e-f) Lv-StB specific staining is shown in cyan, general eubacterial staining in red, and host cell nuclei in blue (DAPI). Overlapping signal is shown in white.

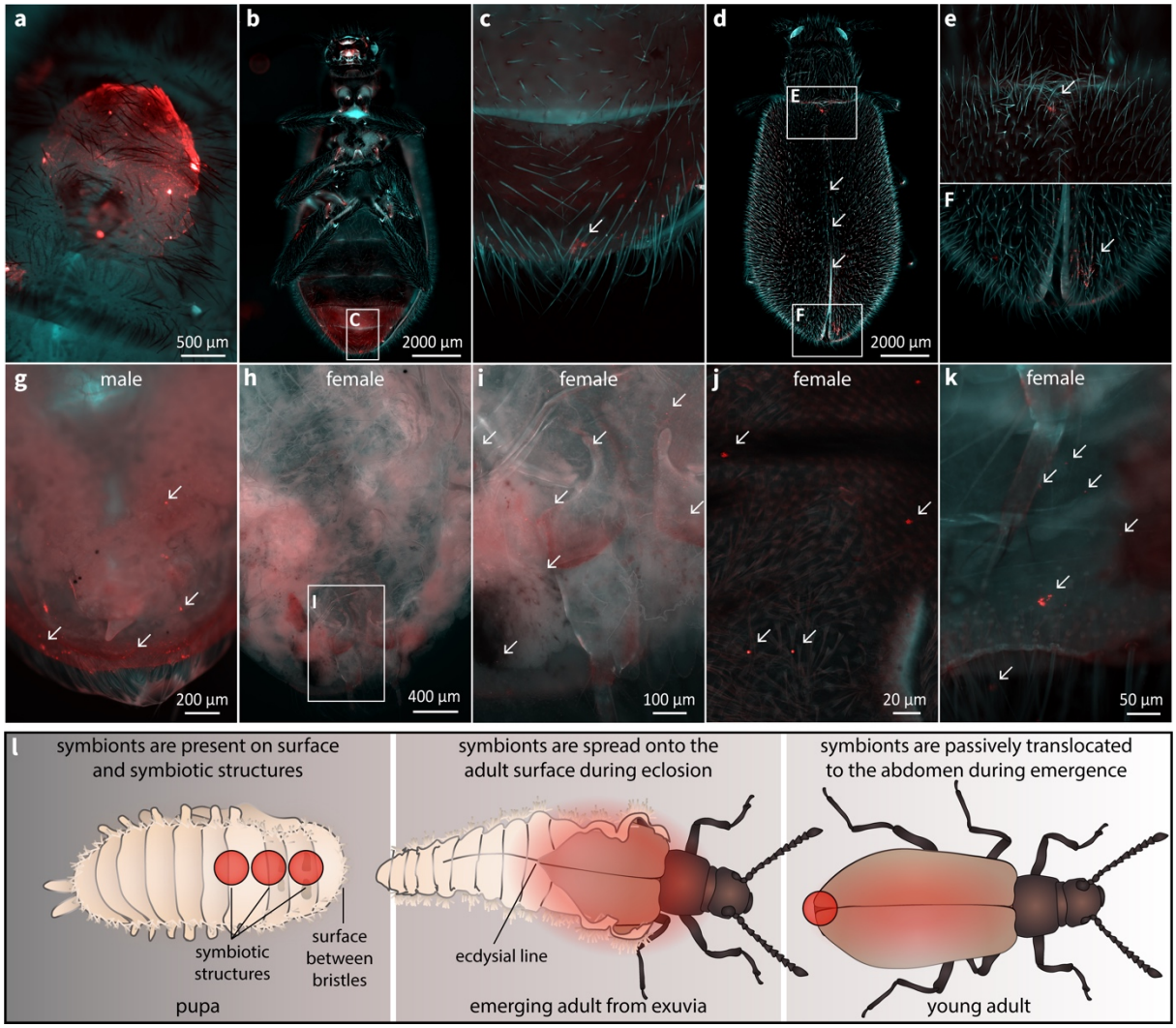

Figure 4: Transmission of immotile beads from the dorsal thorax of pupae to the abdominal region in adults. Fluorescent beads were applied to the region of the dorsal symbiont organ in *L. villosa* pupae and their location was examined on emerged adults. Fluorescent beads (arrows) are shown in red. The general autofluorescence of the insects in the EGFP channel is shown in cyan, while weak autofluorescence was also present in red. **a** Location of the applied beads on the surface of a pupa. **b** Ventral view of an emerged adult showing beads in the **c** caudal abdominal region. **d** Dorsal view of an adult showing beads on the **e** thorax and abdomen and at the **f** caudal part on the elytra. **g** Dorsal view of a male abdomen, where elytra and integument were removed showing accumulations of beads. **h-j** Dorsal view of one female abdomen without elytra and integument, showing multiple beads in the region of the reproductive system. **k** Dissected abdomen of a second female showing several beads in the abdomen. **l** Proposed mechanism of the translocation of beads and symbionts during eclosion. Red circles or areas indicate the presence of symbionts or fluorescent beads observed across our experiments.

4. Discussion

Here we characterize the developmental dynamics of the symbiosis between *Burkholderia* ectosymbionts and the beetle species *L. hirta* and *L. villosa,* specifically during the host pupal stage. By quantifying and localizing the symbionts, detailing the structure of the symbiotic organs, and simulating a possible transmission route for the symbionts during pupation, we gain novel insights into the morphological rearrangements and the role of specialized symbiotic organs during metamorphosis of this insect.

Morphological adaptations to accommodate microbial symbionts have evolved in many insect taxa. Sometimes, these adaptations are associated with the exoskeleton of the insect as modifications of the cuticle [1,14,27,33,34]. Among those, the symbiotic organs in *Lagria* larvae and pupae are unique given their morphology and presence on the dorsal thorax. However, their developmental origin has not been elucidated yet. These are cuticle-lined invaginations associated with glandular cells [27], which likely aid in nourishing the symbionts via secretions. Similarly, mycangia are structures accommodating mainly fungal symbionts in bark and ambrosia beetles [35,36], lizard beetles [37], ship-timber beetles [38], stag beetles [39], leaf-rolling weevils [40] and also wood wasps [41]. In ambrosia beetles, large variation in anatomy and location of mycangia is described, while their purpose is generally symbiont cultivation and transmission [33]. Glandular cells, likely for nourishing the symbionts, are associated with the mycangia [42]. Some mycangia occur in a similar region as the *Lagria* organs at the dorsal thorax [42], but seem to be only present in adult beetles.

Unlike the nutritional benefit in mycangial symbioses in beetles, other cuticle-associated symbionts protect their hosts, food source, or offspring against pathogens. Adult attine ants harbor defensive *Pseudonocardia* symbionts within cuticular crypts [34,43,44], antibiotic-producing *Streptomyces* symbionts are located within antennal gland reservoirs and on the pupal cocoon of beewolves [12–14,45], and protective *Penicillium* fungi colonize mycangia of the leaf-rolling weevil *Euops chinensis* [40]. In these systems, immature host stages lack specialized structures to accommodate their symbionts, which might be due to the sheltered lifestyle within burrows, galleries, chambers, or leaf cradles during development. In those habitats, there might be no selective pressure to evolve or retain specialized structures, if symbionts can be deposited along with the offspring and taken up later.

Storing the symbionts outside the host in the environment can be a way to circumvent problems associated with symbiont transmission during complete metamorphosis [3,46], which often requires symbiont translocations across morphologically different life stages. In eusocial insects, such as bees [47] or ants [48] with overlapping generations in a shared environment, social transmission allows the host to have aposymbiotic life stages without losing the association to symbionts [49]. For solitary holometabolous hosts without this opportunity, maintaining beneficial symbiotic organs and symbionts can be problematic, especially during pupation, when the larval tissue is remodeled into the completely distinct adult stage [3,50]. Symbionts that are located internally or associated with the gut epithelium can be especially affected by this reorganization. Degradation and remodeling of the gut

might lead to elimination or shifts in microbial communities [50,51], however, a core microbiota can sometimes be maintained even under drastic conditions [52]. *Lagria* pupae circumvent the risk of losing symbionts during metamorphosis through specialized structures that enable maintenance and direct transmission of symbionts during female development. In addition, their defensive symbionts stay connected with the host in every life stage. This might facilitate free foraging in the environment without constraints of building or finding specialized habitats for environmental symbiont transmission during delicate phases, like molting or pupation.

Although *Lagria* beetles continuously house *Burkholderia* symbionts, they offer different habitats during their life cycle. The conditions for the symbionts might change not only due to occasional horizontal transmission through the plant environment [30] but also within the beetle. Starting from direct exposure to the environment on the egg stage for around six days, they colonize a more confined habitat with contact to the environment in the dorsal structures of larvae. Then, they inhabit an again more exposed stage on the surface during pupation, which usually lasts six days, until they reach their final and probably most sheltered condition in the female accessory glands. Changes in the environment, even if minor, impose selective pressures on the symbionts and might force them to adapt to different abiotic and biotic factors, e.g. temperature, pH, nutrients, or host immune factors [53]. While we cannot draw conclusions on the molecular factors that are involved in symbiont colonization and relocation, we can suggest potential mechanisms for translocation of the bacteria based on our results on the morphology and development of the symbiotic organs across life stages. It is plausible that motile members of *Lagria* beetle microbiota can actively move longer distances during translocation to another organ, yet Lv-StB [32] can colonize despite being presumably immotile. Here, we show that accessory glands of early emerged adult females are already highly infected with the symbionts, including Lv-StB. Therefore, it is possible that colonization by a few cells is sufficient, and they can rapidly grow within the glands in the first hours after emergence. However, the precise growth rate of Lv-StB during this period has not been determined.

Because we have not detected symbionts within the guts of female pupae and never within host cells, an internal transmission to the reproductive system during pupation seems unlikely [1]. The transfer of immotile beads into the region of the symbiotic organs suggests that host movements might contribute to translocation of symbionts without fully relying on bacterial motility (Figure 6 l). However, the relevance of motility to finally colonize the structures remains uncertain. In many vertically transmitted symbionts, relaxed selective pressures on genes involved in motility and chemotaxis have led to the loss of these genes [54]. For intracellular symbionts, motility is not needed for transmission when symbionts are consistently present in germ line cells [55] or they can rely on host-mediated transport mechanisms [56]. Endosymbionts that are only located in bacteriomes can also be transmitted via migrating bacteriocytes [24,57]. Also, many pathogens are non-motile, yet able to infect hosts [58], often by being transported via hitchhiking on other bacteria [59]. For gut symbionts, host behaviors such as peristaltic movements might also help symbionts reach their niche

after they are ingested orally, making motility expendable [22]. Migration via the molting fluid, i.e. the liquid accumulating between old and new cuticles before each molt, is also possible and could exempt symbionts of specialized motility. This has been described in bacterial symbionts of *Haematopinus* lice, harbored in three symbiotic "depots" that develop in the dorsal area of female larvae, close to the midgut [1]. Before the final molt leading to adulthood, the bacteria are released from these depots into the molting fluid, and thereby reach an opening to the developing reproductive organs. This allows the symbionts to colonize ovarial ampullae and later facilitate vertical transmission to the eggs [1,60]. While apparently similar, the dorsal depots of lice have a different histological origin to the symbiotic structures of *Lagria* larvae, as they are not cuticular. Also, based on our histological observations, the dorsal crypts of *L. villosa* are unlikely to have access to the molting fluid (Fig. 4 b-f). Instead, immotile *Lagria* symbionts are possibly translocated passively during emergence from the exuvia. Exuviae open at the cranial end of the dorsal ecdysial line exactly at the location of the symbiotic organs, from where the freshly emerged adults crawl out. This breaking point might facilitate symbiont transmission, ensuring that the host is infected with the symbionts from the exuvial surface during emergence (Figure 6 l). However, it is possible that some kind of motility is necessary to finally reach the accessory glands in adult females.

The defensive function of *Burkholderia* symbionts was previously described via manipulative bioassays for *L. villosa* eggs [29,31] and young larvae [Chapter I]. A defensive role of the symbionts during the pupal stage is also possible, since bioactive compounds could be detected in organs of *L. villosa* pupae and the respective exuviae [Chapter I]. However, since our current results show that *L. villosa* and *L. hirta* female pupae contain higher symbiont titers than the male counterparts, the latter may be less well defended. While low titers of symbionts in addition to remnants of the defensive metabolites may be sufficient for aiding in protection during male pupation, this requires further investigation. Plausibly, transmission to the female accessory glands might be the major selective pressure to maintain symbionts during pupation.

Although metamorphosis is a key driver of adaptability and diversity in holometabola, it comes with the constraints of higher vulnerability during pupation and the need to relocate beneficial symbionts [3,50]. While numerous taxa within most holometabolous insect orders are described to harbor mutualistic microbes, their maintenance and relocation in immature life stages (i.e. larvae and pupae) and specifically during metamorphosis are only rarely studied. Here, we elucidate the morphology of peculiar symbiotic structures of the exoskeleton in pupae of two *Lagria* species and propose the transmission route of ectosymbionts during metamorphosis. The morphological modification of the dorsal cuticle allows the beetle to retain the valuable symbionts in a reservoir despite remodeling of internal structures during metamorphosis.

5. Methods

5.1. Insect collecting and rearing

L. hirta individuals were collected in Germany in 2020 (Table S1) and reared in a terrarium (80 cm×120 cm×135 cm) consisting of a mesh cage and a plastic container filled with watered soil and live blackberry plants. The terrarium was maintained outside under trees and a canvas cover to protect it against heavy rain and to simulate semi-natural rearing conditions over the winter. The offspring of these individuals were collected in 2021. *L. villosa* individuals were collected in Brazil in 2019 (Table S1), reared in plastic containers in a climate chamber (16:8 L:D light regime at 26 °C and 60% humidity), and were fed with leaves of lettuce, soybean, and kidney bean.

5.2. Sex determination in *Lagria* pupae

The sex determination of *Lagria* pupae was done by a combination of approaches. In both species, sex can be roughly estimated by size, since females are often bigger and heavier than males (Figure S1 a). An accurate sex determination was possible in early pupae based on the caudal abdominal region and the morphology of the genital papillae in both *Lagria* species (Figure S1 b-d). For *L. hirta* pupae, the sex can also be determined based on the last antennal segment, which is notably longer in males (Figure S1 e).

5.3. Determination of symbiont titers using DNA extraction and qPCR

L. villosa individuals were collected in the field or as offspring of field-collected females and preserved dry at −80 °C or in ethanol at −20 °C. Adult females were dissected to obtain the symbiont-bearing structures, whereas entire individuals were used for all other life stages. Samples were homogenized with liquid nitrogen, and DNA extraction was carried out using the Epicentre MasterPure Complete DNA and RNA Purification Kit or the innuPREP DNA/ RNA Mini Kit (Analytik Jena) following the manufacturer's instructions. To analyze symbiont titers across *L. villosa* life stages, qPCRs were carried out targeting the gyrase B gene of all *Burkholderia* strains using the primers Burk_gyrB_F (5'-CTCGAGAAGCTGCGCTATCA-3') and Burk_gyrB_R (5'-GCGATAGAGGAACGTGAGCA-3'). To determine differences in symbiont titers between sexes in *L. villosa* and *L. hirta* pupae, qPCRs were conducted targeting the 16S rRNA gene targeting all known *Burkholderia* symbiont strains of *Lagria* beetles, using the primers Burk16S_1_F (5'-GTTGGCCGATGGCTGATT-3') and Burk16S_1_R (5'-AAGTGCTTTACAACCCGAAGG-3'). qPCRs were carried out using the 5x HOT FIREPol EvaGreen HRM Mix EvaGreen (Solis BioDyne) on a RotorGene-Q cycler (Qiagen) in 10 µL reactions including 0.5 µL of each primer and 1 µL template DNA under the following conditions: Initial activation at 95 °C for 15 min, denaturation at 95 °C for 15 s, annealing at 62 °C for 15 s and elongation at 72 °C for 15 s for 50 cycles. Standard curves were created by

amplifying the fragment, followed by purification and determination of the DNA concentration using a Qubit fluorometer (Thermo Fisher). A standard containing 1 ng/µL was generated and 1:10 serial dilutions down to 10^{-8} ng/µL were prepared. All standards and no-template controls were included in the qPCR run for absolute quantification.

5.4. Morphology of the cuticular structures of female and male pupae (µCT)

For each *L. villosa* pupal stage (early, middle, and late), two female and two male individuals were prepared for micro-computed tomography (µCT) analysis. To this end, samples were fixed in 4% PFA in 80% ethanol at room temperature for 48h and then washed twice with 80% ethanol for 1h each time under agitation. After samples were dehydrated in denaturated ≥99.8% ethanol for 48h, contrasting was performed in freshly prepared 1% resublimated iodine (Carl Roth, Karlsruhe, Germany) in pure methanol at room temperature for 24h. Subsequently, the samples were washed three times for 1h each using denaturated ≥99.8% ethanol and under shaking conditions, followed by three times in pure ethanol. Drying was performed in an automated EM CPD300 critical point dryer (Leica Microsystems, Wetzlar, Germany) at medium speed CO_2 supply with a delay of 20 minutes, with 20 exchange cycles, followed by heating at medium speed and slow gas exhaust. The dry specimens were attached upright to an approximately 5 mm piece of fishing line using a UV-curable adhesive Fotoplast Gel (Dreve Otoplastik, Unna, Germany) and mounted on the specimen holder.
All X-ray scans were performed using a SkyScan 1272 microtomograph (Bruker, Kontich, Belgium) with 360° rotation, 0.2° rotation steps, and a frame averaging of 4. The average source voltage and current were adjusted to 39-100 kV and 100-200 µA, respectively, to generate consistent signal attenuation of approximately 35%. The average pixel size was 5-6 µm. The NRecon software (Bruker, Kontich, Belgium) was used for reconstruction and ring artifact correction.
Image analysis was performed using Dragonfly 2020.2 [Object Research Systems (ORS) Inc, Montreal, Canada, 2020; software available at http://www.theobjects.com/dragonfly]. TIFF image stacks were imported with an X:Y: Z ratio of 1:1:1 and precisely aligned within the clipping box. The segmentation and reconstruction of symbiotic organs was performed manually. 2D Videos were created in Dragonfly with a speed of 40 FPS.

5.5. Evaluation of symbiont presence, localization, and host morphology using fluorescence *in situ* hybridization (FISH)

Lagria larvae and pupae were fixated in 4% formaldehyde for at least 3 days. Embedding, semithin sectioning, and FISH were performed as described previously [61]. One pupal-adult exuvia was placed on a glass slide with double-sided adhesive tape and fixated with 70 % ethanol. The Cy3- or Cy5-labeled Burk16S probe (5'-TGCGGTTAGACTAGCCACT-3') was used to mark all *B. gladioli* strains and the Cy3-labeled EUB338 probe (5'GCTGCCTCCCGTAGGAGT-3') was used for general eubacteria. DAPI (4',6-diamidino-2-

phenylindole) was used to label the host cell nuclei and as counterstaining. Images were taken on an AxioImager.Z2 fluorescence microscope (Zeiss, Jena, Germany). To determine symbiont presence and localization in female and male pupae, sagittal sections of early pupae were imaged and relevant locations or areas containing symbionts were analyzed, while the exuvia was imaged as a whole. To find out when morphological changes of the symbiotic organs occur during female and male development, sagittal sections of a 1st instar larva, two late last instar larvae before pupation, and two pupae were analyzed focusing on the morphology of the symbiotic organs.

5.6. Simulation of immotile symbiont transmission from pupa to adult using fluorescent beads

To simulate symbiont transmission of immotile bacterial symbionts, five early *L. villosa* pupae were inoculated with fluorescent beads (Sigma Aldrich, Latex beads, amine-modified polystyrene, fluorescent red, 1.0 µm mean particle size). Pupae were kept in separate wells of a 24-well plate, which was prepared with moist vermiculite and sterile filter paper. Fluorescent beads were diluted in PBS to a final concentration of 10^6 beads/µL and 5 µL were added to the dorsal thorax at the region of the symbiont-bearing organs. Two freshly emerged female adults and one male as a control were then dissected, starting with removal of the elytra and wings until the reproductive organs were fully visible. In between each dissection step, dissection tools were washed and images were taken to determine the location of the fluorescently labeled beads. Images were acquired on an AxioImager.Z2 fluorescence microscope (Zeiss, Jena, Germany).

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SISGen genetic patrimony access permit A412E4B and shipping permits RD32745 and R41A5BB.

8. Contribution to the field

Metamorphosis in holometabolous insects is characterized by a complete change in body plan and the resulting distinct life stages: egg, larva, pupa, and adult. Although metamorphosis is likely one of the reasons for the success and diversity of major insect groups, it also involves key challenges. Among these, drastic changes in tissues can affect structures harboring beneficial microbial symbionts. These reorganizations can be problematic for maintenance and transmission of specific partners. Despite its relevance in holometabolous insects, symbiont translocation during metamorphosis has been rarely studied. Here, we visually characterize the morphological dynamics of the ectosymbiont-bearing structures of two Lagriinae beetle species during the pupal stage and quantify symbiont titers in both sexes. We found that sex-dependent changes occur in the cuticular symbiotic structures on the dorsal thorax. Female pupae retain these along with their ectosymbionts, likely for later transmission to the offspring, while the organs and symbiont titers are reduced in males. We also used fluorescent beads to simulate a potential symbiont translocation route during pupation, suggesting that immotile symbionts can likely reach the symbiotic organs in adult females via the external surface. Thereby, these cuticle-derived adaptations allow the beetle to maintain and translocate ectosymbionts from pupae to the adult female, despite radical morphological rearrangements during metamorphosis.

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10. Supplement

Video S1: µCT through a female *L. villosa* pupa. Symbiotic structures are visualized in red.

https://www.frontiersin.org/articles/10.3389/fphys.2022.979200/full#supplementary-material

Video S2: µCT through a male *L. villosa* pupa. Symbiotic structures are visualized in red.

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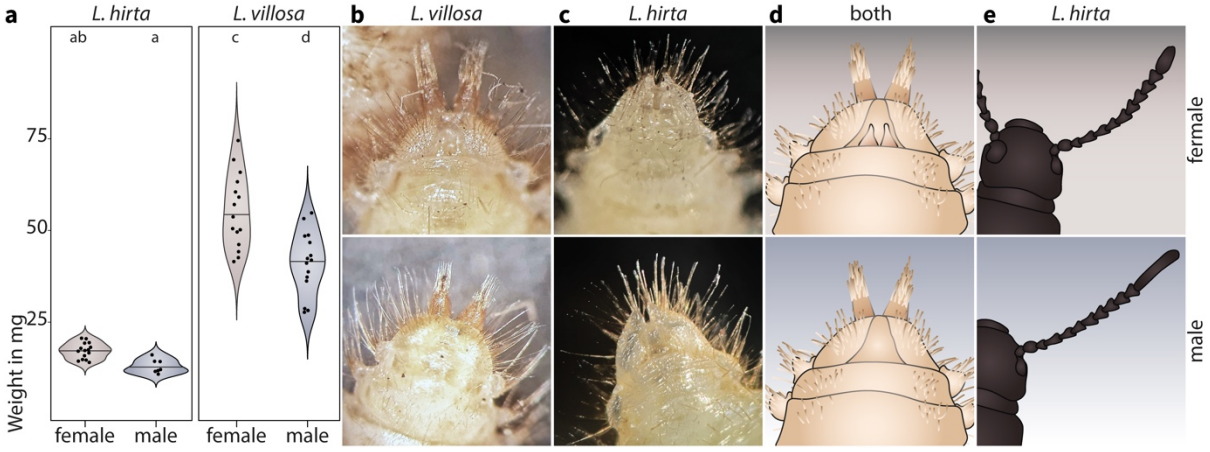

Figure S1: Sex determination of *Lagria* pupae. a Weight of early *Lagria* pupae. **b-e** Morphological characteristics of female (upper row) and male (lower row) pupae. **b, c** Photographs of the ventral side of the caudal region of the abdomen of **b** female and male *L. villosa*, and **c** *L. hirta* pupae. **d** Illustration showing visual differences between female and male pupae. **e** Differences in the length of the last antennal segment of *L. hirta* beetles, which can be observed in the pupal and adult stage.

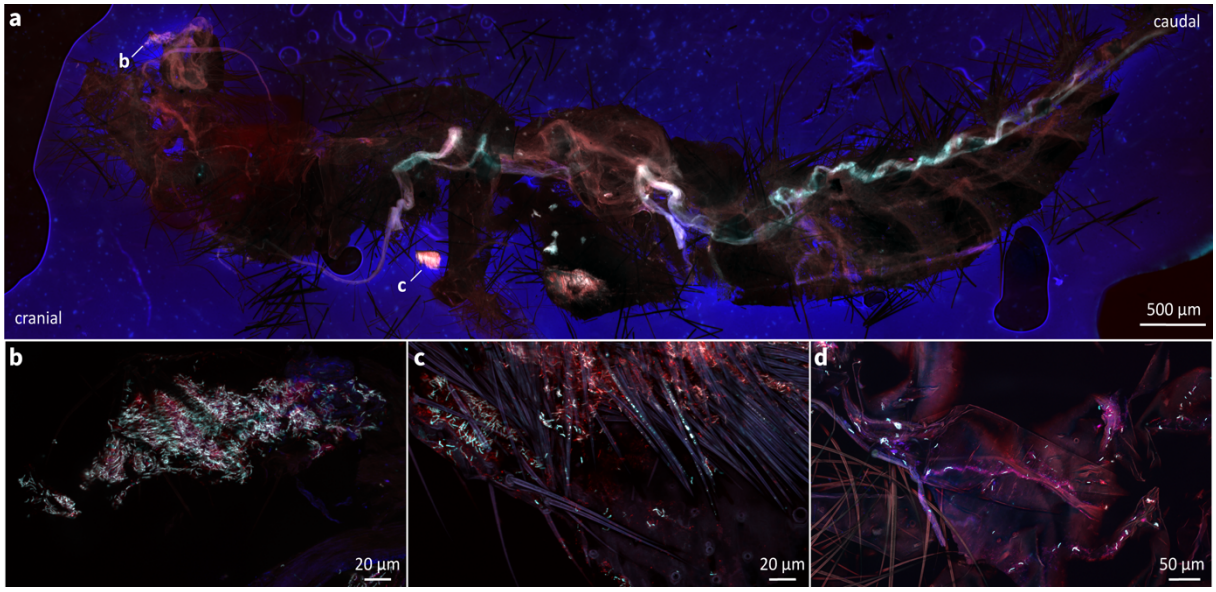

Figure S2. Symbiont localization on the surface of *L. villosa* pupae. Symbionts are generally depicted in white, host cuticle and tissue in purple and the adhesive tape in blue. *Burkholderia-specific* staining is shown in cyan, eubacterial specific staining in red, and overlapping cells in white. **a** FISH on an adult-pupa exuvia collected from the field. **b, c** Dense symbiont accumulations in different parts of the exuvia shown in **a**, including the external surface, as evidenced by the insect bristles in **c**. **d** Symbionts spread out across the exuvia.

Table S1: Original localities of collected beetles and their use for this study

Time	State	Locality	Coordinates	Habitat	Species
July, 2020	Rheinland-Pfalz, Germany	Höhr-Grenzhausen	50°26'12.8"N, 7°40'46.7"E	Mixed coniferous forest	*L. hirta*
July, 2020	Rheinland-Pfalz, Germany	Höhr-Grenzhausen	50°26'26.4"N, 7°40'50.2"E	Mixed coniferous forest	*L. hirta*
May 2019	São Paulo, Brazil	Jundiaí	S23° 8' 3.732", W46° 58' 47.352"	Manioc plantation	*L. villosa*
May 2019	São Paulo, Brazil	Jundiaí	S23° 7' 42.06", W46° 59' 25.296"	Starfruit tree	*L. villosa*
May 2019	São Paulo, Brazil	Cordeirópolis	S22° 30' 11.376", W47° 25' 40.08"	Sugar cane plantation	*L. villosa*
May 2019	São Paulo, Brazil	Cordeirópolis	S22° 30' 13.32", W47° 25' 28.092"	Soybean, radish plantation	*L. villosa*
May 2019	São Paulo, Brazil	Itirapina	S22° 15' 15.84", W47° 50' 43.728"	Corn, coffee plantation	*L. villosa*
May 2019	São Paulo, Brazil	Brotas	S22° 16' 18.516", W47° 56' 4.452"	Manioc plantation	*L. villosa*
May 2019	São Paulo, Brazil	Brotas	S22° 17' 25.98", W48° 3' 12.276"	Rye plantation	*L. villosa*
March 2019	São Paulo, Brazil	Cordeirópolis	S22° 29' 26.88", W47° 25' 58.476"	Soybean plantation	*L. villosa*
March 2019	São Paulo, Brazil	Brotas	S22° 17' 25.98", W48° 3' 12.276"	Soybean plantation	*L. villosa*
March 2019	São Paulo, Brazil	Jaú	S22° 15' 49.896", W48° 31' 12.396"	Soybean plantation	*L. villosa*
March 2019	São Paulo, Brazil	Cordeirópolis	S22° 29' 40.776", W47° 23' 48.192"	Soybean plantation	*L. villosa*
March 2019	São Paulo, Brazil	Santa Gertrudes	S22° 27' 56.196", W47° 31' 55.488"	Soybean plantation	*L. villosa*
March 2019	São Paulo, Brazil	Cordeirópolis	S22° 30' 11.376", W47° 25' 40.08"	Soybean plantation	*L. villosa*
March 2022	São Paulo, Brazil	Cordeirópolis	S22°30'11.4" W47°25'40.1"	Soybean plantation	*L. villosa*
March 2022	São Paulo, Brazil	Cordeirópolis	S22°29'27.1" W47°26'04.7"	Soybean plantation	*L. villosa*
March 2022	São Paulo, Brazil	Cordeirópolis	S22°29'13.1" W47°26'34.8"	Soybean plantation	*L. villosa*
March 2022	São Paulo, Brazil	Cordeirópolis	S22°28'42.8" W47°26'38.0"	Soybean plantation	*L. villosa*
March 2022	São Paulo, Brazil	Torrinha	S22°23'41.4" W48°08'43.1"	Soybean plantation	*L. villosa*

CHAPTER III

Severe bottleneck during post-hatch vertical transmission of a defensive beetle symbiont

in preparation

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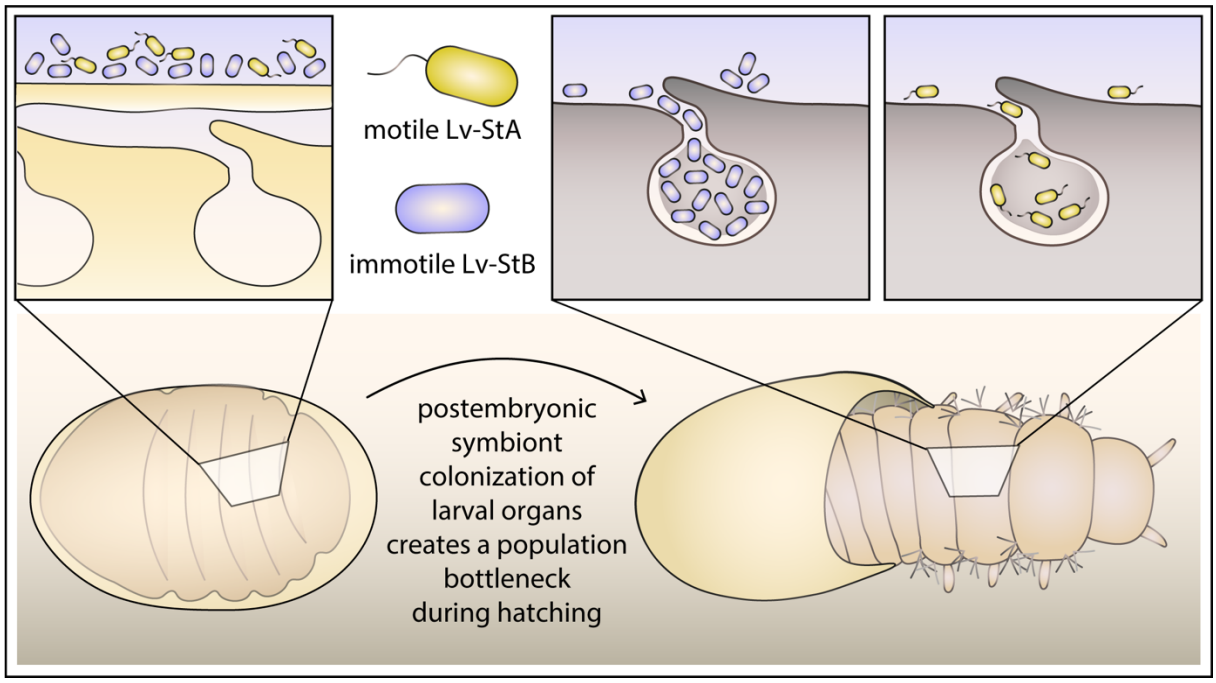

R.G., **R.S.J.**, M.K., and L.V.F. conceived the study and designed the experiments. R.G. carried out main experiments including egg reinfections, CFU-counts, and statistical analyses. R.G. and **R.S.J.** performed light microscopy and FISH experiments, and V.G., **R.S.J.**, and L.V.F. carried out light sheet microscopy experiments. **R.S.J.** generated GFP-mutants of the symbiont strains. R.G. and L.V.F wrote the manuscript and all authors reviewed and commented on the manuscript.

1. Abstract

Beneficial, host-associated microbes are horizontally or vertically transmitted to offspring and usually, a synchronous effort by host and symbiont mediated mechanisms are important for colonization. Additionally, symbionts are subject to a population bottleneck during transmission. The severity of the bottleneck affects genetic diversity and possibly the strength of the symbiotic association with the host. While many studies have investigated the dynamics and population sizes of transovarially transmitted intracellular symbionts, less is known for beneficial microbes that colonize extracellular and external surfaces of hosts, such as the cuticle of insects. Here, we investigate the colonization dynamics of a *Burkholderia gladioli* symbiont of *Lagria villosa* beetles. Symbionts colonize specialized cuticular invaginations on the dorsal surface of *Lagria* larvae that are open to the outside and produce anti-microbial defense compounds. We assessed the bottleneck size and the colonization time-point in the beetles, and investigated the involvement of the host during this process. Previous studies show that the larvae are capable of acquiring symbionts from the environment, however, this appears to be less efficient in younger larvae post-hatching from the eggs. We further hypothesize that the different strains found associated with an individual beetle may have different colonization strategies.

2. Introduction

Transmission of microbial symbionts ensures that symbiont-derived benefits such as provision of nutrients, protection against pathogens or breakdown of harmful products are sustained through generations. Transmission may be horizontal from the environment or unrelated hosts, vertical from parent to offspring or a mixture of both [1] In insects, some symbionts are localized intracellularly in specialized bacteriocytes and are vertically transmitted through the germline [2–4]. By contrast, extracellular symbionts generally experience a phase of environmental exposure during transmission and are acquired after egg hatching [5]. For example, bacterial symbionts can be enclosed in capsules or jelly deposited with the eggs in Plataspid and Urostylidid bugs, respectively, or smeared onto the egg surface during oviposition as occurs in other stinkbugs [6,7], as well as in several beetles and flies [5,8]. . In most of these cases, the freshly hatched nymphs or larvae probe the eggs and acquire the symbionts. Therefore, an active host behavior post-hatching often supports successful colonization. Unusually, in European beewolf wasps, *Streptomyces* symbionts are transmitted post-hatch via the brood cell environment [9,10]. Larvae spin a cocoon and incorporate the symbiotic bacteria while doing so, and emerging adults take up the symbionts into antennal reservoirs [9]. Other extracellular symbionts are horizontally acquired from the environment each generation, like *Caballeronia* symbionts of the *Riptortus* bean bugs [11,12]. There, motility is required for successful colonization [13]. In fact, many horizontally acquired symbionts retain machineries like motility and chemotaxis to enter and reach the symbiotic organs of the host [14,15]. Therefore, host behaviour and symbiont molecular machineries can both contribute to successful colonization and establishment of the symbiosis.

During transmission, the number of colonizing symbiont cells and the timing of entry can have important effects on successful symbiosis establishment as well as on the evolutionary trajectory of the symbiosis. For a number of systems, despite high abundance of symbionts on the eggs or the external environment, only a small number successfully colonizes the symbiotic organs of the host [10,16–20]. The severity of this population bottleneck during colonization can affect microbial community diversity in hosts where multiple strains or species compete for the same niche [21]. The time of colonization can also be restrictive, and a means to enhance specificity in symbiont acquisition and/or synchronize host development with the initiation of symbiosis. For example, symbiont acquisition occurs in young bean bug nymphs only until they reach the second instar, after which colonization is not possible any longer [22]. Similarly, transmission of defensive *Pseudonocardia* symbionts of fungus-growing ants is restricted to a 2h time window after the larvae hatch. Preceding competitors might also be key for symbionts to warrant dominance as observed for colonizing bacteria in squids [23], hydra [24] or humans [25].

As opposed to transovarially transmitted symbionts and those acquired by feeding, the mechanistic basis of colonization for symbionts residing on the insect cuticle has been rarely studied. Symbionts of beewolves or fungus growing ants, which reside in cuticular structures, are known to colonize after egg hatching from maternal provisions or contact with nestmates, respectively. However, how precisely the bacteria invade the cuticular invaginations has not been described. Similarly, *Lagria villosa* beetle larvae carry several strains of *Burkholderia* bacterial in three specialized cuticular invaginations on the dorsal surface of the body [26,27, Chapter I]. However, the process by which these defensive symbionts colonize the cuticular organs of the beetles form the egg surface has remained elusive.

As adults, female *L. villosa* beetles host the bacterial symbionts in accessory glands associated to the reproductive system [26]. Among the different *Burkholderia* strains found in these beetles [26,28], *Burkholderia* Lv-StB (henceforth "Lv-StB") is the most abundant and prevalent symbiont strain across individuals [27, Chapter I]. Yet, this strain remains uncultivated *in vitro* and has a reduced genome in comparison to its close relatives [29]. It is presumed to be immotile, as functional genes for flagellar biosynthesis are absent in the genome [29]. The closely related strain *B. gladioli* Lv-StA (henceforth "Lv-StA") that has been isolated from *L. villosa*, is capable of flagellar motility and as Lv-StB, can produce bioactive compounds that protect the insect host [26]. However, this strain is only sporadically present in the beetles [Chapter I]. About 2×10^6 *Burkholderia* cells are smeared by a female onto the egg surface during oviposition, where they produce antifungal agents that protect the embryo from entomopathogens [26,27]. In the congeneric species *L. hirta*, it was proposed that the symbionts enter the egg before hatching and are able to colonize the dorsal structures as part of a vertical transmission route [30]. Direct evidence for this route is however lacking. Notably, the dorsal structures in *L. villosa* larvae are open to the outside [Chapter I] and recent studies show that the larvae are also capable of acquiring Lv-StA from the environment, which successfully make it to the adult female glands during metamorphosis [31].

Here, we carried out manipulative assays using the culturable strain Lv-StA [26], to estimate the size of the population bottleneck during colonization, determine the timing of symbiont entry into the dorsal structures, and investigate the efficiency of acquisition of this strain during different time point in early in early larvae. We additionally tested host involvement during the colonization process, aiming to better understand the colonization dynamics of a cuticle-associated symbiont strain of *L. villosa* beetles.

3. Results and discussion

3.1. When and how many cells enter the larval symbiotic organs?

Female *L. villosa* adults vertically transmit on average 2×10^6 symbiont cells onto the egg surface [26], where they remain over a period of five to six days until first instar larvae (L1) hatch. At this state, the symbionts are housed in three invaginations of the dorsal cuticle [26, Chapter I]. After transmission onto the egg surface, it is conceivable that either i) symbionts enter the late egg to colonize the dorsal surface structures of the embryo as described for the related species *L. hirta* [30], or ii) larvae acquire the symbionts from the egg surface during or after hatching. To evaluate which of these routes Lv-StA follows in *L. villosa*, we infected freshly laid eggs and estimated the number of cells internalized in mid and late-stage eggs or larvae by CFU counting. We did not detect any colonies on plate when we sampled from the eggs containing the developed embryo (Figure 1 a). However, CFU counts were obtained from L1 and the number of cells that colonize the dorsal glands was estimated to be 2044 ± 3641 cells (Figure 1 a). In second instar larvae (L2), CFU counts amount to 9431 ± 7814 (Figure 1 a). This indicates that symbionts colonize during or after hatching and increase in titers over time. Also, it suggests that there is high variation in the number of colonizing cells across different individuals. A complementary FISH experiment on a dissected embryo previous to hatching (Figure S1 a), and an early L1 support the finding that bacteria are only present within the structures after hatching (Figure S1 b).

Around 0.1 % of Lv-StA cells are present in the dorsal symbiotic organs of L1 after hatching from the egg (Figure 1 a, b), representing a narrow bottleneck for this symbiont. Colonization occurs post-hatching in *L. villosa* and differs from observation of symbionts in *L. hirta* [30], however, it is plausible that other strains associated with *L. villosa* colonize at an earlier time-point. Although speculative, the variation in the bottleneck size during colonization may be related to low stringency in the regulation of symbiont entry by the host at this stage, which would be in line with the presence of multiple bacterial strains within the same host. In L2, the cell number may be overestimated since in the lab set-up we observed that they consume infected exuviae after molting and FISH images show cells adhering to the exuvia lying within the gut (Figure S1 c). Therefore, CFU counts in infected second instars possibly represent the sum of cells that colonized the dorsal organs and are present in the gut. However, since the cells in the gut are not present in all the samples and do not seem to adhere to the gut lining, they are most probably transient.

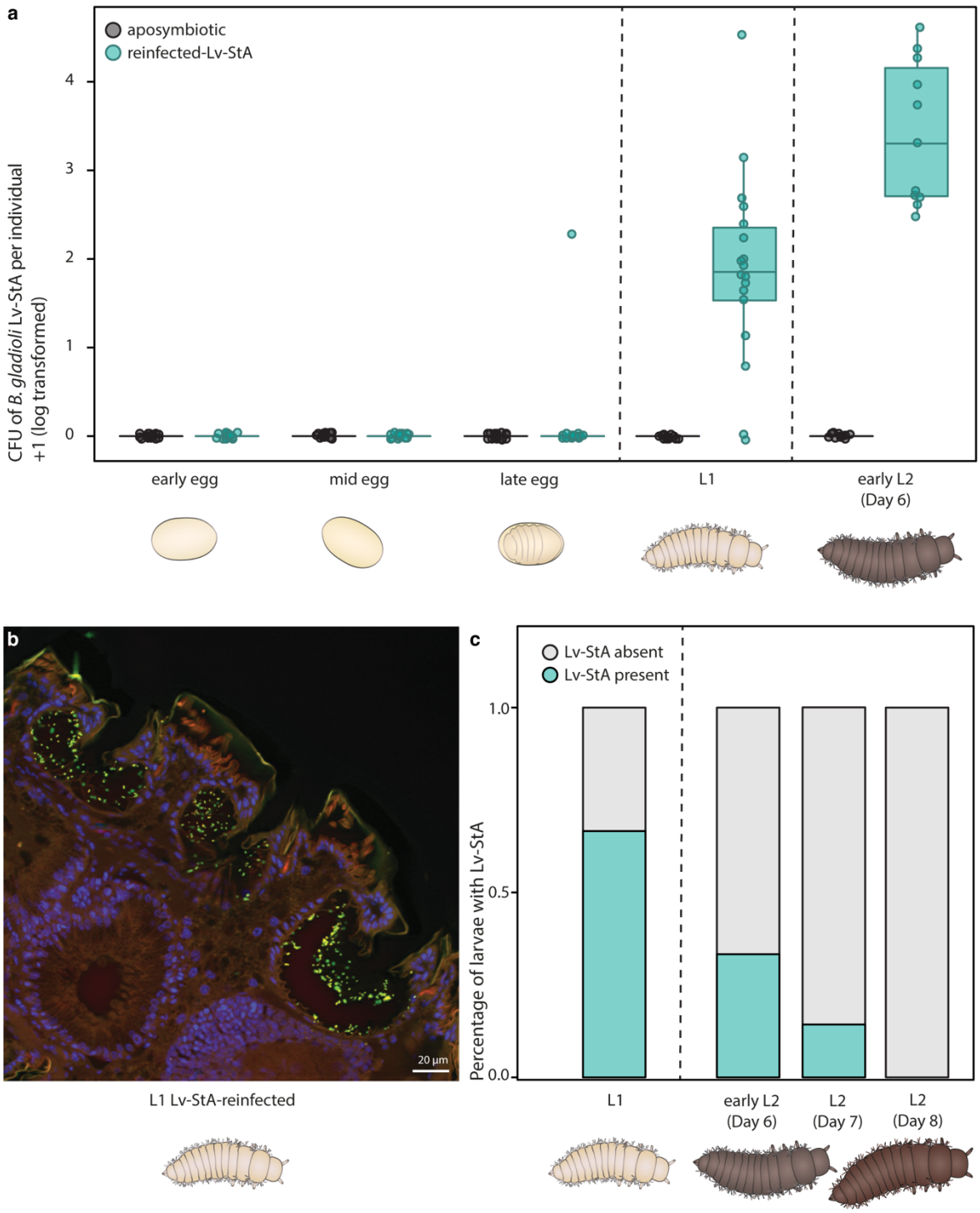

Figure 1: Lv-StA colonizes during or immediately after hatching and the success rate of colonization declines shortly after. a Titers (CFU counts) of Lv-StA per individual egg or larva (CFU count + 1, Log transformed) on eggs from Day days 0, 3 and 4, as well as L1 and L2 larvae. **b** Representative overview image of a L1 larva (surface washed with SDS) hatched from LvStA-reinfected eggs show Lv-StA presence in the three dorsal symbiotic organs. **c** Presence or absence of Lv-StA in the dorsal organs based on FISH pictures from larvae on days 5, 6, 7 and 8, 24 hours after infection on eggs or larvae. At least three cells in the dorsal organs were counted as symbiont presence.

3.2. Vertical transmission vs. post-hatch horizontal acquisition

Larvae are capable of acquiring Lv-StA symbionts when repeatedly exposed to infected leaf-litter for several days [31]. To understand the efficiency of symbiont acquisition in younger larvae in a defined timeframe after hatching, we performed infections on late eggs (day 4), L1 (day 5) and L2 (day 6 and 7) and assessed the presence or absence of Lv-StA, 24 hours

post-infection. There is a trend for lower efficiency of symbiont acquisition on day 7 and especially on day 8 in L2 (Figure 1 c). This suggests that symbiont acquisition from the egg surface is more likely to succeed in comparison to horizontal acquisition.
In contrast to the *Cabelleronia* symbionts in bean bugs [22] and *Pseudonocardia* symbionts in leaf-cutter ants [32], a strict temporal acquisition window may not exist in *L. villosa* larvae. Instead, exposure of larvae to an environment with high strain abundance, and increased frequency of exposure may encourage horizontal acquisition [31]. The presence of other strains in the dorsal organs and priority effects [33] may also result in a different outcome during post-hatch acquisition.

3.3. Is motility or the interaction with live cells important for colonization?

The dominant symbiotic strain, Lv-StB is likely immotile, lacking genes for motility and chemotaxis [29]. It is puzzling how a strain lacking a flagellum can successfully colonize and dominate the host-associated community, given that translocation to a specific site in the host seems necessary Chapter I. By simulating symbiont transmission from the egg to the larval stage using fluorescent beads, we tested whether motility is indeed required, or if the larva's movements while hatching are sufficient to direct the cells into the dorsal structures (Figure 2 a-b). After infecting *L. villosa* eggs with a concentration of beads similar to natural symbiont infections [26], they successfully adhered to the egg surface (Figure 2 c-e). Posteriorly to hatching, the surface of first instar larvae were covered with the fluorescent beads, including regions close to the symbiotic organs (Figure 2 f-i). This suggests that immotile particles can be successfully translocated from eggs to larvae. However, we could not detect beads within the dorsal symbiont organs in whole larvae nor in sections of one individual (Figure 2 j) suggesting that the fluorescent beads did not colonize the symbiotic organs but can reach the larval outer surface despite being immotile.
To visualize the process of colonization, GFP-tagged Lv-StA were infected on eggs (Figure S2 a) and hatching larvae were imaged under the light-sheet microscope (Figure S2 b). Although we could not yet observe the transfer of cells from the egg surface to the dorsal surface structures, we visualized motile Lv-StA in the dorsal surface structures of L1 shortly after hatching (Figure S2 b, Video S1). It is conceivable that the two different strains associated with the beetles, Lv-StA and Lv-StB, may have different strategies to reach the larval symbiotic organ once they are smeared over the larval surface. We suspect that Lv-StB might make use of an alternative approach, like inter-bacterial hitchhiking [34] or a host-controlled mechanism to navigate into the organs, whereas, Lv-StA relies on an intact flagellar machinery. Similar differences in navigation strategies exist in two different *Synechococcus* species associated with *Petrosia ficiformis,* marine sponges. As in the beetle symbionts, contrasting symbiotic lifestyles, transmission modes and the degree of genomic erosion could underlie these abilities [35].

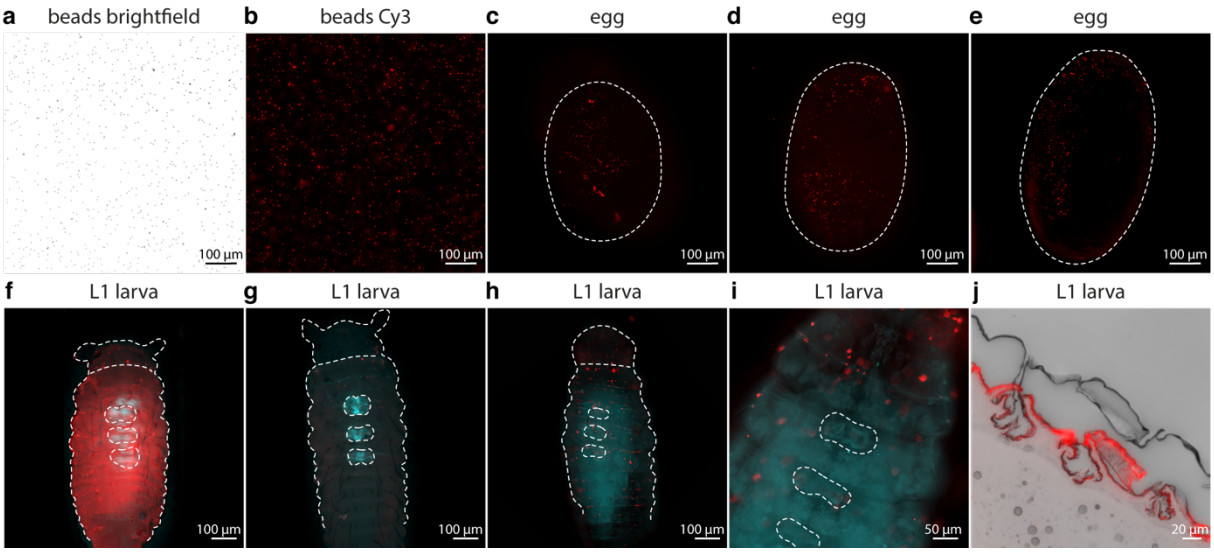

Figure 2: Infection of *L. villosa* eggs with fluorescent beads simulates translocation onto larval surface during hatching. a-b Fluorescent beads (red) in a PBS suspension imaged with **a** brightfield or **b** in the Cy3-channel. **c-e** Individual eggs after infection with fluorescent beads. **f-i** Whole L1 larvae hatched from infected eggs carrying beads on the outer surface, but not within the dorsal symbiont organs. **j** Sagittal section of a hatched L1 larva after bead infection showing no presence of fluorescent beads within the dorsal symbiont organs. Overlay of brightfield (black and white) and Cy3-channel (red). Red signal shows the autofluorescence of the cuticle. Dotted lines were manually added to indicate specimen profiles and dorsal symbiotic organ location based the DAPI signal (not shown) or EGFP channel (cyan) visualizing autofluorescence of the tissue in larvae.

4. Conclusion

Symbiont colonization of a host usually involves considerable changes in effective population size, translocation to a new habitat and re-establishment of the host-microbe interaction. As such, it is a key stage determining symbiosis evolution and stability. We show that *L. villosa* larvae can acquire a few tens to more than a thousand cells from the surface of the egg, most likely when the larva brushes against the egg surface as it hatches out. In these beetles, direct transfer of symbionts from the egg surface is a more effective colonization strategy, compared to horizontal acquisition of later larval instars. However, further experiments investigating the interactions between the multiple symbiont strains during colonization are necessary to understand the natural symbiont colonization dynamics. It is also evident that host movements are sufficient for symbionts to spread over the larval external surface, but navigation into the specialized dorsal structures is most probably mediated by symbiont molecular factors that are yet to be described.

5. Methods

5.1. Insect collecting and rearing

L. villosa beetles were collected in soybean plantations within the state of São Paulo, Brazil, in 2019 and 2020. Adults were fed soybean leaves, lettuce, and kidney bean leaves and kept at 26-28°C and 55-60% humidity under a 16/8 h day/night regime. Water was provided in centrifuge (50 mL) or microcentrifuge (1.5 mL) tubes closed with a cotton plug.

5.2. Preparation of Lv-StA culture for infection

B. gladioli Lv-StA was grown in King's B (KB) liquid media or agar (Soybean peptone 20g/L, K_2HPO_4 1.5 g/L, $MgSo_4.7H_2O$ 1.5 g/L, Agar 15 g/L) and incubated at 30°C, 300 rpm. Before infection on eggs, cells grown in the medium were pelleted by centrifuging at 10,000 rpm for 6 min and the cell pellet was washed with 1 ml 1x PBS twice. The final pellet was resuspended in 1x PBS and cell concentration was determined using a Neubauer cell counting chamber. The concentration was adjusted to 2×10^6 cells/µl in 1x PBS before infecting 2.5 µl of the cell suspension per egg or larva.

5.3. Egg sterilization and infection

Egg sterilization and infection were performed as previously described [26]. An egg clutch containing 200-300 eggs collected upon egg laying and surface sterilized with 200 µl of 70% ethanol for 5 mins and 200 µl of 12% NaClO for 30 seconds. Eggs were washed with sterile water in between each step and after sterilization to remove any remaining bleach. The surface-sterilized (aposymbiotic) eggs were incubated in petri plates lined with wet vermiculite and filter paper.

5.4. Construction of GFP-labelled symbiont strain Lv-StA-GFP

The GFP-labelled strain Lv-StA-GFP originated from the symbiont Lv-StA strain previously isolated from *L. villosa* [26]. It was generated via a mini-TN7 transposon insertion containing P_{lac}:GFP into the symbiont chromosome by triparental mating, as previously described [36,37] using the strains listed in Table S1. Diaminopimelic acid (DAP) auxotroph *E. coli* donor strains WM3064 containing either the plasmid for the MiniTn7KsGFP transposon (pURR25 [36]) or the plasmid for the Tn7 transposase genes tnsABCDE (pUXBF13 [38]) were individually grown on Luria-Bertani (LB) medium (Tryptone 10 g/L, Yeast extract 5 g/L, NaCl 10 g/L) containing, 300 µg/mL DAP and ampicillin (Amp, 100 µg/mL) at 30 °C. The recipient symbiont strain Lv-StA was grown in LB at 30 °C. Donor and recipient strains were conjugated by triparental mating on LB-agar containing 300 µg/mL DAP for 24 h at 30 °C. Afterwards, GFP-labelled recipient cells were selected on LB-agar containing kanamycin

(Km, 50 µg/mL) and screened for GFP-fluorescence using an AxioImager.Z2 fluorescence microscope (Zeiss, Jena, Germany). LvStA-GFP cells were preserved as frozen glycerol stocks at -80 °C and cultured either in LB or KB medium.

5.5. Estimating the time point and CFU of cells that colonize larvae

Lv-StA was infected on one half of a sterilized, freshly laid egg clutch (day 0). The other half was not infected and used as aposymbiotic control. Six individual mid time eggs (day 4), late eggs (day 5), L1 (day 6) and L2 (day 7) were collected from three replicate clutches. We then quantified the cells that entered the eggs or the larval cuticular structures. For this purpose, individuals were surface washed with 100 µl 1% SDS (thrice) and 100 µl of 1x PBS (twice) to ensure that cells that did not colonize the egg-interior or larval structures were washed off. Individuals were crushed in 100 µl 1x PBS and diluted before plating on KB agar plates. Lv-StA colonies that appeared on plate were counted after 24 hours to estimate the CFU (Colony Forming Units) per individual.

5.6. Comparing colonization efficiency across post-hatch timepoints

Freshly laid eggs were surface sterilized and split into four groups. Lv-StA cells were infected on eggs on day 4 (group 1), L1 on day 5 (group 2) and L2 on days 6 (group 3) and 7 (group 4). Two replicate larvae were collected from the infected groups 24 hours post infection (days 5, 6, 7 and 8), embedded in 1% Agar and stored in 4% formaldehyde at 4°C before histological sectioning as previously described [39] and evaluated using fluorescence *in situ* hybridization (FISH), as described below.

5.7. Fluorescent *in situ* hybridization (FISH)

Using FISH, we assessed the presence/ absence of Lv-StA in the dorsal surface structures of L1 and L2 [39]. Semi thin sections (8 µm) of larvae were hybridized with the following probes: Eub338_Cy3 (5'-Cy3-GCTGCCTCCCGTAGGAGT-3') specific for Eubacteria [40], Burk_16S_Cy5 (5'-Cy5- TGCGGTTAGACTAGCCACT-3') specific for *B. gladioli* strains, and DAPI (40,6-diamidino-2-phenylindole) was used to counterstain host nuclei. Hybridized sections were imaged using an AxioImager.Z2 fluorescence microscope (Zeiss, Jena, Germany) or a Leica fluorescence microscope. To compare colonization efficiency before and post-hatching, presence of *B. gladioli* cells was assessed single-blind from the microscopy pictures.

5.8. Simulated transmission of an immotile symbiont strain from eggs to larvae using fluorescent beads

To simulate symbiont transmission of immotile bacterial symbionts, individual *L. villosa* eggs were infected with fluorescent beads (Sigma Aldrich, Latex beads, amine-modified polystyrene, fluorescent red, 1.0 µm mean particle size). Fluorescent beads were diluted in sterile PBS to a final concentration of 10^6 beads/µL and 2.5 µL of the suspension were added onto each egg. After infection, presence and localization of the beads were checked on three eggs under an epifluorescence microscope. After hatching five L1 were either imaged after freezing at −20 °C, alive as a whole mount (four individuals) or embedded in 1 % agar (one individual), and fixed in 4% formaldehyde to undergo histological sectioning as described previously [39]. The location and presence of the beads were observed on whole larvae and sections using the bright field and Cy3-channel on an AxioImager.Z2 fluorescence microscope (Zeiss, Jena, Germany).

6. Funding

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7. Acknowledgements

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9. Supplement

Table S1: Bacterial strains and plasmids used in this study

Bacterial strain	Plasmid	Function	Description	Reference
E. coli WM3064	pURR25	Donor containing transposon	Mini Tn7KsGFP, GFP driven by $P_{lac\ (PA1/04/03)}$ promoter, mobilizable $oriT_{IncP}\alpha$, suicide $oriR_{R6Ky}$; Ap^r (bla)	Teal et al. 2006
E. coli WM3064	pUX-BF13	Helper encoding transposases	Tn7 transposase genes tnsABCDE, mobilizable $oriT_{IncP}\alpha$, suicide $oriR_{R6Ky}$; Ap^r (bla)	Bao et al. 1991
B. gladioli Lv-StA	-	Wild type	Isolate from L. villosa	Flórez et al. 2017
B. gladioli Lv-StA-GFP (Lv-StA-GFP)	-		GFP-mutant of B. gladioli Lv-StA	This study

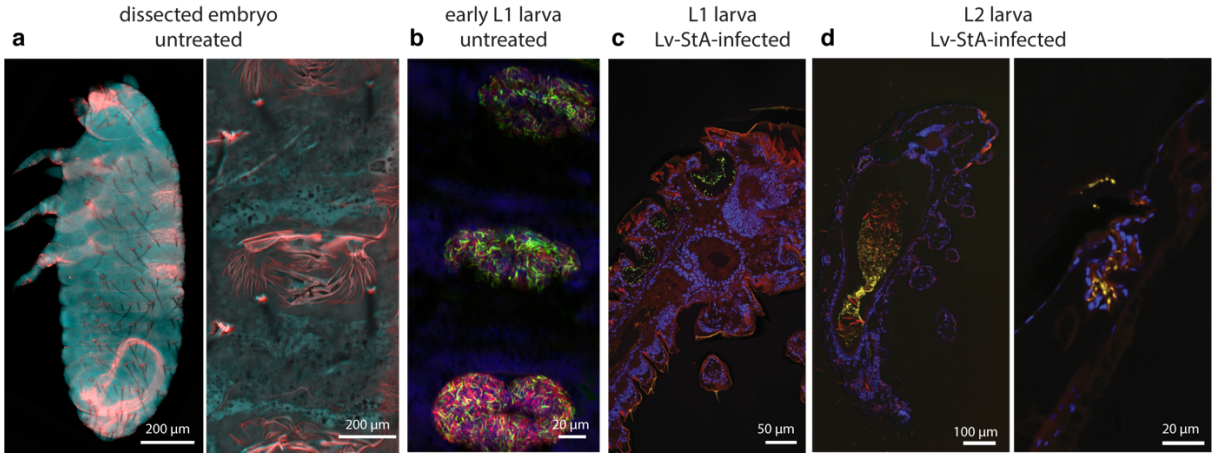


Figure S1: Symbiont localization in different life stages of untreated and infected *L. villosa* specimen. *B. gladioli*-specific staining is shown in green, general eubacterial staining in red, overlapping FISH signal in yellow, and host cell nuclei in blue (DAPI). **a** Dissected embryo from late egg with no symbionts inside the already developed dorsal symbiont organs. Autofluorescence of the host tissue shown in cyan. **b** Whole-mount FISH on an early L1 (untreated) showing numerous symbiont cells inside the three dorsal symbiont organs. *B. gladioli*-specific staining is shown in green, general eubacterial staining in red, overlapping FISH signal in yellow, and host cell nuclei in blue (DAPI). **c** Representative overview image of a L1 larva (surface washed with SDS) hatched from Lv-StA-reinfected eggs show Lv-StA presence in the three dorsal symbiotic organs. **d** Representative image of a L2 larva (surface washed with SDS) hatched from Lv-StA-reinfected eggs show Lv-StA presence in the symbiotic organs, but also transient cells in the gut stuck to exuvia that the larva consumed after molting.

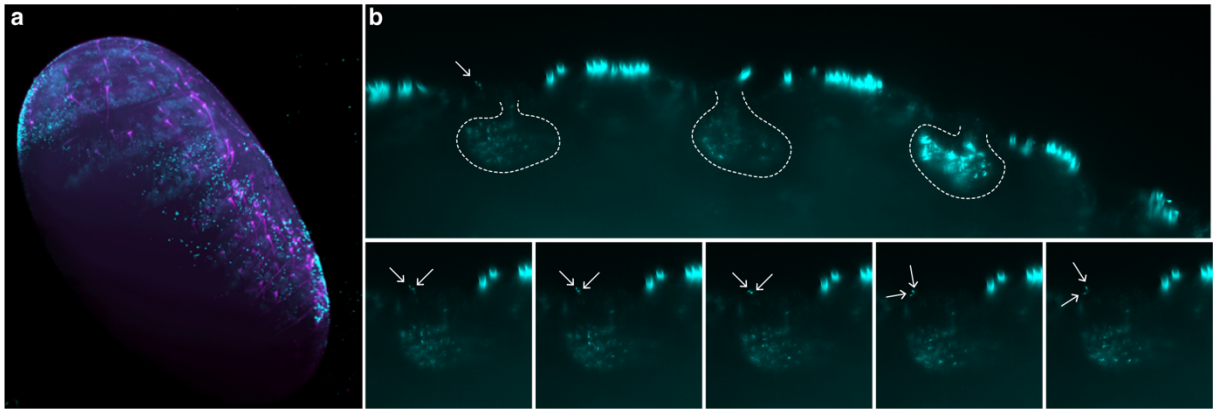
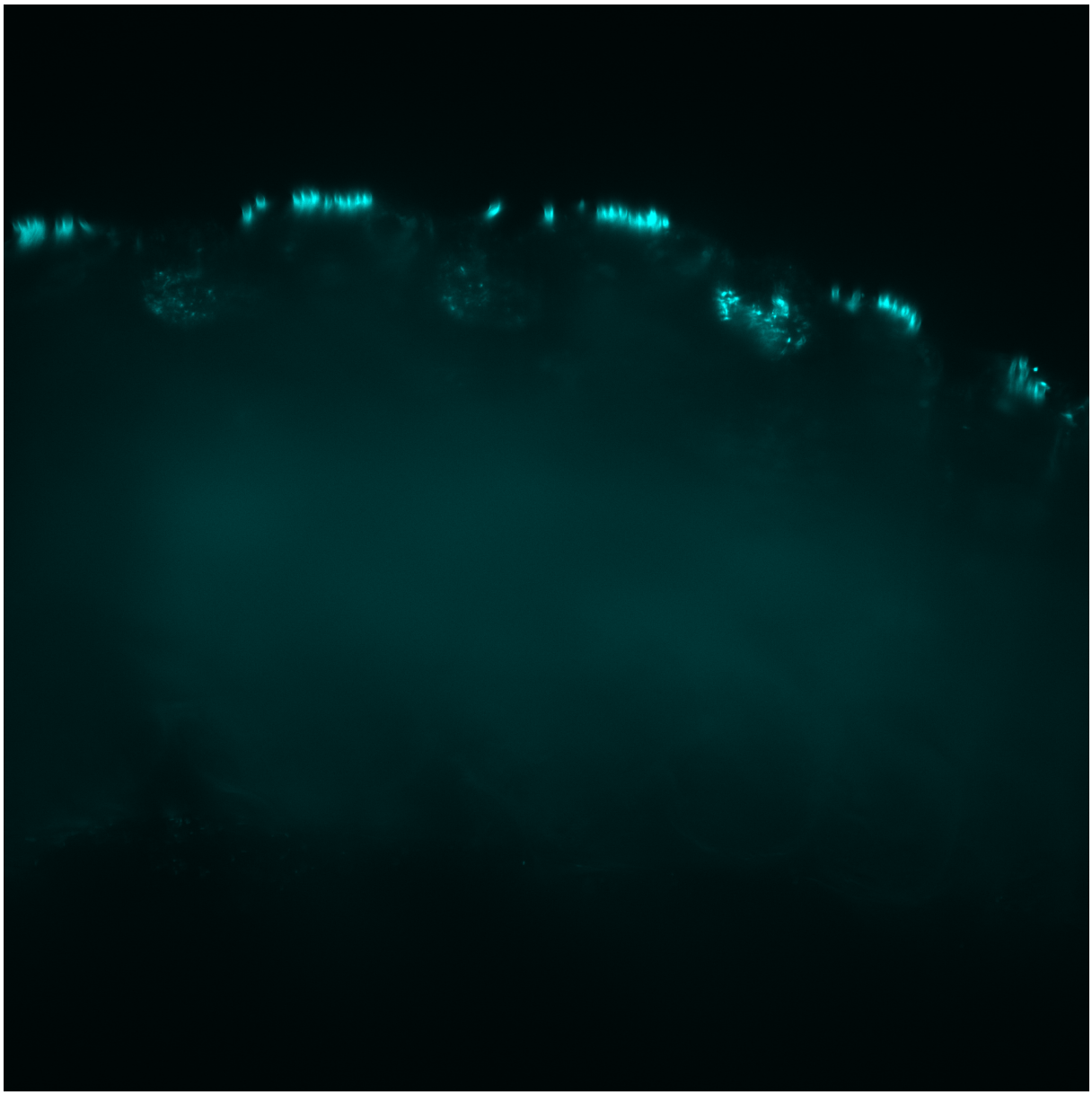


Figure S2: Motile Lv-StA symbiont cells on the larval surface and symbiotic organs. a Lv-StA-GFP cells (cyan) were infected on aposymbiotic *L. villosa* eggs (autofluorescence of embryo in magenta) and imaged with a light sheet microscope. **b** Motile Lv-StA cells were detected moving on the surface and inside the symbiotic organs of a L1 larvae hatched from infected eggs. Arrows indicate two single symbiont cells moving along the surface. Dotted lines roughly represent the outline of the symbiotic dorsal organs.

Video S1: Lv-StA symbiont cells moving on the larval surface and inside the symbiotic organs.



CHAPTER IV

Localization, function and interaction of low-abundance symbionts in *Lagria villosa*

in preparation

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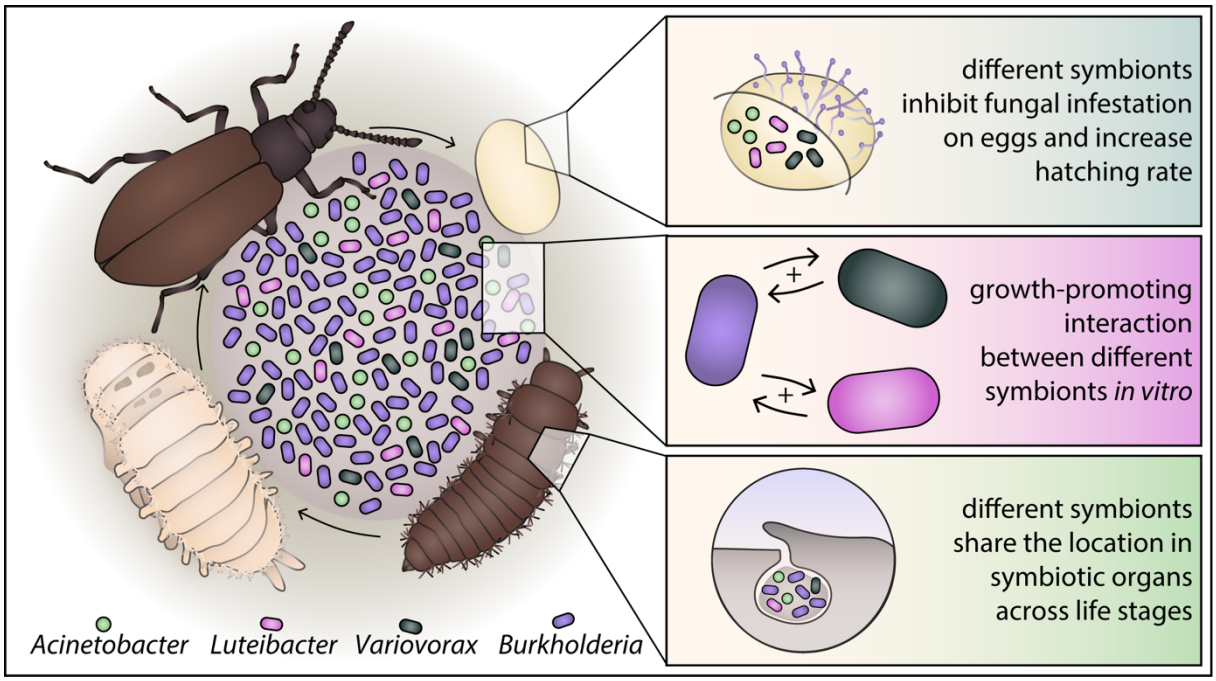

L.V.F., **R.S.J.** and M.K. conceived the study. I.K. and S.R. isolated symbionts. L.V.F. designed FISH probes. B.W. and **R.S.J.** carried out histological sectioning. J.A., **R.S.J.** and S.R. carried out FISH experiments. H.V. carried out MinION sequencing and genome assembly. L.V.F., D.G. and **R.S.J.** analyzed sequencing data. S.R. carried out manipulative bioassays. S.B. carried out *in vitro* assays. **R.S.J.** evaluated microscopy results and wrote the original draft. L.V.F and M.K. reviewed and commented on the manuscript.

1. Abstract

Symbiotic associations between insects and microorganisms are ubiquitous in nature. Often multiple symbionts can be found in a community of the same host. Their individual function and potential interaction with each other can have important effects on their abundance in the host, and on the host itself. *Lagria villosa* beetles accommodate defensive *Burkholderia* symbiont strains in symbiotic organs, which protect the immature life stages against fungal pathogens by producing several antimicrobial compounds. Here, we elucidate the presence, potential role, and interaction between Burkholderia and non-*Burkholderia* symbionts of the microbial community in *L. villosa* beetles. By combining microbial community sequencing, FISH, *in vivo* and *in vitro* assays, as well as genome analysis, we identified that at least three non-*Burkholderia* symbionts are consistently associated with the beetle in lower abundance. They share their localization with *Burkholderia* within the symbiotic organs of all life stages and are potentially coexisting by positively influencing each other's growth. Preliminary experiments also showed their potential for protection against a fungal pathogen of *L. villosa*, complementing the defensive traits of the *Burkholderia* symbionts, potentially also through secondary metabolites.

2. Introduction

Many insects are associated with symbiotic microorganisms and often more than one species can persist within individual hosts. In multi-species symbioses, different symbionts can be separated in parts of the insect body, by colonizing different organs, tissues, or life stages [1,2]. However, in some insects, a symbiont community shares a niche within its host, which can lead to interactions between microbes that might even disrupt the association [3,4]. In general, there is not much experimental evidence on consequences or costs of multiple infections for the hosts, since it demands systems that offer the opportunity to separate and re-assemble the community.

Defensive symbioses are often dynamic and allow for the presence of different symbionts across populations [3,5,6]. Due to context-dependent benefits for their hosts, which can for example differ in the presence or absence or the type of natural enemies, symbionts might differ or fluctuate in their abundance among insect populations or life stages [5]. Defensive symbionts might only be maintained, as long as they are beneficial and outweigh the costs of accommodation for the host [6,7].

Lagria villosa beetles accommodate different defensive *Burkholderia gladioli* strains in cuticular symbiotic organs, which aid in protection of immature life stages [8–10, Chapter I]. Although one *Burkholderia* strain (*Burkholderia* Lv-StB, henceforth "Lv-StB") dominates the community, co-infections with other strains can occur in natural conditions [8,9, Chapter I]. Besides the strain diversity within *Burkholderia*, the bacterial community of the beetle consists of several other taxa belonging to multiple families, some of which consistently occur across individuals and populations [11, Chapter I]. These bacteria were found in all life stages including female accessory glands, eggs and whole individuals of larvae and pupae

Chapter I. However, whether these bacteria are commensal or mutualistic members of the community, if they are localized within the symbiotic organs in all life stages, and whether they have an impact on host fitness currently remains unknown.

Here, we investigated the symbiotic potential of three isolated bacterial associates (*Acinetobacter*, *Luteibacter*, *Variovorax*, henceforth collectively termed "non-*Burkholderia* symbionts") of *L. villosa* beetles by assessing their abundance, localization, metabolic potential, possible functional role for the host, and their interaction with *Burkholderia* symbionts. Microbial community profiling across different host life stages and fluorescence *in situ* hybridization (FISH) revealed that all three bacteria are consistently present in the microbial community along with *Burkholderia* and reside within the symbiotic organs of the host. Furthermore, the presence of multiple secondary metabolite gene clusters in the genomes of the non-*Burkholderia* symbionts and their ability to inhibit fungal growth on the insect egg by preliminary bioassays suggest that these bacteria also contribute to host-defense. When kept in *in vitro* co-cultures with the defensive *Burkholderia* strain *B. gladioli* Lv-StA (henceforth "LvStA"), the isolates positively influenced the growth of Lv-StA and vice versa, suggesting collaborative rather than competitive interactions between *Burkholderia* and the other community members.

3. Results

3.1. Non-*Burkholderia* are consistently present and located within the symbiotic organs

The relative abundances of the bacterial community on a family level across different life stages show that besides the already described Burkholderiaceae, also Comamonadaceae, Moraxellaceae, Pseudomonadaceae, Rhizobiaceae, Rhodanobacteraceae, and Xanthomonadaceae were present, albeit in lower abundances Chapter I (Figure 1, Pseudomonadaceae, Rhizobiaceae and Xanthomonadaceae not shown). From these non-*Burkholderia* bacteria obtained from the beetle microbiota, we could isolate three that coincided with bacterial taxa previously found to be most consistent across life stages according to 16S rRNA based bacterial community profiling Chapter I (Figure 1). We isolated each one strain of Moraxellaceae (*Acinetobacter*Lv1), Rhodanobacteraceae (*Luteibacter*Lv2), and Comamonadaceae (*Variovorax*Lv3) from accessory glands of field-collected females and sequenced their genomes.

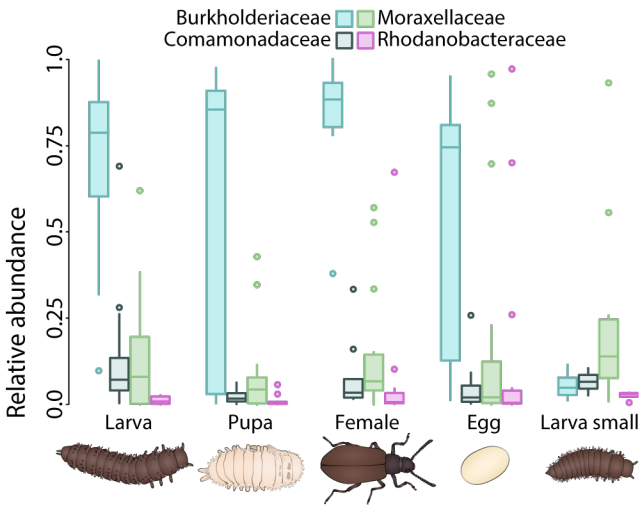

Figure 5: Consistent families present in the bacterial community of *L. villosa* life stages. Relative abundance of four consistently present bacterial families (cyan: Burkholderiaceae, green: Moraxellaceae (including *Acinetobacter*), magenta: Rhodanobacteraceae (including *Luteibacter*), dark-teal: Comamonadaceae (including *Variovorax*)) of different *L. villosa* life stages obtained by high-throughput amplicon sequencing of the bacterial 16S rRNA V4 region.

To find out whether the non-*Burkholderia* members in the community are sharing the same habitat as the *Burkholderia* symbionts and if they are maintained within the beetles, we carried out fluorescence *in situ* hybridization (FISH) across different life stages of *L. villosa*. We combined a probe specific for one of the non-*Burkholderia* bacteria (Figure S1), with a *Burkholderia*-specific probe within a single sample to assess potential spatial structuring. For most of the probe-combinations, we sampled one replicate per life stages from young larvae (L1, L2), older larvae (larva medium, larva big), as well as a female pupae and adult. Overall, all non-*Burkholderia* members were only found within the symbiotic organs of the beetles and were usually evenly distributed within the organs. *Burkholderia* dominated the microbial community in every sample except for a single symbiotic organ in a L2 larva (Figure 2 a), where only a low number of *Burkholderia* cells could be detected, and more *Acinetobacter* cells were observed (Figure 2 a). In another section of the same individual, we observed the *Burkholderia* symbionts to be separated from other bacteria including *Luteibacter* (Figure 2 b). A bulk of *Burkholderia* cells was separated from *Luteibacter* along with other bacteria and a few

Burkholderia cells, which were located at the cranial and caudal sides of the organ (Figure 2 b, L2). While *Acinetobacter* seemed to be present across life stages (Figure 2 a & d, except pupal stage), *Luteibacter* was only detected in the larval stages (Figure 2 b & d) and *Variovorax* was only found in one of the larval stages (Figure 2 c & d). However, it should be noted that the *Variovorax* probe was not used on young larvae, and only one individual per stage was tested in total for all probe-combinations. In addition, the morphology of the pupal organs including strongly autofluorescent bristles potentially impeded detection of single cells.

These results show that although *Burkholderia* is highly abundant within the symbiotic organs, multiple other bacteria are consistently present in the community and can stably colonize the organs and, particularly for *Acinetobacter*, are maintained throughout host development.

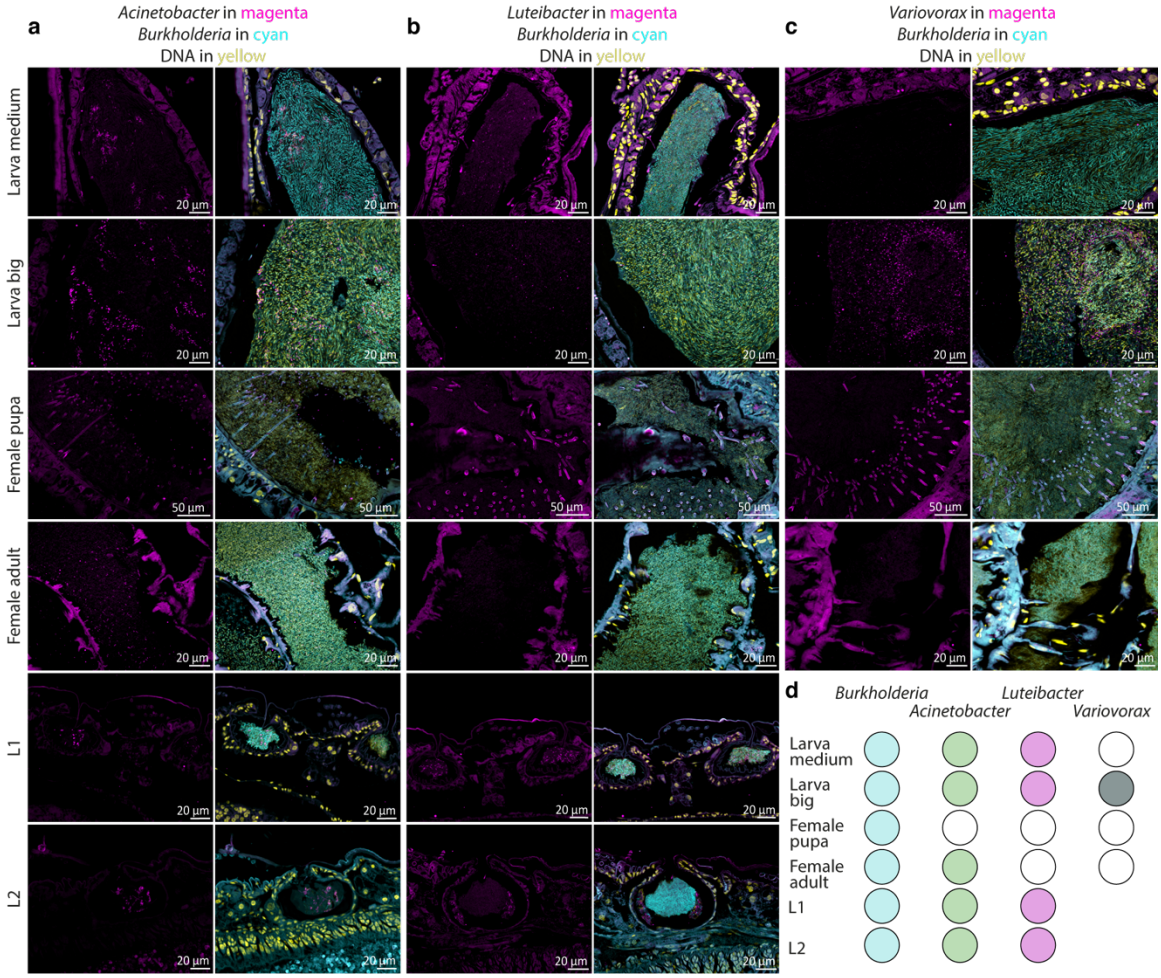

Figure 2: Localization of symbiotic bacteria across *L. villosa* life stages. Rows show different life stages from field-collected (Larva medium, Larva big, Female pupa, Female adult) or offspring of field-collected individuals (L1, L2) and columns show the signal of different FISH-probes or combinations. DAPI shows nuclei staining of host and bacteria in yellow, a Cy-3 labeled probe shows ***Burkholderia***-staining in cyan and a Cy5-labeled probe shows staining of **a** ***Acinetobacter***, **b** ***Luteibcater*** or **c** ***Variovorax*** in magenta. Signal of the Cy-5 probe is shown in the first column and the second columns shows the merged channels. All individuals were sectioned sagittally except for the female adult (transversally). **d** Summary of a-c indicating detection (full circle) or no detection (empty circle) of the different bacteria across tested life stages.

3.2. Non-*Burkholderia* symbionts confer protection against a fungal pathogen

Because the non-*Burkholderia* symbionts were consistently present across life stages including the egg stage (Figure 1), we carried out bioassays exposing eggs reinfected with each of the isolates individually to a known pathogen of *L. villosa* (*Purpureocilium lilacinum*) [8,9,Chapter I] and compared the fungal growth to aposymbiotic eggs. All isolated bacteria significantly inhibited fungal growth on the eggs (Figure 3a, Cox mixed effects model, *p* values: *Acinetobacter*-Lv1 = 0.00530, *Luteibacter*Lv2 = 0.00003, VariovoraxLv3 = 0.00150). Eggs reinfected with *Luteibacter*Lv2 were most likely to be protected, which was also reflected in the reduced amount of fungus growing on the eggs (Figure 3 b). In addition, the reinfected eggs had a higher chance of hatching after exposure to the fungus than aposymbiotic individuals (Figure 3 c). FISH of the hatched larvae (Figure S2 a-c) revealed the isolates *Acinetobacter*-Lv1 and *Luteibacter*-Lv2 could successfully colonize the dorsal symbiotic organs of larvae after reinfection in the egg stage, which indicates a vertical transmission route.

Because we found the isolates to be protective on the *Lagria* eggs, we aimed at characterizing the symbionts' potential to produce secondary metabolites, based on draft genome sequences that we obtained for all three isolates. The draft genomes had a completeness of 93.3 % for *Acinetobacter*-Lv1 (4.7 Mbp, 0.37 % GC content, 1 contig), 99.03 % *Luteibacter*-Lv2 (5.3 Mbp, 0.65 % GC content, 3 contigs), and 100 % *Variovorax*-Lv3 (9.3 Mbp, 0.69 % GC content, two contigs). We looked at the predicted production of secondary metabolites via antiSMASH [12] in the draft genomes of *Acinetobacter*-Lv1, *Luteibacter*-Lv2, and *Variovorax*-Lv3 in comparison to the cultivable *B. gladioli* strain from *L. villosa* LvStA, whose secondary metabolites were previously described (Figure 3 d). The analysis revealed that the genomes of *Variovorax*-Lv3 and *Luteibacter*-Lv2 encode multiple candidate biosynthetic gene clusters (BGCs) (13 and 8, respectively) across several different types of secondary metabolites (8 and 7, respectively), while the *Acinetobacter*-Lv1 genome was predicted to encode only for one arylpolyene and two siderophores. Only three of the BGCs in the non-*Burkholderia* symbiont genomes exhibited a similarity of >70% to known BGCs (percentage of genes within the closest known compound that have a significant BLAST hit to genes within the current region), and these were predicted to encode for rhizomides (100% similarity) and xanthoferrin (85%) in *Luteibacter*-Lv2 and acinetoferrin (74%) in *Acinetobacter*-Lv1.

These results suggest that members of at least three bacterial families besides Burkholderiaceae contribute to protection of the egg stage potentially by the production of secondary metabolites.

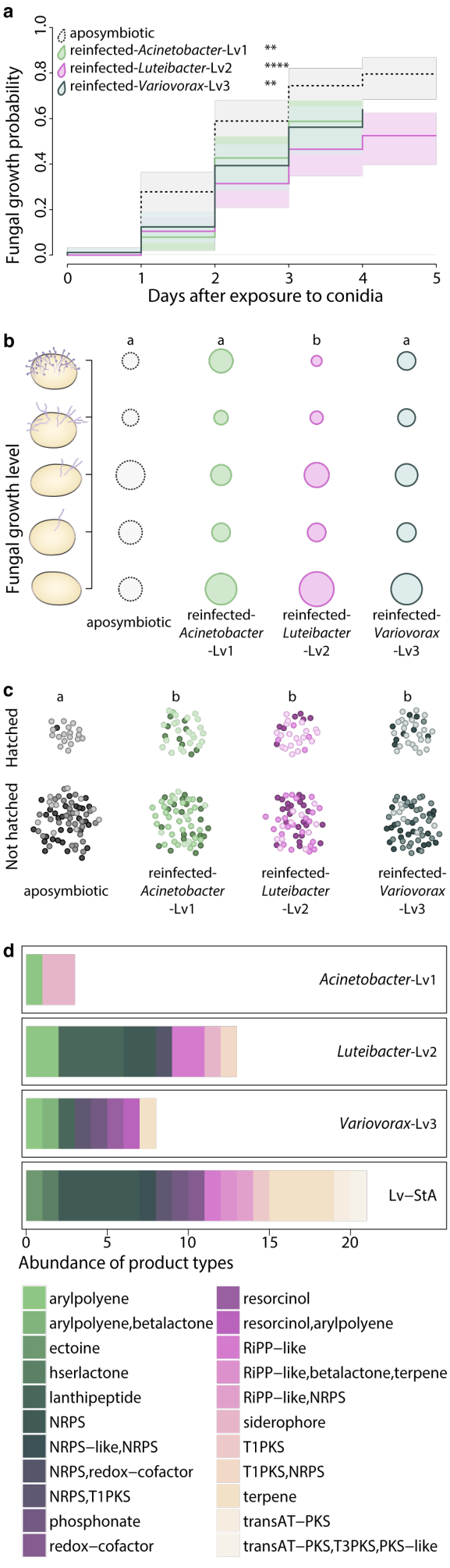

Figure 3: Non-*Burkholderia* isolates indicate defensive traits towards *L. villosa* eggs. a Fungal growth probability for differently treated eggs exposed to the conidia of the fungus *P. lilacinum* was assessed single-blindly until the eggs hatched: aposymbiotic (dotted line, n=90), reinfected-***Acinetobacter****-Lv1* (green line, n=89), reinfected-***Luteibacter****-Lv2* (magenta line, n=86) and reinfected-***Variovorax****-Lv2* (dark-teal line, N=89). Asterisks indicate significant differences compared to the aposymbiotic treatment (Cox Mixed-Effects Model, ** p<0.01, **** p<0.0001). **b** Fungus level was single-blindly monitored and assessed on the last day before hatching. Different letters indicate significant differences between experimental treatments (Generalized linear mixed model fit by maximum likelihood, $\alpha \leq 0.05$). **c** Hatching rate of differently treated eggs used in the bioassay. Eggs originating from different clutches are depicted by colored dots in a different brightness throughout the treatments. Different letters indicate significant differences between experimental treatments (Generalized linear mixed model fit by maximum likelihood, $\alpha \leq 0.05$). **d** Distribution of secondary metabolites predicted to be produced among the *L. villosa* isolates. Colors differentiate between types of secondary metabolites as annotated by antiSMASH.

3.3.Symbiont-symbiont interactions between LvStA and non-*Burkholderia* symbionts

Given the presence of multiple symbionts within the beetle's bacterial community and their potential to produce secondary metabolites, we set out to evaluate their potential for interactions by looking their performance in confrontation assays *in vitro*. Since *Luteibacter*-Lv2 and *Variovorax*-Lv3 encoded for a diverse array of candidate BGCs, we preliminary used them for *in vitro* confrontation assays against Lv-StA to test whether the symbiont strains influence each other's growth on agar plates of different media (Figure 4). First, we observed that the three symbionts grew differently well in axenic cultures on the different kind of media. While Lv-StA and *Luteibacter*-Lv2 grew best on King's B agar, *Variovorax*-Lv3 had the largest colonies on Actinomycete isolation agar (Figure S3). The growth of all isolates was then also observed in co-cultures of each two isolates, and colony size was measured on three timepoints across three

weeks (Figure S4). The growth rate calculated from three timepoints of Lv-StA seemed unaffected when co-inoculated with *Luteibacter*-Lv2 or *Variovorax*-Lv3, apart from a decreased growth rate when inoculated with *Variovorax*-Lv3 on King's B (Figure 4, first row). Notably, when inoculated in co-culture with Lv-StA, *Luteibacter*-Lv2 grew significantly better on both King's B and Actinomycete isolation agar, while colonies seemed to be unaffected by other strains on Sf-900 agar (Figure 4, second row). *Variovorax*-Lv3 grew also significantly better on King's B (Figure 4, third row). Overall, none of the isolates grew worse in co-culture with another symbiont, except for one case. These results imply that the symbionts benefit each other's growth rather than competing, potentially through cross-feeding, underpinning their ability to cohabitate in the same niche within a host.

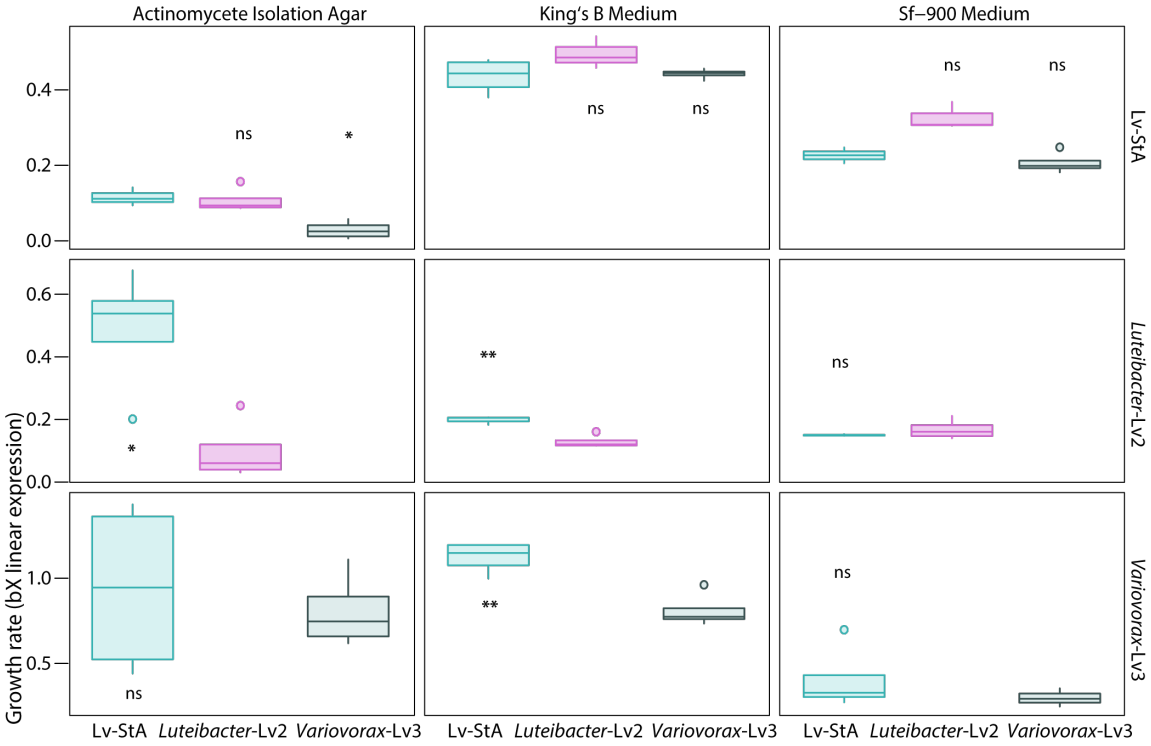

Figure 6: Growth rate of *L. villosa* symbionts in confrontation to another strain. Growth rate (bX value of linear regression) of different focal strains (LvStA: first row, *Luteibacter*-Lv2: second row, *Variovorax*-Lv3: third row) was assessed by measuring colony size of three different timepoints when inoculated in co-culture on an agar plate with an opposing strain including (Lv-StA: turquoise, *Luteibacter*-Lv2: magenta, *Variovorax*-Lv3: dark-teal). The confrontation assays were carried out on different media (Actinomycete isolation agar, King's B medium, Sf-900 medium) and in two to four replicates indicated by the connected dots. Asterisks indicate significant differences between the strains and "ns" indicates no significant difference. For statistical analyses different tests were carried out as described in the method section.

4. Discussion

In contrast to nutritional symbioses, defensive symbioses are often more flexible, and their ecological relevance can be disguised due to context-dependent benefits, especially under natural conditions. The defensive association between *Burkholderia* bacteria and *Lagria* beetles was described previously, with multiple coinfecting *Burkholderia* strains protecting their host against fungal pathogens in at least two host species [8,9,11, Chapter I, Chapter V]. Despite *Burkholderia* being the most abundant symbiont in *L. villosa*, other bacterial genera are consistently present [8,13, Chapter I]. However, their impact on the microbial community and potential contributions to the host remained elusive. Here, we localized three low-abundance members of the community within the symbiotic organs, showed the consistent presence of the corresponding families across different life stages, demonstrated their defensive abilities on the egg stage against a natural antagonist of the host and observed potential cooperation between one *Burkholderia* and three non-*Burkholderia* culturable strains. These results shed light on a multipartite mutualism in *L. villosa* beetles, in which different protective symbionts share a localization in the host, and contribute to egg defense as well as successful hatching of the insect.

Burkholderia symbionts are present in at least six different species of the Lagriini tribe (Coleoptera, Tenebrionidae) including *L. villosa* [8]. Additional bacteria might be associated to this beetle group, however, a comparison of the 16S rRNA profile of *L. villosa* and the related species *Lagria hirta* suggests that other than the Burkholderiaceae, the bacterial community is mostly different in composition. This divergence suggests that the association with the *Burkholderia* symbionts likely originated much earlier. However, the presence throughout the beetle's life stages, the localization within the symbiotic organs and the successful colonization of the larval organs from the egg surface suggest that the non*Burkholderia* symbionts are likely vertically transmitted along with the *Burkholderia* strains during oviposition. When inoculated alone, non-*Burkholderia* symbionts colonized the symbiotic organs of first instar larvae in considerable amounts, which does not seem to occur under natural conditions when *Burkholderia* is present [Chapter I] (Figure 2). Since we did not detect all non-*Burkholderia* symbionts in every tested life stage via FISH (except for *Acinetobacter*), it is possible that those strains are either lost in some individuals or only present in very low abundances. Future experiments including a larger set of replicates in combination with quantitative PCR on tracked life stages would be useful to detect possible fluctuations and coinfections of single strains in the symbiont community.

Facultative symbioses can be dynamic regarding transmission and maintenance, and how a diverse community is stabilized is not well understood, especially for defensive symbioses [6]. Multiple infections are known for the pea aphid *A. pisum* that harbors its obligate nutrient-supplementing endosymbiont *Buchnera aphidicola* and can additionally accommodate up to seven heritable facultative symbionts, including *Hamiltonella defensa*, *Serratia symbiotica*, *Regiella insecticola*, *Spiroplasma*, *Rickettsia* or *Rickettsiella viridis* which confer resistance to biotic or abiotic stresses [14–18]. Although multiple facultative symbionts can occur in

individual aphids, the outcomes of multiple infections for the host are variable, and not always positive [14,19,20]. Thus, as in other cases of multiple infections in insects [21,22], including *L. villosa*, the costs and benefits of coinfecting facultative symbionts are generally unclear.

While *Luteibacter* (Rhodanobacteraceae, Xanthomonadales) and *Variovorax* (Comamonadaceae, Burkholderiales) are described to be associated with plants [23–25] or fungal endophytes [26–28], the genus *Acinetobacter* (Moraxellaceae, Pseudomonadales) is more widespread in nature and found in the environment, animals, humans, and plants. Different *Acinetobacter* strains are also present in the gut community of various insects, such as tsetse flies [29], diamondback moths [30], saturniid moths [31], red postman butterflies [32] and cabbage white butterflies [33]. In stable flies, *Acinetobacter* is required for larval development [34], while it is involved in suppression of plant defenses in the Colorado potato beetle [35] and in promoting growth of another community member in the gut of tiger mosquitoes [36]. Although these genera are generally not known as defensive symbionts, it was previously reported from metagenomics datasets that *L. villosa*-associated strains of Rhodanobacteraceae, Pseudomonadaceae, Xanthomonadaceae and Moraxellaceae have the potential to produce multiple bioactive compounds [13]. Our results indicate that the isolates of *Acinetobacter*-Lv1, *Luteibacter*-Lv2 and *Variovorax*-Lv3 inhibit growth of *P. lilacinum* on the egg surface of *L. villosa* and increase hatching of the larvae in presence of the fungus, although the protection is less strong than that by single *Burkholderia* strains or the whole natural community [8,9]. However, to evaluate if the protective effect is comparable to the *Burkholderia* strains, it is necessary to test the isolates and natural community in the same assay. Additionally, it would be interesting to evaluate if host protection is enhanced due to possible synergistic effects, reduced due to competition on the eggs, or unaltered when multiple defensive symbionts are present. Furthermore, testing the isolates in the presence of various pathogens of *L. villosa* would inform on their activity against specific antagonists or their ability for broad range protection.

Although LvStA produces several bioactive secondary metabolites, some shown to be antibacterial [8], it enhances the growth of *Luteibacter*-Lv2 and *Variovorax*-Lv3 *in vitro* when inoculated in co-culture. This positive interaction was also observed vice versa, indicating potential cooperation between the community members. However, to gain better insights into microbe-microbe interactions of *L. villosa* symbionts, it would be necessary to also evaluate the missing strain combinations from the available isolates (Lv-StA vs. *Acinetobacter*-Lv1, *Luteibacter*-Lv2 vs *Variovorax*-Lv3, *Luteibacter*-Lv2 vs. *Acinetobacter*-Lv1, *Variovorax*-Lv3 vs. *Acinetobacter*-Lv1), as well as in communities including more than two isolates, and include more isolates if available. For comparisons of multiple interacting pairs or communities, metabolic modelling could be useful, since positive interactions among bacteria might be more common than generally thought [37]. It was suggested that positive interactions between culturable bacteria appear through shared metabolites or secretions and can enable the growth of otherwise absent species [37,38]. Observing this in a symbiotic context could also shed light on the ability of some unculturable symbionts to be present and co-transmitted in multi-species symbioses. Although all combinations in our study

resulted in positive interactions, this might not necessarily be the case *in vivo*, given the observed dominance of *B. gladioli* within the beetles [8]. Therefore, it will be important to evaluate their interaction also under natural conditions inside the symbiotic organs, since competition between symbionts might impact the abundance in the host and possibly their transmission [19]. For this purpose, eggs could be reinfected with different combinations of the isolates, and their presence and abundance in later life stages could be evaluated via FISH and qPCR.

Taken together, *L. villosa* beetles are associated with multiple potentially defensive symbionts, some of which can be cultivated *in vitro*. The possibility of manipulative experiments offers the opportunity for further elucidating general questions on symbiont-mediated defense, symbiont interactions, as well as the stability and costs of a multi-partner symbiotic community.

5. Methods

5.1. Insect collecting and rearing

L. villosa individuals were collected in 2019 on several soybean plantations in Brazil and reared in plastic containers in a climate chamber (16:8 L:D light regime at 26 °C and 60% humidity). Beetles were fed with fresh and dry leaves from lettuce, soybean, and kidney bean.

5.2. Isolation of symbionts

Symbiotic organs of field-collected individuals (larvae and female adults) were dissected and either directly placed in sterile PBS for direct use or stored in 70% glycerol at -80 °C for later use. Symbiotic organs were crushed with a pipette tip to release the bacteria in the PBS suspension. The cell concentration was counted with a Neubauer Cell Count Chamber and diluted to three different concentrations (10 cells/µL, 1 cell/µL, 0.1 cell/µL). PBS suspensions were either plated onto Petri dishes (Ø 55 mm) containing solid medium (15 g/L Agar, 250 mL/L CMRL, 150 mL/L Grace's Insect Media (3x), 80 mL/L FBS, 5 mL/L TPB, 400 µl phenol red, pH 6.5) or inoculated into 96-well plates containing 100 µL of liquid medium (500 ml/l H_2O dest., 250 mL/L CMRL, 150 mL/L Grace's, Insect Media (3x), 80 ml/l FBS, 5 mL/L TPB, 1 mM ATP, 1 mM CTP, 1 mM GTP, 1 mM TTP, 10 µM lipoic acid, 400 µL phenol red, pH 6.5). Grown colonies were kept as glycerol stocks and replated on King's B medium (15 g/L Agar, 2 g/L peptone, 1.5 g/L K_2HPO_4, 1.5 g/L $MgSO_4 \cdot 7H_20$) until pure cultures were obtained. To identify the isolates, single colonies were shock-frozen with liquid nitrogen and DNA was extracted using the MasterPure DNA extraction kit (Epicentre Technologies, Madison, USA) following the manufacturer's instructions. PCRs were carried out using the gDNA extract using the primers fD1 (5'AGAGTTTGATCCTGGCTCAG'3) and rP2 (5'ACGGCTACCTTGTTACGACTT'3) under the following conditions: 3 min at 94 °C, 40 s at 94 °C for denaturation, 60 s at 50 °C for annealing and 60 s at 72 °C for elongation. Successfully amplified PCR products were purified with the innuPrep PCRpure Kit (Analytik Jena), sent to StarSEQ GmbH (Mainz) for sequencing and compared to the NCBI database via Nucleotide BLAST.

5.3. Fluorescence *in situ* hybridization (FISH)

Specimens were fixated in 4% formaldehyde in PBS for at least 3 days. Embedding, semithin sectioning and FISH were performed as described previously [39]. The Cy3-labeled Burk16S probe (5'TGCGGTTAGACTAGCCACT'3) was used to mark *B. gladioli*. The 16S rRNA gene sequences obtained from selected bacterial isolates were used to design the Cy5-labeled Acineto_16S probe (5'AGAGCCTCCTCCTCGCTTAA'3) for *Acinetobacter*, the Cy5-labeled Lutei_16S probe (5'CGCACATCGGTCCATCCAAC'3) for *Luteibacter* and the Cy5-labeled Vario_16S probe (5' ACTCCAGCAATGCAGTCACA'3) for *Variovorax*. DAPI (4',6-diamidino-2-

phenylindole) was used to label the host cell nuclei and as counterstaining. Images were taken on an AxioImager.Z2 fluorescence microscope (Zeiss, Jena, Germany).

5.4. Bacterial isolate genome sequencing, assembly, and analysis

High molecular genomic DNA from *Acinetobacter*-Lv1, *Luteibacter*-Lv2, and *Variovorax*-Lv3 from pure liquid cultures using the Genomic Tip Kit 20/g (Qiagen). The corresponding genomes were sequenced on a MinION sequencer (Oxford Nanopore Technologies) and assembled using Flye. These and the genome of Lv-StA were annotated with RAST using KBase. For the comparison of candidate biosynthesis gene clusters, annotated genomes were analysed using antiSMASH (Version 6) [12]. Identified clusters were compared to antiSMASH-predicted clusters and the MIBiG database [40] with a relaxed direction strictness.

5.5. Bacterial community profiling via 16S rRNA amplicon sequencing

A previously compiled community profiling dataset obtained by 16S rRNA amplicon sequencing Chapter I was re-analyzed for the relative abundance of the newly isolated symbionts *Acinetobacter*, *Luteibacter* and *Variovorax* and their respective bacterial families across life stages. Amplicon sequence variants (ASVs) were inferred using the R package DADA2 [41] with default parameters including dereplication, chimera removal, and trimming lengths of 250 and 140 nt for forward and reverse reads, respectively. Taxonomy was assigned using the pre-trained classifier SILVA 132 [42,43] with subsequent removal of reads classified as chloroplasts, eukaryotes or mitochondria.

5.6. *In vivo* bioassays

Bioassays were carried out in 96-well plates that were prepared as previously described [8]. 360 eggs from three egg clutches of laboratory-reared females were divided into four groups, then surface sterilized and either reinfected with 2.5 µL of a bacterial suspension or sterile PBS to obtain aposymbiotic eggs [8]. For reinfections, $2x10^6$ cells/µL of a symbiont suspension (*Acinetobacter*-Lv1, *Luteibacter*-Lv2, *Variovorax*-Lv3) in PBS was added to each egg, and individual eggs from all four treatments were randomly assigned into the wells which were treated with 5 µL of a 25 conidia/µL suspension of the fungus *P. lilacinum*. Plates were kept at 25°C and single-blindly monitored daily for fungus infestation, amount of fungus growing, and larval hatching. Statistical analyses were carried out in RStudio V5 Version 1.1.453 using the coxme (Version 2.2-16) and lme4 (Version 1.1-23) packages. A Cox Mixed Effects Model was used to assess fungal growth probability and Generalized Linear Mixed Effects Models to evaluate fungal growth level and hatching rate, in each case using clutch as a random factor and fungal treatment as a fixed factor. Plots were created in RStudio using the ggplot2 package (Version 3.3.0) and were further adapted using Adobe Illustrator.

5.7. *In vitro* confrontation assays

Single colonies were transferred to liquid King's B medium for two days in 50 mL falcon tubes at 30°C, and 5µL of a bacterial suspension of 10^6 cells/µL was used for the co-culture. Plates of Actinomycete isolation agar (22 g of Fluka actinomycetes agar, 5 mL glycerol, 1 L distilled water), King's B agar, and Sf-900 agar (Sf-900 medium Gibco®, 3 % agar) were each co-inoculated with different strains in a 2 cm distance. Plates with double inoculations of the same strain were used as a control. Plates were kept at 30°C, monitored and photographed every second day within three weeks. The growth area of each focal strain was determined at three timepoints (for King's B agar: day 6, 12, and 20, for Actinomycete isolation agar day 6, 14, and 21, and for Sf-900 agar day 6, 14, and 21) using ImageJ (Version 1.53a) and the bX value of a linear regression was used to compare growth rates across groups. Statistical analysis was carried out in RStudio using car (Version 3.0-10), and PMCMRplus (Version 1.9.0). Data were tested for normality and homogeneity of group variances using Shapiro-Wilk test and Fligner-Killeen tests respectively. According to the outcome Student's *t*-test or Welch's *t*test were carried out for comparisons of two groups, and ANOVA or Kruskall-Wallis test with posthoc Dunn's test for more than two groups. Plots were created using the ggplot2 package (Version 3.3.0) with further adaptation in Adobe Illustrator.

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9. Supplement

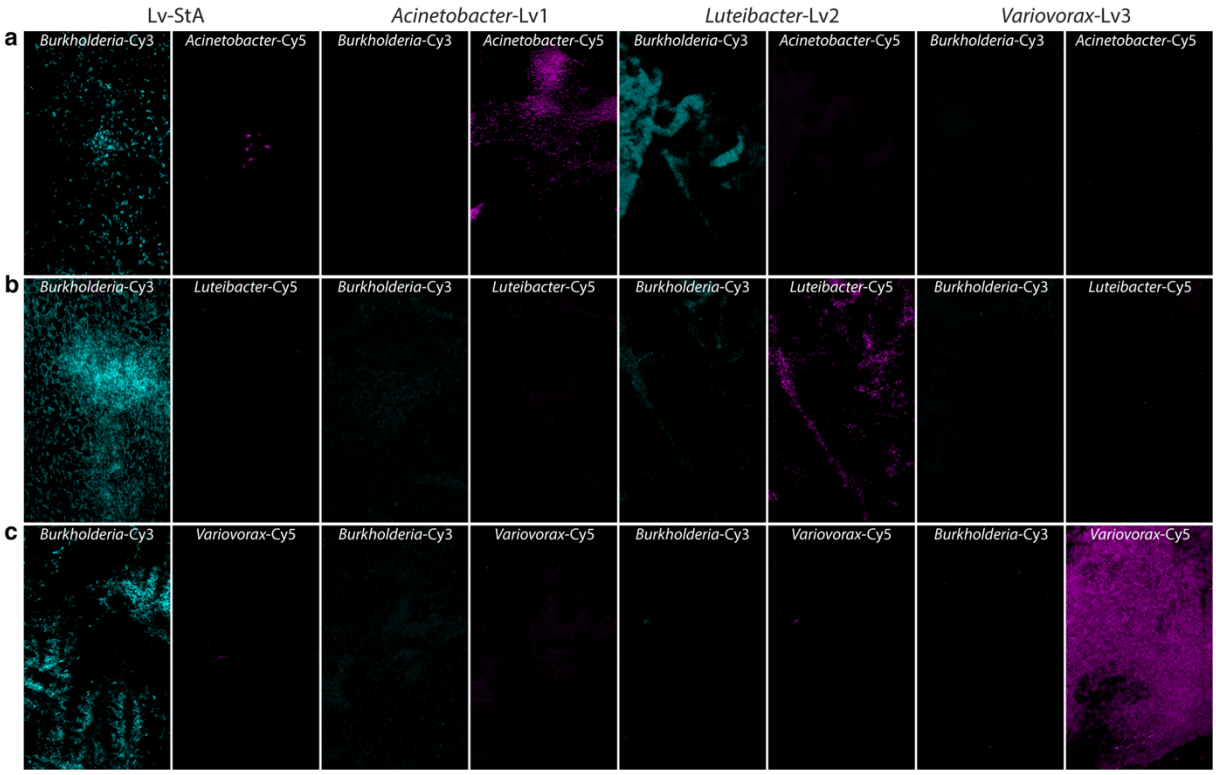

Figure S1: Specificity test of FISH probed designed to target different non-*Burkholderia* symbionts. Bacterial cultures of Lv-StA, *Acinetobacter*-Lv1, *Luteibacter*-Lv2, and *Variovorax*-Lv3 were hybridized with each a probe combination including a probe specific for *Burkholderia* (*Burkholderia*-Cy3 in cyan) and one of each non-Burkholderia isolates (*Acinetobacter*Cy5, *Luteibacter*Cy5, *Variovorax*Cy5 in magenta). **a-c** The Burkholderia-specific probe labels *Burkholderia*-Lv-StA cells specifically and shows partial unspecific staining on Luteibacter-Lv2 culture. **a** The *Acinetobacter*-specific probe labels *Acinetobacter*-Lv1 specifically. **b** The *Luteibacter*-specific probe labels *Luteibacter*-Lv2 specifically. **c** The *Variovorax*-specific probe labels *Variovorax*-Lv3 specifically.

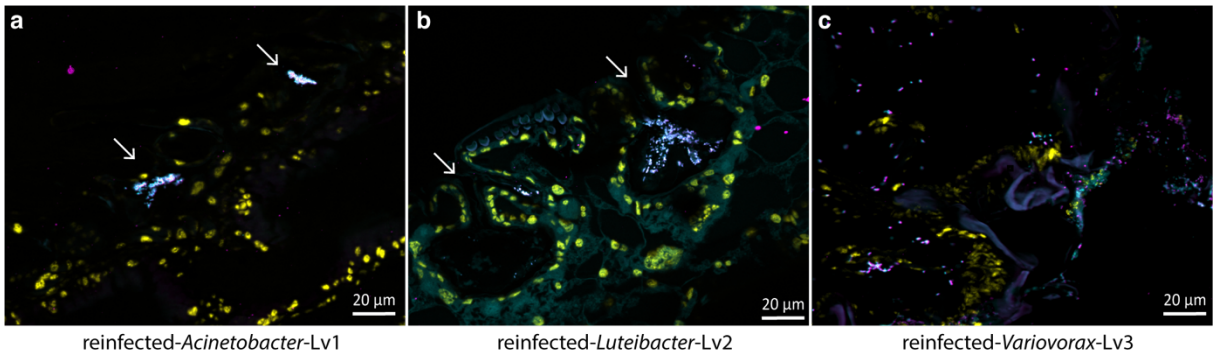

Figure S2: FISH on reinfected larvae hatched from bioassay. Eubacteria were stained with a Cy3-labeled probe shown in cyan and each of the symbionts were stained with a Cy5-labeled probe shown in magenta, while overlapping signal appears white. **a** Individual reinfected with *Acinetobacter*-Lv1 harbored cells inside the symbiotic organs (arrows). **b** Individual reinfected with *Luteibacter*-Lv2 harbored cells inside the symbiotic organs (arrows). **c** Individual reinfected with *Variovorax*-Lv3 showed cells across the larval surface and mouth region.

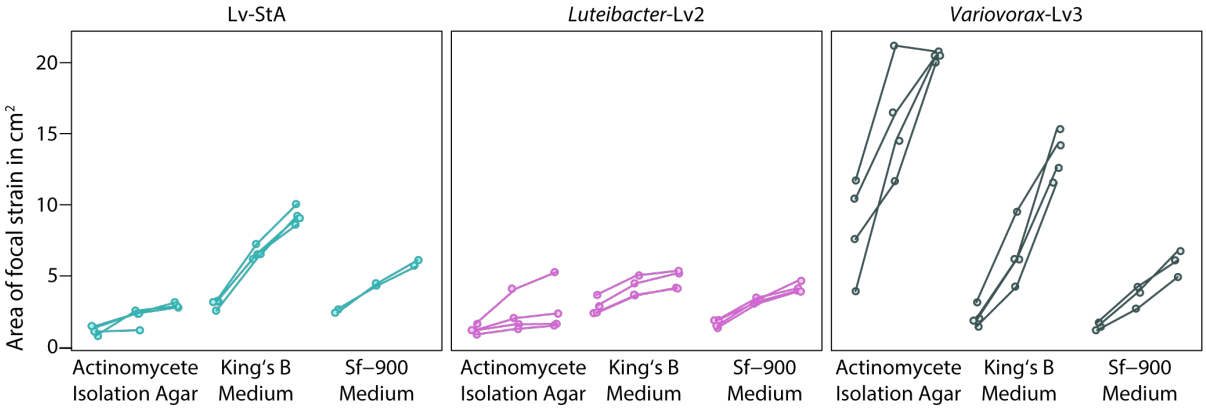

Figure S3: Growth area of the different isolates in axenic plates on different media. Lv-StA: turquoise, *Luteibacter*-Lv2: magenta, *Variovorax*-Lv3: dark-teal. Lines connect three timepoints of each replicate plate at day 6, 12, and 20.

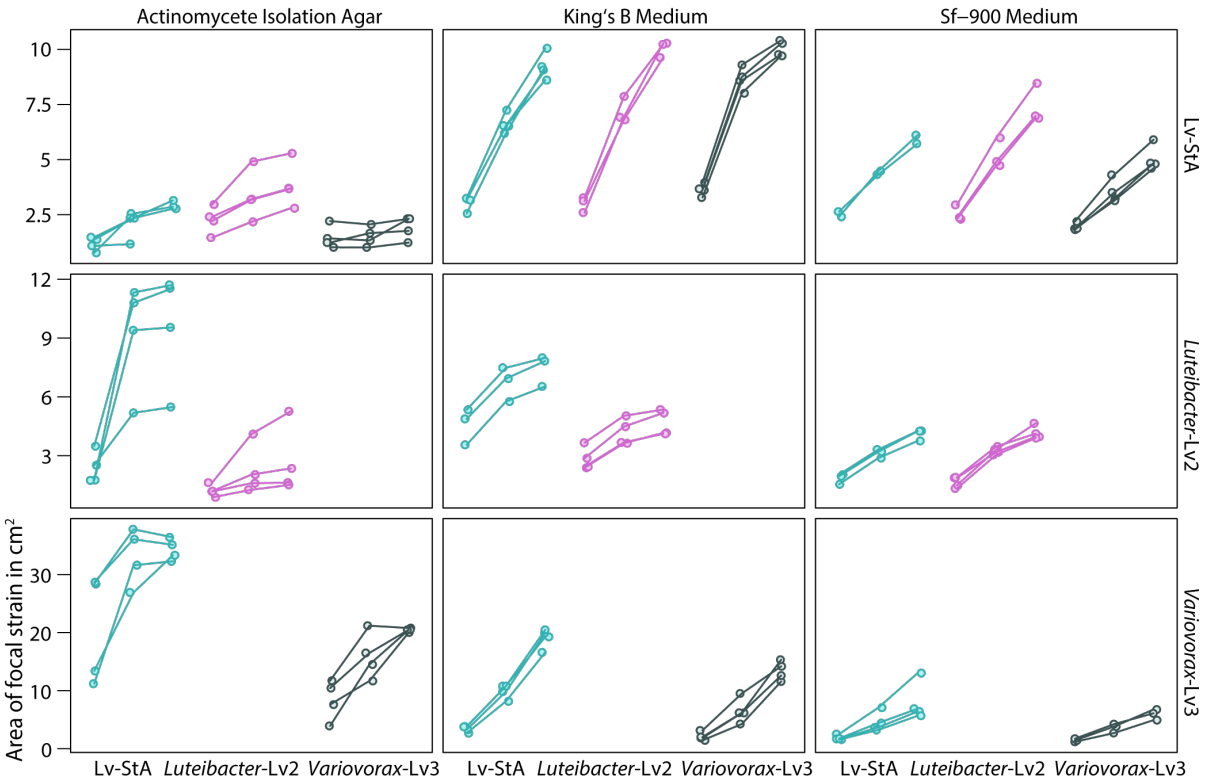

Figure S4: Growth of *L. villosa* symbionts in confrontation to another strain. Growth of different focal strains (LvStA: first row, *Luteibacter*-Lv2: second row, *Variovorax*-Lv3: third row) was assessed by measuring colony size of three different timepoints when inoculated in co-culture on an agar plate with an opposing strain including (Lv-StA: turquoise, *Luteibacter*-Lv2: magenta, *Variovorax*-Lv3: dark-teal). The confrontation assays were carried out on different media (Actinomycete isolation agar, King's B medium, Sf-900 medium) and in two to four replicates indicated by the connected dots.

CHAPTER V

Coexistence and functional differentiation of cultivable and non-cultivable defensive symbionts in *Lagria* beetles

in preparation

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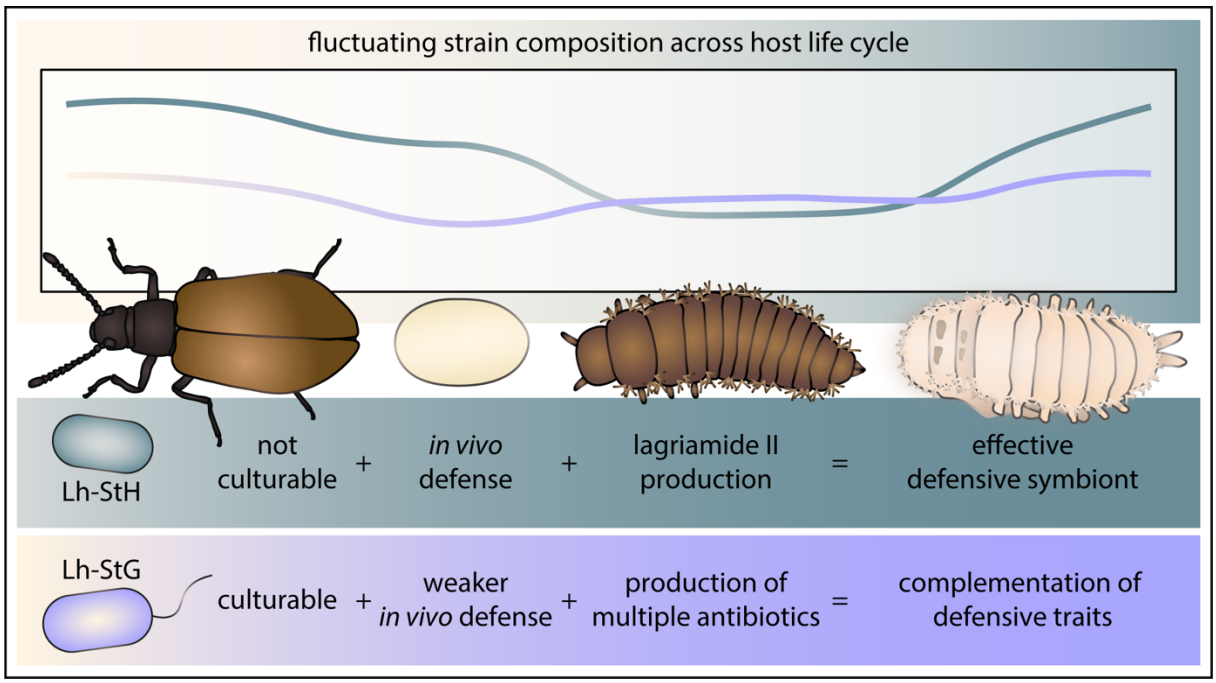

R.S.J., M.K., and L.V.F. conceived the study and designed the experiments. **R.S.J.** carried out manipulative assays, high throughput amplicon sequencing, and the corresponding analysis and illustrations. S.B. and **R.S.J.** carried out qPCR and FISH experiments. K.S. carried out chemical quantification of the compounds. Samples were collected by R.S. J., L.V.F., and M.K. Experiments and data analysis were done with input from C.H., M.K., and L.V.F. **R.S.J.**, L.V.F., and M.K. wrote the manuscript and all authors commented on the final draft.

1. Abstract

Strain diversity and the coexistence of multiple symbiont strains is a frequent phenomenon in natural environments, including animal hosts. Multiple infections of closely related strains are often considered to outcompete each other and to occur in host populations, but not within individuals. Here, we demonstrate the coexistence of two *Burkholderia* strains within individuals of its host *Lagria hirta* and examine their functionality and dynamics across different life stages. We found that both strains are highly abundant across populations and individuals and fluctuate in their relative abundance across host development, despite potentially different levels of host dependence. Both strains defend their hosts eggs against fungal growth likely through the production of antimicrobial compounds, which show similarities to chemical mediators or a related beetle species Lagria villosa. By comparing symbiont dynamics, secondary metabolite profiles and *in vivo* protection, we sought to shed light onto the successful relationship of Lagria beetles with multiple defensive *Burkholderia* symbiont strains.

2. Introduction

Strain diversity within bacterial populations is ubiquitous in nature and is also increasingly recognized as an important aspect of symbiotic microbial communities associated with eukaryotic hosts. However, theory predicts that within individual hosts, intraspecific symbiont variation can cause competition between strains and lead to imbalances in the symbiosis [1]. Concordantly, competing strains can be observed in the squid-*Vibrio* symbiosis, where either a dominant D-strain singly colonizes the light organ by outcompeting others, or other so-called niche-sharing S-strains can persist in the same host [2–4]. However, overcoming competition can be achieved by fine-scale genomic variation between strains that can result in functional divergence and niche differentiation [5]. Therefore, related symbiont strains might be able to take over different roles and coexist in the host. That functional diversity can enable the coexistence of distinct strains within a host was recently shown across different deep-sea mussels [6] and a shrimp [7]. Similarly, honey bees are associated with coexisting closely related strains that diverge in their metabolic capabilities, which may facilitate coexistence [8,9].

Characterizing symbiont strain dynamics and their functional roles throughout host development is key to understand the causes and consequences of strain diversity in host-associated microbial communities, and why these might coexist. However, in many cases differentiation on a strain level remains challenging and assessing strain functionality is often unfeasible in intimate symbioses, especially when symbionts are not culturable. Hence, experimentally tractable systems with multiple co-infecting symbiont strains are very useful to tackle these questions. These should allow us to better understand the implications of coexistence, as well as the fitness consequences of strain diversity for the symbionts and the host [2–4].

The darkling beetles *L. villosa* and *L. hirta* are both associated with multiple closely related *Burkholderia gladioli* strains, which fall into distinct phylogenetic clades within the species [10]. Previous studies in *L. villosa* showed that one *Burkholderia* strain (*Burkholderia* Lv-StB, henceforth "Lv-StB") dominates the community across life stages while other strains can also be present among some individuals [11,12, Chapter I]. One of these is *B. gladioli* Lv-StA (henceforth "Lv-StA"), which – in contrast to Lv-StB – has been cultured *in vitro* and has an almost four-fold bigger genome [11]. Lv-StA is generally capable of living independent from the beetle host and infect plants [11,13]. Both strains can protect *L. villosa* eggs and larvae against pathogenic fungi through different sets of antimicrobial compounds [11,12, Chapter I]. While Lv-StB produces the antifungal compound lagriamide [12], Lv-StA produces an array of antifungal and antibacterial substances [11,14–16]. Despite its metabolic versatility, Lv-StA is only rarely found in in natural *L. villosa* populations and seldom coexists with Lv-StB[12, Chapter I]. Like *L. villosa*, the congeneric species *L. hirta* is associated with multiple *Burkholderia* strains [10,17], but the dynamics of strain coexistence and their functional relevance for the host remain unknown.

Here, we investigate the dynamics and functionality of the *Burkholderia* community associated with *L. hirta*. We compared the presence and consistency of two *B. gladioli* strains, the culturable *B. gladioli* Lh-StG and the unculturable *B. gladioli* Lh-StH (henceforth "Lh-StG" and "Lh-StH", respectively) throughout different life stages. We also assessed their functional potential in defending the beetle host via manipulative bioassays, high-performance liquid chromatography-mass spectrometry (HPLC-MS), and genome analysis, and compared them to the strains of the related host species *L. villosa*. Our results reveal that both strains coexist across host development in varying relative abundances and protect the beetle eggs from fungal infestation, but likely do so via different chemical mediators. These findings shed new light on the dynamics and functional differentiation in multi-strain symbiotic communities and question the prevailing view that stable coexistence of closely related microbial symbionts is unlikely in animal associated microbiomes.

3. Results

3.1. Coexistence of a culturable and a non-culturable *Burkholderia* strain in *L. hirta* beetles

We characterized the composition of the bacterial community associated with *Lagria hirta* using 16S amplicon sequencing of offspring from field-collected females, focusing on *Burkholderia* ASVs (Figure 1). Therefore, we collected pools of individuals from different clutches for each sample. After quality filtering an removal of chimeric sequences, we obtained a minimum of 2268, a maximum of 113300, and on average 54537 reads among all samples, and in total 1570 ASVs. As observed across *L. villosa* life stages [11, Chapter I] and for adult *L. hirta* females [10], Burkholderiaceae were consistently present in eggs and early larval instars of *L. hirta* in varying abundance (Figure 1 a). Within the genus *Burkholderia*, two ASVs were prevalent across the evaluated life stages and individuals (Fig. 1 b). One of these likely corresponds to a strain previously cultured *in vitro* (Lh-StG), as the sequences show 100 % similarity in the evaluated 16S rRNA gene region. The second predominant ASV matches a strain that is so far not culturable (Lh-StH), yet is present in high relative abundance, particularly in the egg stage (Figure 1 b). ASVs labeled with an asterisk (LhStG* and LhStH*) indicate potentially very closely related strains corresponding to LhStG and LhStH respectively. The proportions of the two dominant Burkholderia ASVs changed throughout host development, with the eggs being dominated by Lh-StH, whereas LhStG was usually present in higher abundance in larvae (Figure 1 b). One ASV corresponding to a strain highly similar to Lv-StC and Lh-StG was only present in two individuals of one clutch (one egg and one L3 larva, clutch 3, Figure 1 b). Besides Burkholderiaceae (present in 100 % of clutches), the most consistent families present among life stages and clutches were Xanthomonadaceae (91 % of clutches), Yersiniaceae (91 % of all clutches), Oxalobacteraceae (82 % of all clutches), Enterococcaceae (64 % of all clutches), Enterobacteriaceae (55 % of all clutches) and Comamonadaceae (45 % of all clutches) (Figure 1 c, Figure S1).

The abundances of the different strains recorded by 16S barcoding are based on 16S copy numbers. While the Lh-StG genome carries five 16S copies, it is unknown how many 16S copies Lh-StH carries. Therefore, we carried out quantitative PCR targeting the single-copy gene gyrase B (*gyrB*) of each *Burkholderia* strain to determine absolute abundance across different life stages and reinforce strain identification based on a second gene marker in addition to the partial 16S rRNA gene sequence. We compared the symbiont titer of Lh-StG and Lh-StH with strain-specific primers in field-collected pupae and females as well as in the egg and larval samples assessed by microbiota profiling. Comparisons of the ratios between LhStG and LhStH per life stage showed highest LhStH titers in adults, followed by female pupae and eggs, while the larval stages had significantly more LhStG than the other stages (Figure 1 d). This indicates that Lh-StG titers increase after colonizing the larval organs, coexisting in equal abundances with LhStH.

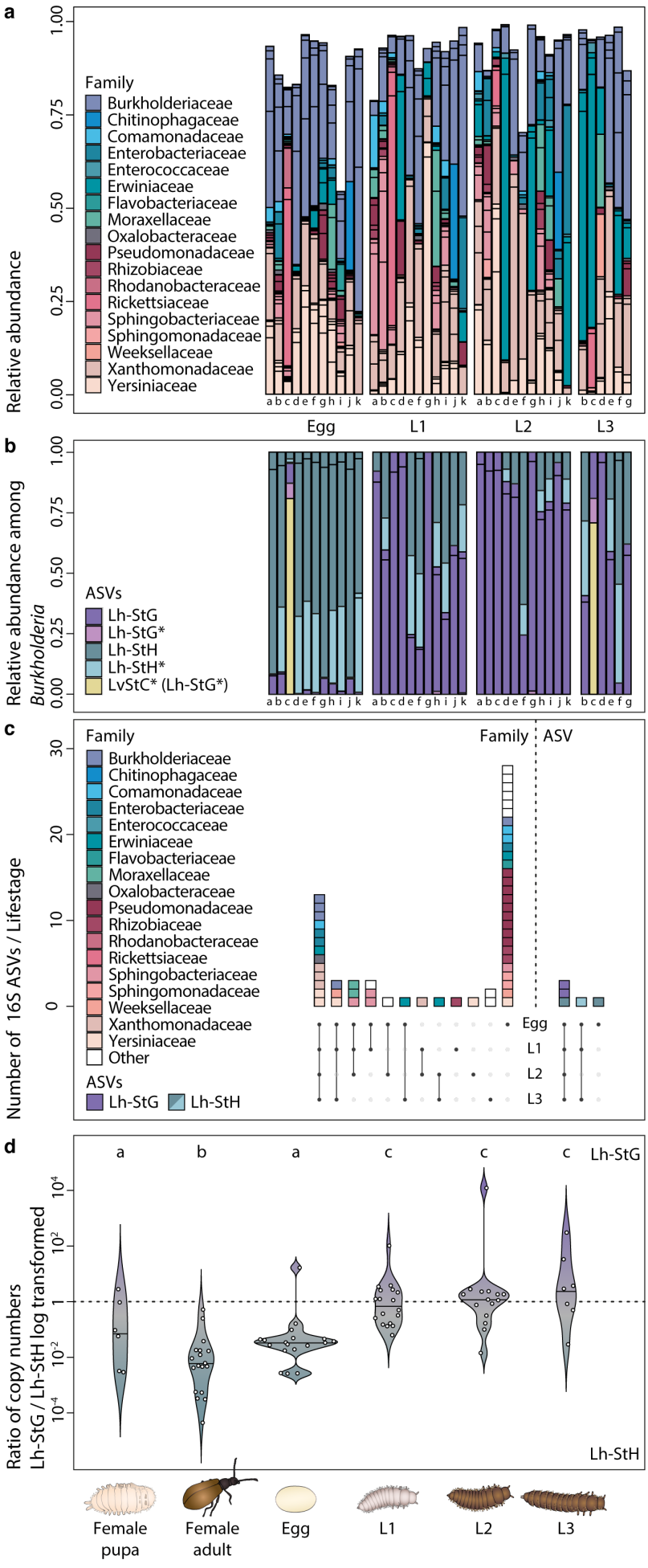

Figure 1: Two *Burkholderia* strains are highly abundant across life stages of *L. hirta*. Egg and larval stages (L1 – L3) from 11 clutches (a-k) were collected as offspring from field-collected females. **a** Relative abundance of bacterial families across early *L. hirta* life stages based on Illumina sequencing of the 16S rRNA V4 region. Each bar corresponds to an egg clutch or a pool of individuals from a same clutch. The 50 families with highest abundances across the dataset are represented. **b** Relative abundance of Burkholderia ASVs. An asterisk (*) in the legend denotes pairwise identity of ASV and reference sequence above 98 % but below 100 %. For the graphical representation, the relative abundance of Lh-StG assigned ASVs was corrected according to the number of 16S copies in this strain (5 copies). **c** Number of shared and individual 16S ASVs present across life stages. Dark dots and lines show presence and grey dots absence in at least 50 % of the samples. Differently colored bars on the vertical axis represent single ASVs corresponding to bacterial families (left) or strains (right) present in each set of life stages. White bars correspond to other bacterial families, which were not in the top 50. **d** Ratio of copy numbers between *Burkholderia* strains measured by qPCR with primers targeting the single-copy gene gyrB specific for each strain. Female glands were dissected from field-collected females. A linear mixed effects model was carried out to compare the ratios between LhStG and LhStH titers $p < 0.05$.

To visualize and evaluate if Lh-StH and Lh-StG share the same environment in the beetle, we carried out fluorescence *in situ* hybridization (FISH) and localized the symbionts in histological sections in different life stages. In a larva, female pupa and an adult female, *Burkholderia* was the main bacterial genus found in the organs or on the surface, while only a few other

bacteria were present (Figure 2 a-c). While we could localize Lh-StH and Lh-StG within the symbiotic organ in the larva (Figure 2 d), we could only clearly identify Lh-StH-labeled cells in the pupa and adult female (Figure 2 e, f). In the pupa, symbiont cells could only be found on the dorsal surface, while they are present in high abundance in the two accessory glands in females as previously reported [10] Both glands were densely filled with the *B. gladioli* symbionts and show a homogenous symbiont distribution (Figure S 2).

These results suggest that two *B. gladioli* strains coexist in the symbiotic organs of *L. hirta*, albeit in fluctuating ratios across different life stages. While the non-culturable Lh-StH dominates in pupae, adult females, and on the eggs, the culturable Lh-StG is present in higher abundance in the early larval stages, suggesting putative differences in colonization success, competitive abilities, and/or their functional roles across different life stages.

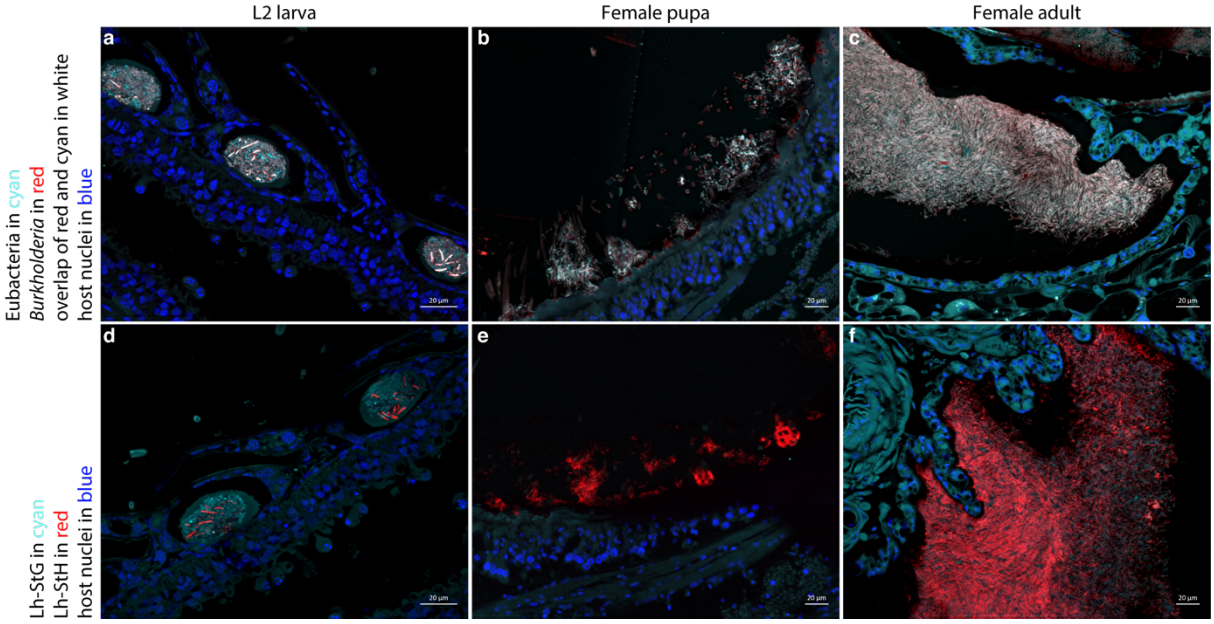

Figure 2: Presence and localization of two *Burkholderia* strains in *L. hirta* larva, pupa, and adult female. Columns show different life stages and rows show different probe combinations. In **a-c** Eubacteria are shown in cyan and Burkholderia in red, while overlapping cells occur in white. In **d-f** Lh-StG is shown in cyan and Lh-StH in red. Host cell nuclei are generally depicted in dark blue. **a** Sagittal section of an L2 larva showing the three dorsal symbiotic organs between the segments being mainly colonized by ***Burkholderia*** (white cells) while a few other bacteria are present (cyan cells). **b** On the surface of pupae and in **c** female accessory glands, ***Burkholderia*** cells are also highly abundant. **d** In an L2 larva, Lh-StG (cyan) and Lh-StH (red) are co-exist in the symbiotic organs. In e) pupae and f) females Lh-StH predominates. Scale bars correspond to 20 µm.

3.2. Both *Burkholderia* symbionts inhibit fungal growth on eggs and increase the hatching rate

In the related species *L. villosa*, eggs most often carry a single dominant *B. gladioli* strain, but occasionally house multiple strains that can defend the egg stage against fungal pathogens. The defensive function of the co-infecting *L. hirta* symbiont strains was however unknown so far. Therefore, we conducted bioassays exposing differently treated eggs laid by field-collected *L. hirta* females to the fungus *Purpureocilium lilacinum*. We quantitatively and

qualitatively evaluated fungal growth on the eggs and analyzed the hatching rate of first instar larvae. As described for *L. villosa*, eggs from *L. hirta* were defended by their natural symbiotic community present on the egg surface (Figure 3 a, b), and both the culturable symbiont from *L. hirta* (Lh-StG) and the one from *L. villosa* (Lv-StA) provided protection. However, eggs reinfected with Lh-StG ("reinfected-Lh-StG") were more vulnerable to fungal growth compared to the natural community ("untreated" and "reinfected-egg wash") or Lv-StA ("reinfected-Lv-StA"). This strain does hold some protective potential though, as fungal inhibition was significantly higher than in the symbiont-free ("aposymbiotic") eggs. Similarly, hatching rates of fungus-exposed eggs were generally enhanced by the presence of *Burkholderia* symbionts, although this effect was only significant for the untreated and Lv-StA-reinfected eggs, respectively, but not for the eggs reinfected with natural community or Lh-StG (Fig. 3 c). Nevertheless, the degree of fungal growth had a strong effect on egg hatching rates across treatments, with heavily infected eggs having significantly lower chances of survival (Fig. 3 d).

To assess whether the degree of symbiont-provided defense depends on the host species, we reinfected aposymbiotic eggs of *L. villosa* with either the *L. hirta*-derived Lh-StG or its native LvStA. As described previously [11], Lv-StA showed strong protective activity. Interestingly, however, *L. villosa* eggs reinfected with Lh-StG showed fungal growth probabilities (Figure S3 a) and levels (Figure S3 b) that were indistinguishable from aposymbiotic eggs. This indicates that Lh-StG does not provide protection to *L. villosa* eggs, while it does reduce fungal growth on eggs of its native host species *L. hirta* (Figure 3 a, b).

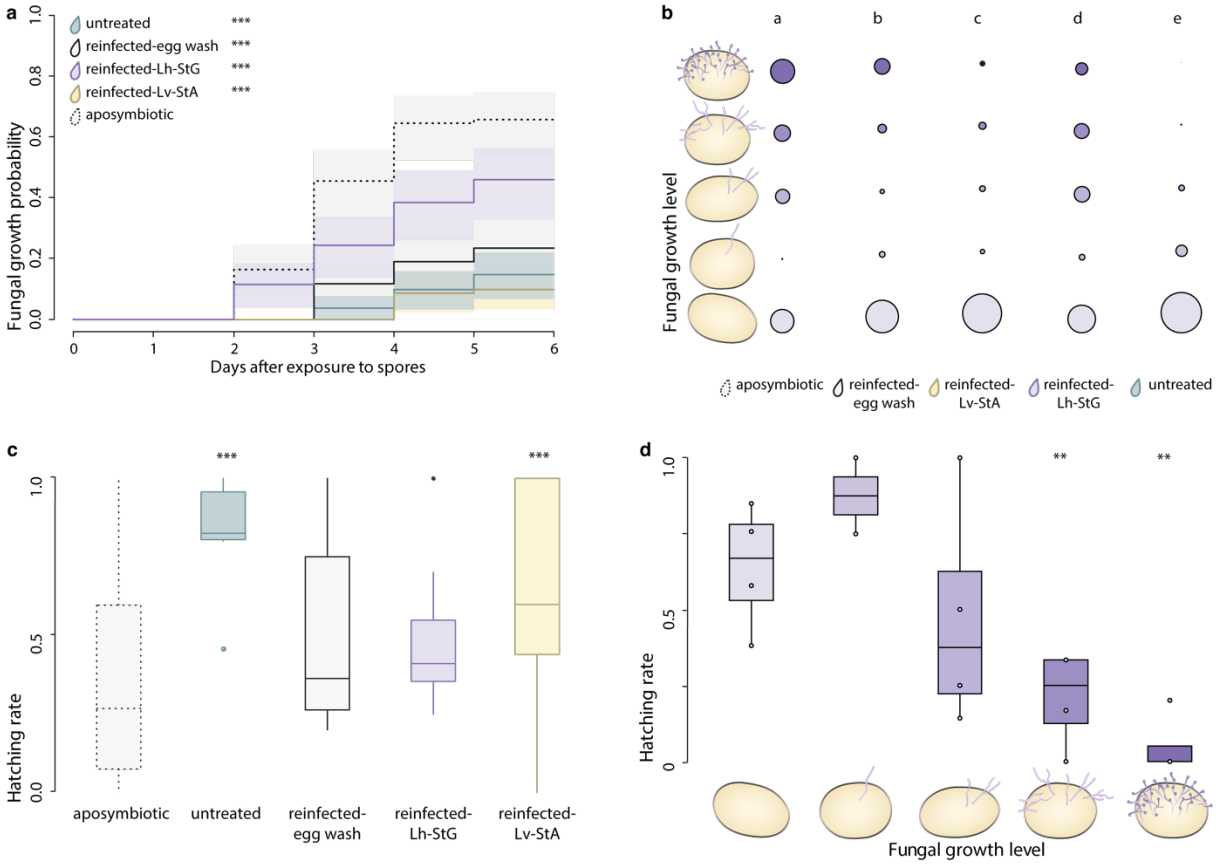

Figure 3: Both *Burkholderia* symbionts protect *L. hirta* eggs against fungal infestation and enhance larval hatching. a Fungal growth probability of eggs exposed to the conidia of the fungus ***P. lilacinum*** was assessed single-blindly until the eggs hatched in the following treatments: untreated (teal line, n=87), reinfected-egg wash (black line, n=70), reinfected-Lh-StG (purple line, n=70), reinfected-Lv-StA (yellow line, N=81) and aposymbiotic (dotted black line, n=79). Cox Mixed-Effects Model compared to the aposymbiotic treatment (a & b, dotted line or boxplot) or fungus-free eggs (c, first boxplot), *** p<0,001. **b** Fungal growth was estimated single-blindly during monitoring and assigned to levels according to different categories as described 11 (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). **c, d** Larval hatching rates were evaluated for four of the monitored clutches and analyzed according to **c** treatment or **d** the amount of fungus infesting the eggs. Different letters and asterisks indicate significant differences between the groups (Generalized linear mixed model with a Poisson distribution and clutch as random factor ** p<0,01,*** p<0,001).

3.3. Defensive and metabolic capabilities of symbiont strains

To investigate potential factors causing the observed differences of defense on *Lagria* eggs and the fluctuating abundances of the different strains, we analyzed the culturable strains from each of the *Lagria* species on a genomic level. We used the previously published whole genome of the *L. villosa*-associated Lv-StA [11] and a draft genome from Lh-StG obtained from a pure culture. Metabolically, both strains are highly similar judging from the gene counts in different SEED categories (Figure S4 a). Also, both Lv-StA and Lh-StG carry multiple putative biosynthetic gene clusters (BGCs) for the production of secondary metabolites (Figure S4 b). In the genome of Lh-StG, we found genes or variants corresponding to all the BGCs that were previously reported for Lv-StA, including toxoflavin [11], terpenes [16], sinapigladioside [11,15], lagriene [11], icosalide [15], haereogladin [15], gladiofungin [16], gladiobactin [18], caryoynencin [11] and burriogladin [15] (Figure 4 a). However, production *in vitro* was only observed for icosalide, caryoynencin, haereogladin, and burriogladin, while sinapigladioside, lagriene, toxoflavin, and gladiofungin were not detected under the tested conditions (unpublished data). Additionally, we found the monobactam sulfazecin in the genome of both culturable strains (Figure 4 a).

To find out whether these compounds are also produced *in vivo* in *L. hirta* individuals, we analyzed crude extracts of female accessory glands, eggs, larval organs, and exuviae by HPLC-HRESI-MS. Icosalide and lagriene could be detected in 50 % of the larval samples, while no other compounds derived from Lh-StG were found in any of the life stages (Table 1). Interestingly, we found that a highly similar compound of the antifungal polyketide lagriamide was present across all tested life stages (Table 1). Lagriamide has so far only been described from the unculturable *Burkholderia* symbiont Lv-StB of *L. villosa* [12, Chapter I]. Therefore, we analyzed the relative abundance of the lagriamide relative (henceforth “lagriamide II”) by comparing retention time, UV absorbance, and high-resolution mass spectra to an authentic reference in different life stages (Figure 4 c). We found that lagriamide II was present in symbiotic organs of field-collected larvae, exuviae from larvae and larva-pupa exuviae, and symbiotic organs of female adults, as well as in eggs from field-collected females (Figure 4 b). Since we did not find a lagriamide biosynthesis gene cluster in the genome of Lh-StG, and *L. villosa* lagriamide is produced by the dominant but unculturable strain Lv-StB [12], we assume that Lh-StH is likely producing the lagriamide II in *L. hirta*.

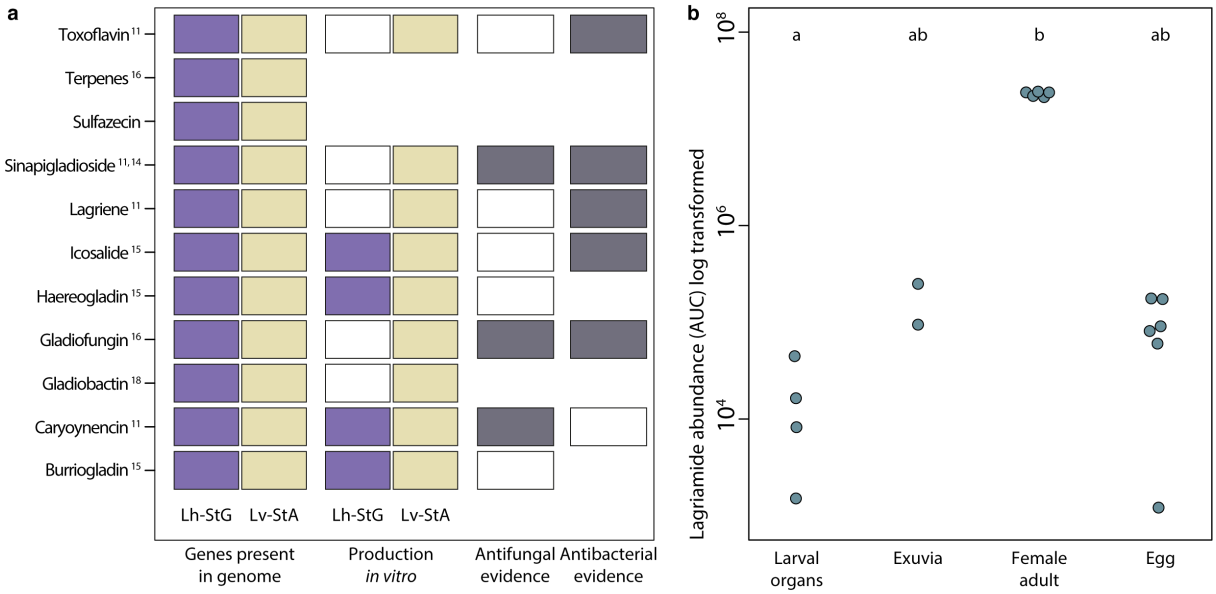

Figure 4: Bioactive compounds produced by different *Burkholderia* symbiont strains of *Lagria* beetles. a Summary of the secondary metabolites produced by the culturable *Lagria* strains and their bioactivity. Filled bars indicate the presence of a certain parameter, white bars show their absence and missing bars indicate not available information. Antibacterial and antifungal evidence was previously evaluated *in vitro* and *in vivo* assays, the presence of genes was assessed via antiSMASH, and *in vitro* production was assessed under specific conditions (unpublished data). **b** Area under the curve (AUC) of the extracted ion chromatogram (EIC) of lagriamide II (m/z = 731 [M-H]-) representing abundance across different life stages, as quantified from crude methanol extracts. Eggs correspond to offspring from field-collected females. All others correspond to field-collected specimens. Different letters indicate significant differences between life stages (Kruskal-Wallis $\chi 2=$, df =, *p* value =, posthoc Dunn's Test, $\alpha \leq 0.05$; dissected larval organs n = 4, exuvia n = 2, female adult n = 5, egg n = 6).

4. Discussion

Lagria beetles harbor different *Burkholderia* strains that are vertically transmitted from female accessory glands onto the eggs, and occasionally from the environment [10,11]. In *L. villosa,* a species occurring in Africa and South America, it was previously demonstrated that different symbiont strains can protect the immature life stages of their hosts against pathogenic fungi, although only one strain – Lv-StB – is consistently abundant under natural conditions [1012, Chapter I]. Here, we show that in the related European species *L. hirta,* two strains (Lh-StH and Lh-StG) coexist throughout host development and are also able to protect the eggs against a fungal pathogen. The strains produce different compounds for protection and vary in their abundance across life stages, indicating a flexible defense strategy for the host. Thereby, we show that symbiont-mediated defense by co-infecting strains with different biosynthetic capabilities can be a successful strategy to combat antagonistic microorganisms.

In both *L. hirta* and *L. villosa*, the unculturable strains Lv-StB and Lh-StH are always present in field populations, indicating their importance for the symbiosis. This is underlined by their production of the antimicrobial compound lagriamide or its relative lagriamide II, which is presumably a crucial compound for antifungal defense [12, Chapter I]. By contrast, the culturable strain Lv-StA is only sporadically present in natural *L. villosa* populations, despite its versatility in producing an arsenal of antifungal and antibacterial compounds *in vitro* and its ability to protect eggs and larvae [11,12,14,16, Chapter I]. *L. hirta*'s culturable symbiont strain Lh-StG is highly similar to LvStA on the genomic level, but we could only detect the production of some of the compounds *in vitro*, and even fewer *in vivo*. This may explain why Lh-StG protects less well *in vivo* on *L. hirta* eggs and shows no defensive activity on the eggs of *L. villosa* at all. It remains enigmatic, however, why Lh-StG but not Lv-StA is consistently abundant in its respective host beetle populations in the field. Whether this reflects adaptation to local natural enemies remains to be assessed.

One prevailing observation is that strain diversity of highly similar microorganisms leads to competition [1]. This conflict might cause that single individuals are associated with only one strain, although multiple closely related strains are present within a population. This can be observed for example in the symbioses of squids [19], bees [9], or bean bugs [20]. It is however also possible that multiple symbiont strains coexist in individuals, when their capabilities are functionally diverse, such as described in deep-sea mussels [6]. We showed that the chemical mediators for defense differ between the coexisting strains of *L. hirta* beetles, which likely enables their living together. In both *Lagria* species *Burkholderia* strains can coexist, albeit with different dynamics. LvStB and LhStH seem to be efficient defenders likely because of their ability to produce lagriamide. Both possibly both coevolved with their hosts, given by the reduced genome of LvStB indicating stable vertical transmission across generations. Both strains are highly abundant across their hosts life stages, while LvStB solely dominates in *L. villosa*. In *L. hirta*, however, LhStG is also frequently present in individuals and even equally abundant in larvae, although its defensive capabilities are putatively poor, at least

against one fungus. One reason for its common association with the beetle and the coexistence with LhStH could be lower costs for the hosts due to its prototrophic lifestyle with potentially little nutritional demands. LhStH on the other hand, might require more nutrients from the host, being more costly to maintain. By that, the cost-benefit ratio might be similar for both strains, granting their coexistence within hosts. Alternatively, the host could also benefit from harboring both symbionts. By the greater variety of secondary metabolites, hosts might be better defended against individual antagonists through synergistic effects [21], or they collectively inhibit a broader spectrum of antagonists [22]. In the case of *L. hirta*, a higher diversity of antimicrobial compounds might also decouple defenses in different life stages, aiming at different predominant antagonists. Therefore, investigating the defensive potential of LhStG in the presence of other pathogens and in different life stages would be valuable. By contrast, LvStA is uncommon in field-collected *L. villosa*, but a very good defender. It is imaginable that LvStA is transmitted less efficiently across generations, possibly due to differences in symbiont or host traits. It currently remains unknown whether host or symbiont factors, or symbiont-symbiont interactions are responsible for the poor maintenance and/or transmission of LvStA in natural populations. It is therefore essential to better understand the molecular factors underlying establishment and maintenance of the symbiosis across different coinfecting strains [23].

We demonstrated that Lh-StG has the genomic potential to produce the compounds sinapigladioside, lagriene, toxoflavin, and icosalide, which can also act against other bacteria [11,14,15]. We also showed *in vivo* that Lh-StG produces icosalide and lagriene in the organs of larvae. This might indicate direct competition against other bacteria, which is in line with LhStG's higher abundance in this life stage. It remains however elusive, whether LhStG produces competitive compounds that could affect other strains and whether Lh-StH might has genomic features responding to competition, or if its constancy across certain life stages is regulated by the host. It is generally unclear in this system, whether the beetle has any mechanisms to select its partner(s) or if the symbiont-produced compounds are recognized by the host. Furthermore, it is imaginable that the antifungal compound lagriamide II is also acting as an agent against other bacteria as it was hypothesized for LvStB [24]. However, since lagriamide II is also produced in the larval stages where it coexists with LhStG, it would be crucial to assess its specificity against different bacteria to support this hypothesis.

Co-infecting strains can provide broad-spectrum protection, and due to their fluctuating abundances in different life stages, this might be a versatile strategy to cope with different antagonists. Combining different compounds might especially be favorable for systems where hosts are exposed to variable pathogens, which is often the case in soil environments [25]. Retaining a long-term stable association with symbionts that produce a diverse mix of antibiotic compounds can be observed in the symbiosis between *Streptomyces* symbionts and beewolves [22]. Since they lack a known co-evolving enemy but are rather surrounded by a community of opportunistic mold fungi, beewolves are protected by symbionts with versatile broad-spectrum antifungal compounds [22,26]. Attine ants on the other hand are in an

evolutionary arms race with fungal antagonists and were able to evolve a mixed defense strategy of harboring co-evolving *Pseudonocardia* and horizontally acquired *Streptomyces* symbionts [27–29]. The symbiosis of both described *Lagria* species is characterized by a more dependent relationship with a so-far unculturable and lagriamide-producing strain (Lv-StB, Lh-StH) and a potentially more or less flexible association to other culturable defensive strains (Lv-StA, LhStG), with different genomic features for defense. This combination might represent a defense strategy similar to the one in ants, to tackle different antagonists from the soil environment, although the presence of a co-evolving enemy is unclear.

Interestingly, LhStG conferred protection on eggs of its own host species *L. hirta*, while it did not inhibit fungal growth on *L. villosa* eggs. Lh-StG might not be able to survive well on heterospecific eggs in contrast to LvStA, although both strains can be grown *in vitro*.

Alternatively, although protection provided by LhStG might be dependent on the antagonist or life stage as discussed above, this observation could be a first piece of evidence for host effects on defense by the symbiont. It would be interesting to find out, whether the host provides metabolites, which are utilized by LhStG for the production of secondary metabolites or if the symbiont requires cues from the host to produce its compounds. If defense of LhStG is host-specific and host-dependent remains yet elusive and requires further investigation.

Lagria beetles evolved a successful relationship with *Burkholderia* symbionts conferring increased resistance to fungal infections from the environment and facilitating successful development of their offspring. It remains puzzling, however, how and why the unculturable lagriamide-producing strains persist within the beetle, although chemically more potent strains with much larger genomes are also present and protective. Broader phylogenetic analyses of the *Burkholderia* strain dynamics and the presence of the lagriamide-producing symbionts across multiple *Lagriinae* species are needed to understand the evolutionary stability of the symbiosis. Furthermore, molecular analyses of host and symbiont factors determining colonization success would allow for understanding the mechanistic basis underlying differential establishment and maintenance of different symbiont strains, yielding insights on the processes determining the dynamics of multi-partner symbiotic communities.

5. Methods

5.1. Insect collecting and rearing

L. hirta individuals were collected in 2012, 2013, 2018, 2019, and 2020 in Germany. Individuals were reared in plastic containers in a climate chamber (16:8 L:D light regime at 21 °C and 60% humidity). Adult beetles were fed with fresh and dry leaves from blackberry, hazelnut, and *Impatiens* sp. while larvae were fed only with dry leaves. Centrifuge tubes with autoclaved tap water and centrifuge tube lids with moist cotton were provided for humidity control and as egg-laying substrate. *L. villosa* individuals were collected in Brazil in 2015. The 3rd generation of this lab culture was used to perform the *in vivo* bioassays on the eggs. Adult beetles were fed on fresh and dry leaves from soybean, pea, cabbage, and lettuce and reared under the same conditions as *L. hirta* adults.

5.2. Bacterial community profiling via 16S rRNA amplicon sequencing

L. hirta eggs and the first three larval stages were collected in the lab as offspring of field-collected females, preserved in ethanol, and extracted as fractions of a single clutch (5-10 individuals per clutch and life stage). For DNA extraction, the Epicentre MasterPure Complete DNA and RNA Purification Kit was used following the manufacturer's instructions and including lysozyme treatment before protein digestion. Samples were randomized according to life stage and clutch in the different rounds of extraction, and no-template-extractions were included to control for possible contamination. The V4/V5 region of the 16S rRNA gene was sequenced by a commercial provider (StarSeq, Mainz, Germany) on an Illumina MiSeq platform. Amplicon sequence variants (ASVs) were inferred using the R package DADA2 [30] with default parameters, trimming lengths of 250 and 200 nt for forward and reverse reads, respectively, and dereplication and chimera removal. Taxonomy was assigned using the pre-trained classifier Silva 132 [31,32] with subsequent removal of reads classified as Eukaryota, chloroplast, or mitochondria. For the strain-level analysis, ASVs within Burkholderiaceae were manually blasted against a local database containing Lagriinae-associated *Burkholderia* strain sequences. Sequences with a pairwise identity of 98-100% were assigned according to the best hit. For the graphical representation, the relative abundance of Lh-StG assigned sequences was corrected according to the number of 16S copies in this strain (5 copies).

5.3. Quantification of Lh-StH and Lh-StG

Extracted DNA from different life stages was used to determine strain-specific symbiont titers using qPCR. Samples of eggs, larvae, and whole pupae were extracted as previously described using the Epicentre MasterPure Complete DNA and RNA Purification Kit, while adult female symbiont organs were dissected before extraction. qPCRs were carried out

targeting the gyrase B gene of each strain using the primers BurkH_gyrB_F (TTTTCTGGTGAGGAAGCGACT) and BurkH_gyrB_R (TGCTCGCGAATTTCGTCGTA) for Lh-StH as well as BurkG_gyrB_F (TACTCGACCGTGCCGAAGATG) and BurkG_gyrB_R (GACCGCGTGCTGGAGAAG) for Lh-StG. Specificity of the primers was evaluated *in silico* by designing them considering a local database of *Lagria*-associated *Burkholderia* strains and by testing them against extracts of LvStA. qPCRs were run under the following conditions: Initial activation at 95 °C for 15 min, denaturation at 95 °C for 15 s, annealing at 62 °C for 15 s and elongation at 72 °C for 15 s. qPCRs were carried out using the 5x HOT FIREPol EvaGreen HRM Mix EvaGreen (Solis BioDyne) on a RotorGene-Q cycler (Qiagen) in 10 µL reactions including 0.5 µL of each primer and 1 µL template DNA. Standard curves were created by amplifying the fragment, followed by purification and determination of the DNA concentration using a Qubit fluorometer. A standard containing 1 ng/µL was generated and 1:10 serial dilutions down to 10-8 ng/µL were prepared. All standards and no-template controls were included in the qPCR run for absolute quantification. Differences between life stages and strains were analyzed with a linear mixed effects model using RStudio (Version 1.2.5042) and the lme4 package (Version 1.1-23). Plots were created using ggplot2 package (Version 3.3.0) and Adobe Illustrator (Adobe, Version 14.1, CC 2020).

5.4. Symbiont localization by fluorescence *in situ* hybridization (FISH)

FISH was performed on semithin sections of *L. hirta* individuals. Before FISH, *L. hirta* individuals were fixated in 4% formaldehyde in PBS for at least 3 days. Embedding, semithin sectioning, and FISH were performed as described previously [33] using a hybridization temperature of 50 °C. Cy3- or Cy5-labeled Burk16S probe (5'TGCGGTTAGACTAGCCACT'3) was used to mark all *B. gladioli* strains, and the Cy5-labeled Burk16S_StH2.1 probe (5'GCACTCCTAGATCTCTCCAGGA'3) for the symbiont strain Lh-StH, the Cy3-labeled Burk16S_StC-G probe (5'GCACTCCCAGATCTCTCTAGGA'3) for the symbiont strain Lh-StG, and the Cy3-labeled EUB338 probe (5'GCTGCCTCCCGTAGGAGT'3) [34] for general eubacteria. DAPI (4',6-diamidino-2-phenylindole) was used to label the host cell nuclei. Images were taken on an AxioImager.Z2 fluorescence microscope (Zeiss, Jena, Germany).

5.5. Isolation and cultivation of Lh-StG

One accessory gland of an *L. hirta* female was homogenized and suspended in 100 µL of sterile PBS and diluted at 10^{-5} in PBS. 100 µL 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 single colonies 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 MgCl2 , 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')[35]. PCRs were carried out on a Biometra professional 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 cycling 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.

5.6. Fungal inhibition bioassay on *Lagria* eggs

Eggs of *L. hirta* and *L. villosa* were used to test for fungal inhibition of different *B. gladioli* symbiont strains *in vivo* based on an established assay protocol [11]. The experimental set-up (Figure S5) contained 96-well plates that were filled with a layer of moist and sterile vermiculite substrate. Filter paper discs were added separately in each of the 60 inner wells, while the 36 outer wells were excluded to ensure equal humidity in all testable wells. 5 µL of a suspension of fungal conidia from *P. lilacinum* [11] (LV1, Accession number: KY630747, KY630748, KY630749) (75 conidia/µL) were inoculated on each filter paper disc. Eggs from six clutches of *L. hirta* and five clutches from *L. villosa* were tested. To obtain aposymbiotic and differently reinfected eggs, new egg clutches of field-collected (*L. hirta*) and laboratory-raised *L. villosa* females (3rd laboratory generation) were divided into different groups. One part of the clutch was not treated and was used as a control with the natural microbial community ("untreated"). The remaining eggs were first washed with sterile PBS, then slightly shaken for 5 min in 70% ethanol, washed 2 times with sterile water, immersed for 30 s in 12% NaClO, and finally washed three times with sterile water. These eggs were then inoculated with PBS to obtain symbiont-free individuals ("aposymbiotic"), with the previously obtained PBS egg wash that contains the natural microbial community ("reinfected-egg wash", previously named "reinfected-natural") [11], or with a PBS suspension containing 10^6 cells/µL of the *L. hirta* native Lh-StG ("reinfected-Lh-StG") or the *L. villosa* native Lv-StA ("reinfected-Lv-StA"). Eggs were randomly assigned to the inner wells of the 96 well plate and monitored single-blindly for six days. Fungal growth was recorded on each egg, differentiating between eggs that had no fungal growth and eggs that had visible fungal growth. Additionally, the amount of grown fungal hyphae was semi-quantitatively assessed for the last day before hatching, by assigning the following levels: 0 (no visible growth), 1 (minor growth directly on the surface, barely noticeable), 2 (multiple mycelia touching the surface), 3 (egg surface coated with multiple mycelia), to 4 (surface completely covered by mycelia, sporulating fungus). To determine the effect of the different treatments on fungal growth, a Cox mixed-effects model with a random intercept per clutch was fitted using the coxme package (Version 2.2-16) in RStudio (Version 1.2.5042). Plots were obtained with the rms package (Version 5.1-4), making use of the Kaplan-Meier-estimator and Adobe Illustrator (Adobe, Version 14.1, CC 2020). To determine the hatching rate of the *L. hirta*

individuals, four of the six clutches were monitored and evaluated according to treatment and level of fungal growth.

5.7. Lh-StG genome sequencing, assembly, and annotation

B. gladioli Lh-StG was sequenced by a commercial provider (Eurofins MWG Operon/Eurofins Genomics, Germany). 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 the final scaffolds. The previously published genome of Lv-StA was re-annotated with RAST in KBase [36–38] for comparison to LhStG. Both annotated genomes were used to classify all annotated genes according to the SEED Subsystem using the Kbase App View Function Profile for Genomes. The resulting raw count of genes with annotations was visualized as a heatmap using the ggplot package in Rstudio. For the comparison of candidate biosynthesis gene clusters, genomes were analyzed using antiSMASH [39]. Identified clusters were compared to antiSMASH-predicted clusters and the MIBiG database [40] with a relaxed direction strictness.

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9. Supplement

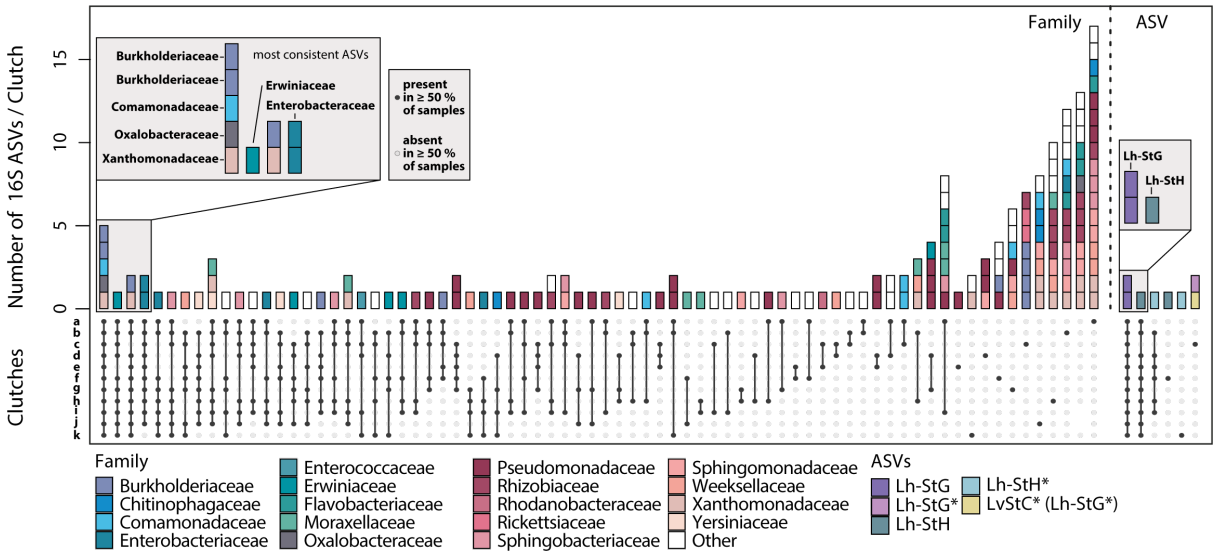

Figure S1: Consistency of different bacterial families in eggs and larvae originating from different egg clutches of *L. hirta*. Egg and larval stages (L1 – L3) from 11 clutches (a-k) were collected as offspring from field-collected females. Several shared and individual 16S ASVs present across clutches. Dark dots and lines show presence and grey dots absence in at least 50 % of the samples. Different colored bars on the vertical axis represent single ASVs corresponding to bacterial families (left) or ASVs (right) present in each set of clutches. White bars correspond to other bacterial families, which were not in the top 50.

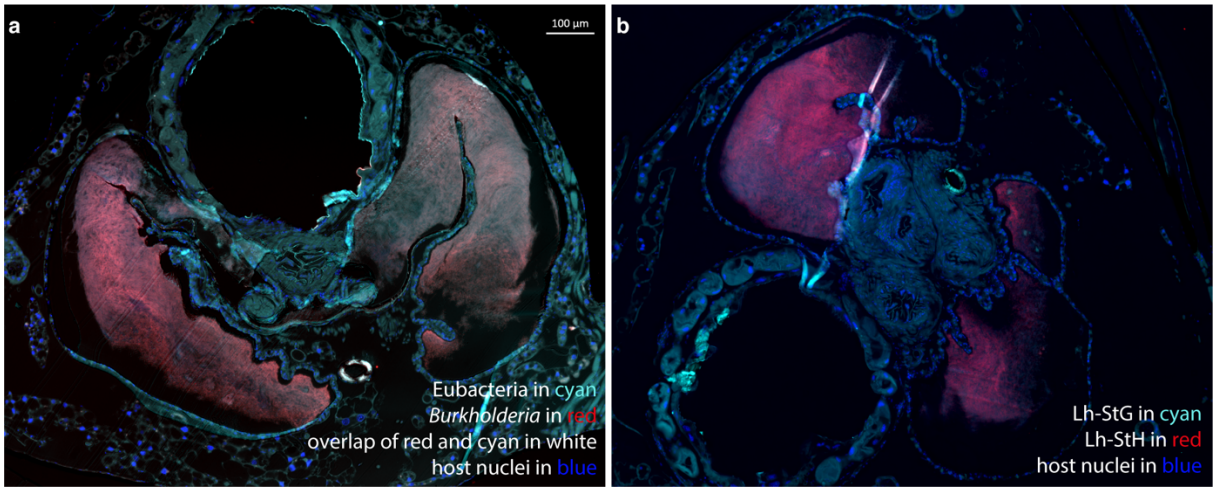

Figure S2*: L. hirta* female accessory glands are equally filled with symbionts. Transversal sections through the abdomen of a field-collected adult female show the symbionts within two accessory glands of the reproductive system. **a** Eubacteria are shown in cyan and Burkholderia in red while overlapping cells occur in white. **b** Lh-StG was labeled with a Cy-3 probe shown in cyan and Lh-StH with a Cy5-probe shown in red. Host cell nuclei are generally depicted in dark blue.

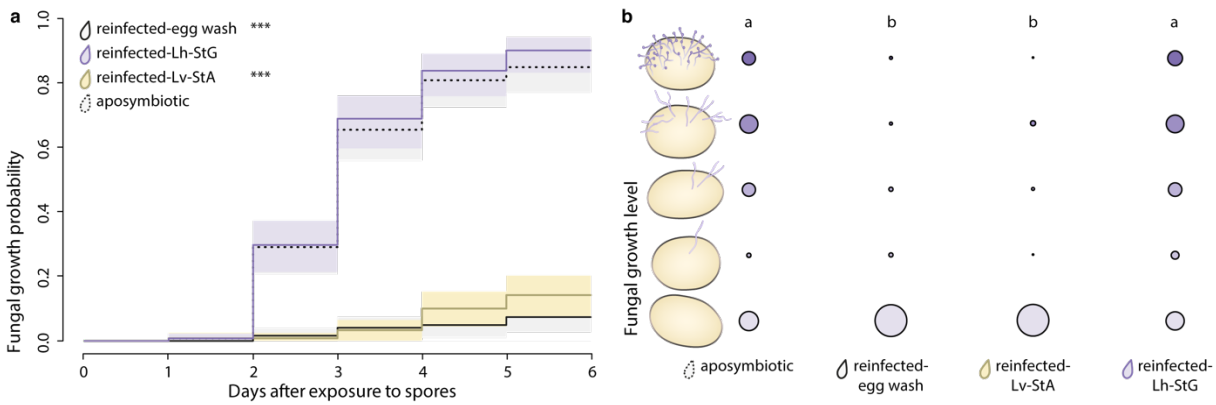

Figure S3: Protective activity of symbiont strains Lv-StA and Lh-StG on *L. villosa* eggs. a Fungal growth probability on *L. villosa* eggs exposed to the conidia of the fungus *P. lilacinum* was assessed single-blindly until the eggs hatched for different treatments: reinfected-egg wash (black line), reinfected-Lh-StG (purple line), reinfected-Lv-StA (yellow line) and aposymbiotic (dotted black line) Cox Mixed-Effects Model compared to the aposymbiotic treatment (a & b, dotted line or boxplot) or fungus-free eggs (c, first boxplot), *** p<0,001. **b** Fungal growth was estimated single-blindly during monitoring and assigned to levels according to different categories as described 11 (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). Different letters indicate significant differences between the groups (Generalized linear mixed model with a Poisson distribution and clutch as random factor $p < 0.05$).

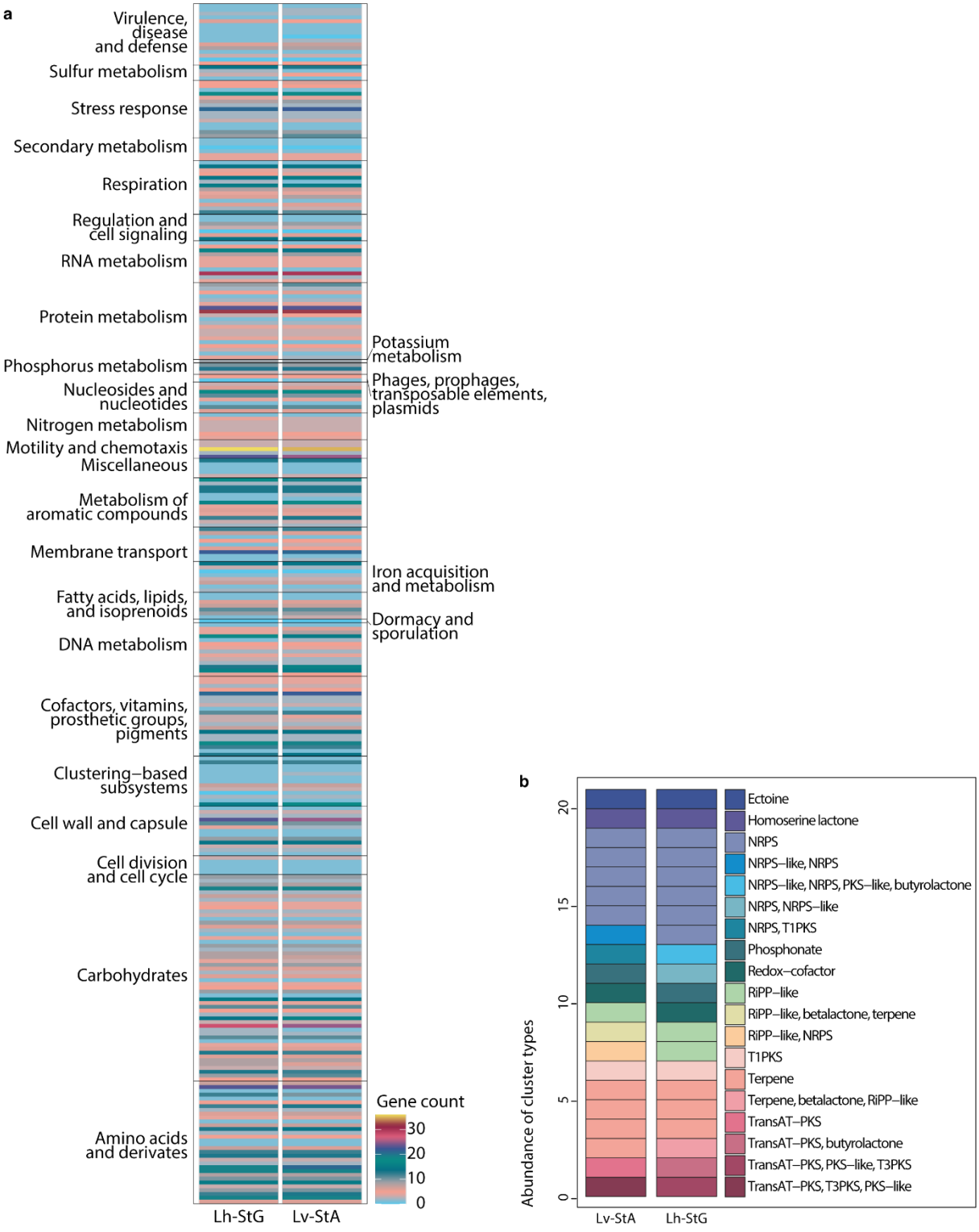

Figure S4: Genomic comparison of Lh-StG and Lv-StA. a Annotated genes of LhStG and LvStA according to functional (SEED) categories. **b** Presence of biosynthetic gene clusters (BGCs) in the genomes of Lh-StG and Lv-StA. Colors indicate the type of BGC annotated by antiSMASH: PKS=polyketide synthase, T3PKS=Type III PKS, NRPS=nonribosomal peptide synthetase, RiPP=ribosomally synthesized and post-translationally modified peptides.

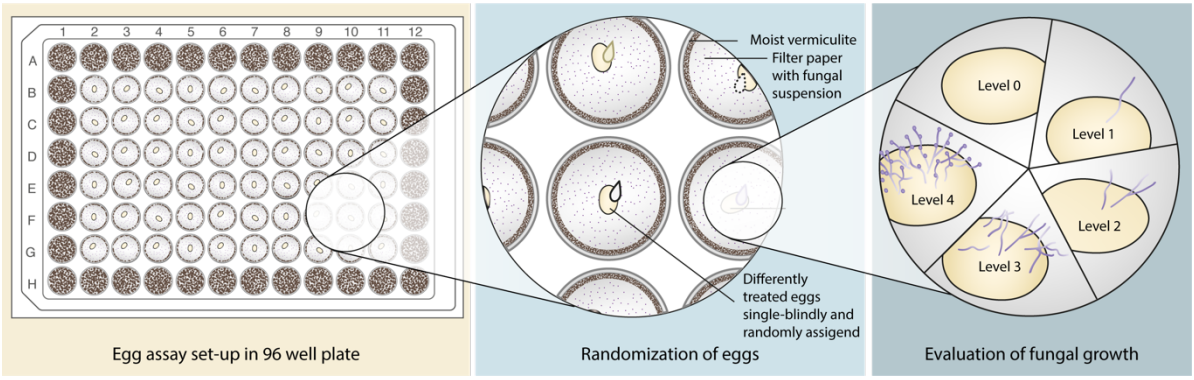

Figure S5: Schematic overview of the *in vivo* assay exposing differently treated eggs to a fungal pathogen.

Table 2: Detection of Burkholderia-produced secondary metabolites in *L. hirta* specimens by HPLC-MS.

Sample	Lagriamide II	Sinapigladioside	Icosalide	Lagriene
egg clutch	yes	no	no	no
egg clutch	yes	no	no	no
egg clutch	yes	NA	NA	NA
egg clutch	yes	NA	NA	NA
egg clutch	yes	NA	NA	NA
egg clutch	yes	NA	NA	NA
exuviae pool L-L	yes	no	no	no
exuviae pool L-P	yes	no	no	no
Female symbiotic organs	yes	NA	NA	NA
Female symbiotic organs	yes	NA	NA	NA
Female symbiotic organs	yes	NA	NA	NA
Female symbiotic organs	yes	NA	NA	NA
Female symbiotic organs	yes	NA	NA	NA
Larval symbiotic organ	yes	no	no	no
Larval symbiotic organ	yes	no	yes	yes
Larval symbiotic organ	yes	no	no	no
Larval symbiotic organ	yes	no	yes	yes

Table S1: Original localities of collected beetles used in this study

Time	State	Locality	Coordinates	Species
June, July, 2012	Thüringen, Germany	Ammerbach	50°54'12.6"N 11°33'02.7"E	*L. hirta*
June, July, 2012	Thüringen, Germany	Ammerbach	50°53'44.1"N 11°33'07.1"E	*L. hirta*
July, 2013	Thüringen, Germany	Ammerbach	50°54'12.6"N 11°33'02.7"E	*L. hirta*
September, 2018	Galicia, Spain	Cabo Udra	42.3383572 , -8.8322441	*L. hirta*
July, 2019, 2020	Rheinland-Pfalz, Germany	Höhr-Grenzhausen	50°26'12.8"N 7°40'46.7"E	*L. hirta*
July, 2019, 2020	Rheinland-Pfalz, Germany	Höhr-Grenzhausen	50°26'26.4"N 7°40'50.2"E	*L. hirta*
January, March	São Paulo, Brazil	Corumbataí	S 22.1838889° W 047.6480556°	*L. villosa*
January, 2015	São Paulo, Brazil	Itaju	S 21.9816667° W 048.8288889°	*L. villosa*
March, 2015	São Paulo, Brazil	São Carlos	S 21.7088889° W 047.9194444°	*L. villosa*
March, 2015	São Paulo, Brazil	Boraceia/Bariri	S 22.2346500° W 048.8073667°	*L. villosa*

GENERAL DISCUSSION

Throughout chapters I – V, I shed light on different aspects of the symbiosis between *Lagria* beetles and their defensive bacterial inhabitants. With the discovery of antibiotic-mediated protection by cuticle-associated ectosymbionts on the larval and pupal surface, we elucidated a novel defense strategy for beetles during the vulnerable stages of molting and metamorphosis. By the examination of the dorsal cuticular invaginations that accommodate the symbionts in larvae and pupae, we could show that these organs enable uptake, release, maintenance, and transmission of the symbionts. Moreover, we found that *L. villosa* beetles harbor beside the already described variety of defensive *Burkholderia* strains also several non-*Burkholderia* symbionts including members of the genera *Acinetobacter*, *Luteibacter*, and *Variovorax* that may complement the protection of immature stages through different secondary metabolites or competitive exclusion.

Here, I aim to provide a synthesis of the discoveries in chapters I – V and discuss characteristics of symbiont and host that might be essential to sustain a defensive symbiosis in the long term. Within the sections, I will discuss the future perspectives and potential of the *Lagria* system to address questions relevant to host-microbe interactions in general.

1. The symbiont side: Being an effective defensive symbiont

Although insects have efficient defense strategies against various kinds of antagonists, these are often not fully available in immature life stages, symbionts can come handy for additional protection. One mechanism on how bacterial symbionts can defend their hosts is by producing antimicrobial compounds that can prevent or allay attacks from natural enemies [1]. In this case, the bacterium should ideally provide chemical weapons that are effective against relevant threats and increase the host's fitness in their presence, colonize the right tissue of the insect, and potentially compete or interact with other colonizing microorganisms, without being pathogenic or harmful to the host. How the symbionts of *Lagria* beetles meet those requirements and why specific strains dominate in a community of defensive symbionts shall be discussed in the following sections.

1.1. Symbiont-mediated protection of immature stages

Immature life stages of insects include the egg stage, for hemimetabolous insects often several nymphal stages and in holometabolous insects the larval and pupal stages. Host defense mediated by microbial symbionts during these stages is progressively acknowledged and investigated [2–7], although some stages remain understudied, such as the pupal stage.
In general, support in protection by microbial symbionts is advantageous when host defenses are lacking or insufficient. The primary barrier, especially against pathogen

infections is the cuticle, and although it is generally acknowledged that the cuticle is most effective once it is sclerotized and melanized [8–11], direct experimental evidence for this is scarce. During larval development, the effectiveness of the cuticle is reduced during molting phases, because the old cuticle is shed and the new one is not matured yet, making immature stages potentially more susceptible for antagonists [8,11,12]. One host mechanism to bridge this time gap is the production of immune effectors within the molting fluid [13,14], but this can be supported by cuticle-associated symbionts as described for *L. villosa* Chapter I. Many insects spend long periods of their life span as larvae and often molt several times, exposing themselves to pathogens in the environment. Protection during and directly after molting might thereby be particularly important, and the involvement of microbial symbionts in this process might be more widespread, especially among ectosymbionts, and should be investigated further across other ecdysozoans.

Another crucial point during insect development is the pupal stage, which is an essential stage that enables holometabolous insects to change from food gatherers to reproductive organisms. However, the general relevance of symbiont-mediated protection remains elusive due to very few studies considering this stage. And yet, many pupae might be especially in need for defending themselves, due to lacking behavioral and structural mechanisms that are often present in other stages, such as mobility or a strongly sclerotized cuticle[15]. In beewolves, it was shown that the late larval stage incorporates *Streptomyces* symbionts into their cocoon enhancing its insufficient structural protection with chemical substances to protect the developing insect [5,16]. These symbionts are placed by the beewolf mother into the brood cell, to be taken up by the late non-feeding larval stage [5,17,18]. In leaf-rolling weevils, also all life stages including the pupal stage benefit from the presence of a mutualistic fungus in the leaf cradle, which supports the behavioral defense strategy of creating the leaf-rolls [7,19]. Recently, it was shown that also a pupa of the leaf beetle *Chelymorpha alternans* is protected by an ectosymbiotic fungus *Fusarium oxysporum*, protecting the immobile stage against predatory ants [20]. With the protection of the *Lagria* pupae, these examples suggest the potential of symbiont-mediated defense also during metamorphosis, however, more studies are needed to evaluate their ecological relevance across insects. Likely, many insects do not solely rely on symbionts during metamorphosis but use their capabilities for defense to complement already existing host mechanisms, e.g. immune responses or behavioral strategies.

While the leaf beetle study shows the direct protection of the pupal stage by specifically removing the symbiont only from the examined life stage [20], the effect of pupal protection in *Lagria* beetles was less direct, since individuals were treated already in the egg stage Chapter I. Also, since male pupae lose a majority of their symbionts during metamorphosis in contrast to females, the relevance of symbiont-mediated protection particularly during pupation remains uncertain and needs surveys more specifically targeting this question Chapter II. Although a washing treatment to remove symbionts as carried out previously Chapter II might be gentler than using fungicides or antibiotics, it cannot be excluded that *Lagria* beetles developing from the differently treated eggs were already affected differently until pupation.

It would be therefore helpful to design an experimental procedure that removes the symbionts in the pupae or before pupation. Since the symbionts of male *Lagria* pupae only reside on the surface and not anymore in deep invaginations, treatment with antibiotics might be feasible. By this, the probability of fungal growth in treated vs. untreated individuals can be compared among males if the treatment does not affect pupal health significantly in the absence of fungi.

1.2. Effect of symbiont status on life history traits of *Lagria* beetles

L. villosa and *L. hirta* harbor multiple defensive symbionts and their protective benefits for the host were shown for different life stages via bioassays [4,21, Chapter I, Chapter IV, Chapter V]. By those studies, also different life history parameters were collected in the absence or presence of fungal pathogens and in individuals infected with natural symbiont community or single-strain-infections, such as (Figure 1 a, b). With those, I want to highlight the effect that defensive symbionts can have on their host under different contexts and in different life stages.

The increased larval and pupal weight (Figure 1 c) of symbiotic *L. villosa* compared to aposymbiotic individuals might suggest a nutritional benefit by the symbionts as previously discussed [22], although results of single-strain infected individuals rather indicate metabolic costs by the symbionts [22]. Decreased weight of aposymbiotic individuals could also represent an effect from missing symbiont-mediated protection against constant exposure to low levels of fungi in the rearing cages. This hypothesis would be in line with the observation of symbiont loss under laboratory conditions [23], the occurrence of smaller individuals, and the eventual collapse of the culture after a few generations. However, if being symbiotic is beneficial in the cages, symbiont maintenance should be selected for. It is therefore rather imaginable that other factors, such as nutritional deficiencies or abiotic factors influence symbiont loss regardless of the presence of fungi.

The clearest benefit for the host of harboring symbionts is visible in the egg stage of *Lagria* beetles. Eggs harboring the natural symbiont community or single strains of *Burkholderia* or other low-abundance symbionts are significantly less infected by fungal pathogens [4, Chapter IV, Chapter V]. Regardless of the symbiont status, unsuccessful fungal infection significantly increases the hatching rate of the larvae, when eggs were exposed to fungi [4, Chapter V]. When differentiating between aposymbiotic and symbiotic individuals, the outcome depends on the species and infected symbiont strains. In *L. villosa*, bioassays showed that the hatching rate is not decreased in aposymbiotic individuals, despite the higher probability of fungal infestation on the eggs, but causes higher larval mortality in young larvae hatching from those eggs [4]. On the other hand, a preliminary bioassay with eggs infected with low-abundance protective symbionts (*Acinetobacter*, *Variovorax*, and *Luteibacter*) shows that the symbiont status can also positively influence the hatching rate, which is also observed for symbionts of *L. hirta* [Chapter IV, Chapter V]. While in *L. villosa*, symbionts significantly improve larval survival in the presence of antagonists, but are not relevant in

the absence of antagonists Chapter I, preliminary assays with *L. hirta* suggest the opposite (Figure 1 b, c) [22]. It is however possible that these results especially of *L. hirta*, are obscured by the experimental conditions like the selected antagonist and non-suitable abiotic conditions, such as temperature, despite strong effects in the egg stage Chapter V. The antagonist was chosen by previous experiences with *L. villosa* due to the lack of knowledge on a known relevant entomopathogen. In fact, many of these assays were carried out or designed under single conditions, which can highly influence the outcome, and might not necessarily reflect the situation in nature.

1.3. Relevance of natural conditions for studying defensive symbioses

It is a general problem while studying defensive symbiosis, to prove the protective benefits of symbionts, especially in vivo [1,24,25]. Due to multiple variables that are harder to control or define like finding the relevant antagonist and its dosage, fine-scale timing of the experiment, mimicking natural parameters like temperature, humidity or pH, availability of antagonists in the lab, finding the correct localization or site, or including all partners of a multipartite symbiosis can be challenging for the experimental design. Since our experimental design in the *L. hirta* assays was lacking a known antagonist, we sought a more natural set-up including soil trying to assess whether the symbiont-mediated protection is relevant in seminatural conditions. By comparing aposymbiotic eggs with symbiotic eggs, we found no difference in the likelihood of fungal growth on the eggs (Figure 1 e). However, the level of fungal growth on the egg surface was significantly lower on symbiotic eggs on day 4 of the experiment (Figure 1 f). In another experiment (data not shown) that was performed in nature, with the access of potential predators and natural weather conditions, unfortunately almost no eggs could be found after three days of observation.

It is desirable and important to mimic natural conditions especially to show the ecological relevance of symbiosis and also to select relevant antagonists [25], however it might not always be feasible in practice. The *Lagria-Burkholderia*-symbiosis might yet be a candidate system to also explore its ecological relevance in the field. Especially *L. villosa* might be suitable for large-scale experiments due to its high abundance on soybean plantations and its multivoltine lifestyle enabling the monitoring of phenotypes across generations. The ability to manipulate the symbiosis from the egg stage, without interfering with the host's health, and to have multiple strains in culture is advantageous. Thus, placing aposymbiotic and symbiotic eggs in cages covering several soybean plants across a plantation or in the lab using multiple terrariums, can give insights into multiple questions in a natural or semi-natural set-up: How relevant are symbiont-mediated defenses under natural conditions? What effect do antagonists have on whole beetle populations? Can aposymbiotic populations be reinfected and stabilized by symbiotic individuals? Is a symbiont association selected for in environments without or low-abundance antagonists? Can the symbiont community be altered through environmental changes, such as differing antagonists? How quickly can new defensive symbionts be fixed or replace other symbionts in the population?

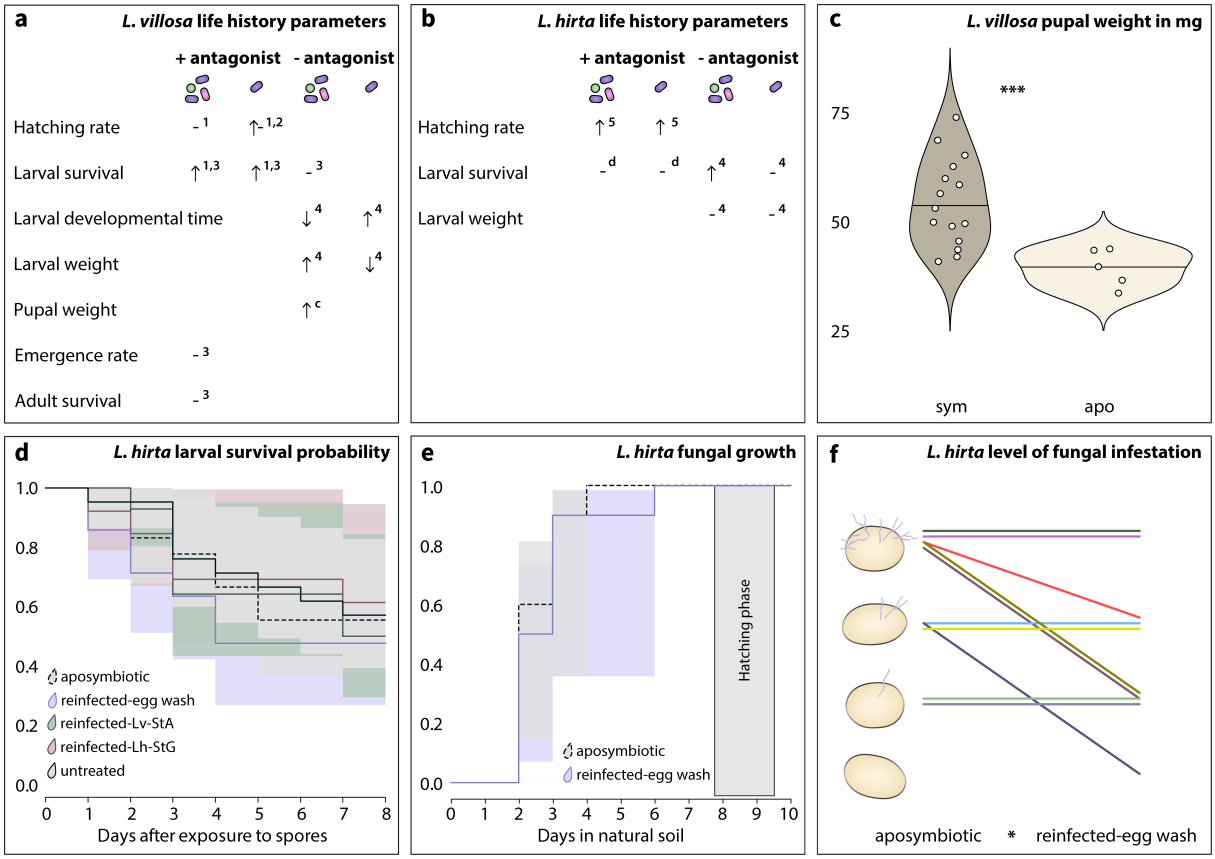

Figure 1: Effect of symbionts on different life history parameters of *L. villosa* and *L. hirta* in the presence and absence of antagonists. Different life history parameters of **a** *L. villosa* and **b** *L. hirta* collected under different conditions and compared to the aposymbiotic individual in the presence (+ antagonist) or absence (-antagonist) of fungal pathogens (including *B. bassiana*, *M. anisopliae*, *P. lilacinum*), and individuals infected with the natural microbiota (multiple cells) or single symbionts (single cells). Upwards arrows indicate an increase, downward arrows indicate a decrease, and hyphens indicate no difference. The presence of two symbols refers to symbiont-specific differences. Numbers and letters indicate study, chapter, or panel showing relevant data: 1 [4], 2 [Chapter IV], 3 [Chapter I], 4 [22], 5 [Chapter V]. **c** In *L. villosa*, female symbiotic pupae (sym) are significantly heavier than aposymbiotic (apo) pupae, when weighed alive. **d** In *L. hirta*, differently treated larvae (aposymbiotic n=18, untreated n=21, reinfected-egg wash n=,14 reinfected-Lv-StA n=14, reinfected-Lh-StG n=13) survive equally well, when exposed to the fungus *P. lilacinum*. **e** When reared in non-sterile soil, aposymbiotic and symbiotic *L. hirta* eggs have the same probability of visible fungal infestation. **f** On day 4, however, the level of fungal growth is significantly reduced in symbiotic eggs. Differently colored lines represent different egg clutches. Asterisks indicate significant differences between experimental treatments (Generalized linear mixed model fit by maximum likelihood, $\alpha \leq 0.05$).

1.4. The dominance of lagriamide-producing strains in the *Lagria* symbiont communities

The bacterial community of *L. villosa* beetles contains several strains of defensive facultative symbionts from at least four families (Burkholderiaceae, Moraxellaceae, Rhodanobacteraceae, Comamonadaceae), with different abundances, host dependences, and metabolic capabilities [Chapter I, Chapter IV]. Among the identified symbionts of *L. villosa*, the *Burkholderia* strain LvStB dominates the community in terms of abundance and infection frequency across life stages in natural populations [4,21, Chapter I]. Similarly, in *L. hirta*, LhStH dominates in adult females and on the eggs, while larvae are also highly infected with LhStG [Chapter V]. Here, I want to discuss why Lv-StB and LhStH might predominate in *Lagria* beetles, despite the frequent infection and defensive potential of other symbionts.

Tightly associated symbionts of insects often show signs of genome erosion, resulting in the loss of certain genes, and retention of genes necessary to keep the symbiosis running [26]. In addition, essential genes for a free-living lifestyle are lost due to relaxed selection inside the host and genetic drift because of smaller effective population sizes and bottlenecks during transmission [27]. In *L. villosa*, LvStB has a severely reduced genome with around 2 Mbp in comparison to other symbionts in the community, which is one-fourth of the genome size of its related *Burkholderia* strain Lv-StA (8.5 Mbp) [21]. Also, it seems to be the only known member of the community that underwent genome reduction by lacking major parts of its primary metabolism and DNA repair pathways [28], likely reflecting its tight association and increased host dependence. The genomes of defensive symbionts are usually larger and retain more complete metabolic pathways [29], however, besides LvStB, also symbionts of other insects have highly reduced genomes and dedicate even 15 % of their genome size to secondary metabolite biosynthetic gene clusters [30]. The gene cluster that was possibly the driver of the association with *Lagria* beetles encodes for the polyketide lagriamide, which comprises 4 % of the symbionts' genome and was horizontally acquired before the degradative genome evolution of Lv-StB [28]. Lagriamide contributes to the inhibition of fungal growth on the beetle's eggs, larvae, and potentially pupae, thereby increasing egg hatching and larval survival in pathogen-rich environments [21, Chapter I, Chapter V]. The acquisition and ability to produce lagriamide seems therefore to be an important trait for its host, and beetles harboring and transmitting lagriamide-producing strains vertically were therefore possibly selected in environments containing fungal antagonists [28].

Due to its evidence in two *Lagria* species [Chapter V], it would be interesting to assess the presence of lagriamide-producing strains across Lagriinae and related species, to gain further insights into its importance on the evolution and stability of the *Lagria-Burkholderia* symbiosis. Furthermore, it would be interesting to elucidate potential differences across localities or even populations, which might reflect adaptations to different antagonists as suggested for the antibiotic-producing symbionts of beewolves [31]. Therefore, comparing populations of *L. villosa* from Africa and South America on their symbiotic community and secondary metabolite profile could give insights into how symbioses adapt to local environments, since the beetles were introduced to South America around 50 years ago [32].

Defensive symbionts are usually not considered to be obligate for their hosts, since they do not take over essential functions, and *L. villosa* beetles survive equally well without their symbionts in the absence of antagonists (Figure 1 a) [22, Chapter I]. Nevertheless, the host significantly benefits from this association by having antimicrobial compounds produced to cope with natural enemies by their symbiotic partners. The benefits for the symbiont, however, are not very clear and easy to evaluate in general [33], but are assumed to include nourishment through host-derived nutrients, a safe space with reduced competition and predation, or the transmission to other environments, and the corresponding expansion of their niche [33,34]. In contrast to the host's perspective, symbionts do not need to benefit from the host to maintain the association, but in some scenarios, the host utilizes the microbe or its by-products, e.g. as a food source [35] or through facilitation [33], or by exploiting its

symbiont [36]. Thus, not all mutualisms from the host's perspective are mutualistic for the symbiont but might be better explained by the interaction termed inverted parasitism [33,37]. Considering that the lagriamide-producing symbionts are not yet culturable outside the beetle, and might not survive for a long time or reproduce outside their hosts [38], dispersal might not anymore be a beneficial service from the host, although it might have been beneficial in the onset of the symbiosis. Since they also share an environment with other symbionts and are as ectosymbionts also exposed to non-symbiotic microbes, competition for a certain niche might still take place. It was already speculated that lagriamide might not only act against fungal pathogens but could also act competitively against other microorganisms [28]. It is therefore conceivable that the host utilizes lagriamide for its purpose as a defensive agent against its natural enemies, while it was originally produced by the symbionts to fight competitors. Its dominance in the symbiotic community would advocate its ability to act competitively, while preliminary confrontation experiments among other symbiont strains suggested facilitative rather than competitive behavior of the other strains [Chapter IV]. However, competitive interaction between the strains may impact the early phases of host-symbiont interactions, which is the colonization phase. There, it can already be determined whether specific strains will dominate in the community. Whether lagriamide plays a role during host colonization, and which other symbionts and host factors are essential for the composition of a symbiont community including closely related strains, are important questions for this system and are yet to be determined.

Lagriamide shows effectiveness against the common pathogen *Aspergillus niger*, the generalist entomopathogens *Beauveria bassiana* and *Metarhizium anisopliae*, and the described natural antagonistic fungus *Purpureocillium lilacinum* [39] *in vitro*, supporting the observed effect of the *in vivo* antifungal assays against additional soil fungi, including *Trichoderma harzianum* [4,21, Chapter I]. This indicates that lagriamide might be able to defend against a broad range of fungal antagonists in the environment, which might underline its dominance in the symbiont community despite the presence of other antibiotic-producing strains. Lagriamide is also consistently produced in every life stage of the beetle, demonstrated by chemical extracts and MS imaging of the compound *in situ* [Chapter I], and its beneficial effect for the host was shown on eggs, young larvae, and pupae [21, Chapter I]. Whether lagriamide is indeed constantly produced or if its production is fine-tuned with specific time points during host development (e.g. higher lagriamide expression upon molting) in response to potential antagonists or colonization events, still needs to be determined.

Preliminary analysis of lagriamide gene expression throughout the early stages of the beetle indicates that lagriamide is among the higher expressed genes in early life stages of *L. villosa* (Figure 2). Lagriamide expression seems higher in late eggs and L1 than early L2, which might suggest that lagriamide production is upregulated in the potentially most vulnerable stages with lesser structural defenses of a melanized cuticle. The individuals collected as early L2 larvae, however, also did not have a fully melanized cuticle yet, but could potentially still benefit from the lagriamide-mediated effect of the L1 stage, after the release of the symbionts and the lagriamide they produced during molting. Some antimicrobial polyketides

derived from insect symbionts can be stable for up to several months if the conditions are suitable [40]. Moreover, it is not known yet, whether there is a time delay between lagriamide expression and the actual availability for the host. It would therefore be useful to know the stability and effectiveness of lagriamide after production and exposure to abiotic and biotic factors occurring in the environment. Since these samples were collected in the absence of any antagonist, it would be interesting regarding the mechanism of defense to collect an additional dataset in the presence of fungi, to evaluate if the production of lagriamide or any other compound of the community is triggered by an antagonist [24]. In the context of lagriamide as a putative competitive agent against other microorganisms, additional coinfections with other bacteria could be of interest, to find out whether it can act against other bacteria. As an alternative, this dataset could be further explored concerning the presence of strains and expression of the secondary metabolite gene clusters, since mechanisms and mediators can be otherwise challenging to elucidate [24], such as for the other so far unculturable symbionts. This might give insights into potential interactions between the community members but would also shed further light on the importance of the lagriamide-producing strain in this system.

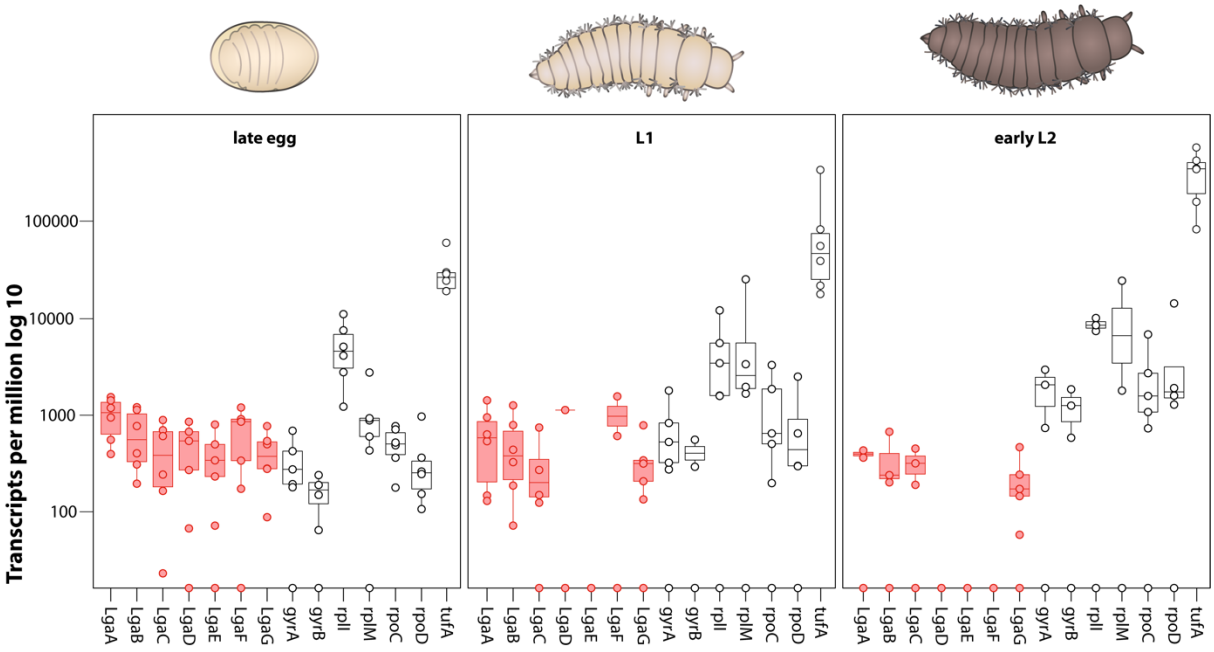

Figure 2: Normalized transcript-level expression of lagriamide and selected genes across early *L. villosa* life stages. Transcripts per million (TPM) derived from RNASeq for lagriamide genes (red) in comparison to selected genes (white) with high TPM values that are usually considered housekeeping genes among bacteria. *gyrA*: DNA gyrase subunit A, *gyrB*: DNA gyrase subunit B, *rplI*: 50S ribosomal protein L9, *rplM*: 50S ribosomal protein L13, *rpoC*: DNA-directed RNA polymerase subunit beta, *tufA*: Elongation factor Tu.

Although the lagriamide-producing symbionts substantially impact their hosts' ecology, they are not the only symbionts providing this benefit. In fact, the close relatives Lv-StA and LhStG can produce multiple antimicrobial compounds and are also capable of defending immature stages against pathogenic fungi [4, Chapter I, Chapter V]. But why does especially LvStA only sporadically infect natural host populations [21, Chapter I], and LhStG seems to be only equally abundant as LhStH in the larval stage [Chapter V] if they could both defend their hosts?

One explanation might be that those strains might face lesser selection pressures on staying associated with their hosts since they can still live outside the beetle and benefit from their ability to escape the host [4,28,38]. LvStB and LhStH may be maintained because of their reduced genomes through the selection for host control mechanisms compared to symbionts with bigger genomes, such as Lv-StA and Lh-StG. Yet, the different strains might also vary in their costs for the hosts. It is, therefore, possible that the small genome strains demand more nutritional input from the host because of their reduced metabolic potential, and it is less costly to maintain strains that have potentially fewer nutritional demands, such as LhStG or LvStA [Chapter V]. Those differences in costs could, however, outweigh the disparity between the varying levels of defense by the two strains. On the other hand, LvStB and LhStH might be cheaper for the host, since they carry less genetic and metabolic burden due to their genome streamlines for defensive compound production. In addition to nutrition from the host, immunity can also play part in costs to the host, which might be higher for the free-living strains if they are not recognized by the host as the right symbiont. This might have led to our observations that the genome-eroded strains generally dominate in *Lagria* beetles, although whether this is cost-dependent needs further validation. Generally in defensive symbioses, the benefits of carrying a symbiont can be context-dependent, and the costs might outweigh the benefits under certain conditions, ultimately leading to fluctuating symbiont infection frequencies, replacements, or losses of symbionts [41,42]. Larvae infected only with Lv-StA showed signs of higher costs for the host, through longer larval developmental time and decreased weight compared to untreated individuals (Figure 1 e) [22]. It is albeit also imaginable that LvStA and especially LhStG are just in the beginning stages of becoming symbionts with higher host dependence. To test whether LhStG shows genomic signs of increased host association, detailed analyses of the genome are needed to find potential signs of degradation and reduction such as pseudogenes, mobile elements, deletions, and chromosome rearrangements [26]. Although speculative, LhStG might be able to eventually replace LhStH under conditions, where defense by particularly lagriamide is not needed but other defenses are still necessary. Replacements or substitutions of small genome-symbionts by novel symbionts with more metabolic capabilities are not unusual to escape potential degradation of symbiont functionality, even in intimate symbioses [43–45]. In a more open system, like in *Lagria*, where symbionts are localized on the outside with the potential for horizontal acquisition, and the presence of multiple strains, might favor symbiont replacements [1]. LhStG can be frequently found in individuals and populations and is maintained in the host, even when other strains like LhStH are lost under laboratory conditions [23, Chapter V]. It is possible that due to its ability to live outside the host, LhStG can adapt better to varying environments, including changes in the host under laboratory conditions.

2. The host side: Providing an ideal place for defensive symbionts

Insects have evolved an incredible diversity of morphological adaptations to accommodate their symbiotic partners and to ensure their establishment and maintenance [46–48]. In defensive symbioses, symbionts can be found in many localities of a host organism, but to perform specific functions for the host and against specific natural enemies, certain localizations might be more favorable than others [1]. Whether an association between host and symbiont can persist long-term is determined by specific attributes from both partners. From the host side, the location should fulfill specifications enabling the symbiont to settle, reproduce, and deliver its beneficial functions. In the next section, I discuss the variety of symbiont localizations in defensive symbioses in relation to their antagonists and which advantages the symbiont-bearing structures of *Lagria* beetles bring for their hosts.

2.1. Host antagonists and symbiont localizations

The main natural enemies of insects are predators, parasites, parasitoids, and pathogens, including bacteria and fungi [1]. Among different insect orders, different microbial symbionts were found to be effective against all these antagonists. Thereby is it important to consider by which means symbionts can defend, such as through competitive exclusion or the production of antimicrobial compounds, but also where the symbionts are localized [1]. It has been suggested that certain localizations are more effective for certain ways of protection, e.g. an external localization of antibiotic-producing symbionts can guarantee direct exposure of symbiont-produced antimicrobial compounds to the antagonist [1,49]. External localizations are the cuticle surrounding all regions of the insect's body [5,6,50,51], cuticular invaginations or crypts across the surface [5,7,51–55], but also the gut [56–62] and regions not physically connected to the insect, like the food sources [61,63,64]. Symbionts residing in these tissues are here called ectosymbionts. Endosymbionts are on the other hand localized inside the host, often intracellularly in bacteriomes [30,65].

Fungal pathogens generally infect the insect by penetrating the integument, meaning they need to breach the cuticle as a first obstacle. Entomopathogens usually can infect anywhere on the cuticle [66], including the gut [67]. It is therefore beneficial for antifungal symbionts to be localized on the outer cuticle of the body or in the gut to fend off fungi before they attach and germinate [1]. This strategy can be observed among many described defensive symbioses, arguably also because many studies were done on a few prominent systems such as fungus-farming ants [68] and termites [69]. Nevertheless, records of antifungal ectosymbionts are found across six insect orders (Blattodea, Coleoptera, Diptera, Hymenoptera, Lepidoptera, and Orthoptera), and appear in strikingly diverse manners. Antifungal ectosymbionts can be found in the domains of bacteria or fungi, can be carried by the host in specialized structures on the body, reside on the host's surface, or be deposited in the environment [1]. Fungal pathogens are a ubiquitous natural enemy exerting strong selective pressures on insect populations [70], which likely lead to multiple independent associations in the evolution of

ectosymbionts among insects as an important and successful defense strategy for insects. By contrast, antifungal protection by endosymbionts appears to be rare and is so far only described for the facultative symbionts of aphids [71] and as a secondary effect for the nutritional symbiont of the grain beetle *O. surinamensis*. There the symbiont improves the overall health of its host by aiding in cuticle biosynthesis, a process that is considered little within defensive symbiosis research [8]. Hence, it is possible that many primarily nutritional symbionts also influence host protection, especially if they are involved in cuticle biosynthesis [8].

For entomopathogenic bacteria, an important route of infection is ingestion, from where they can compete for limited resources inside the gut or disrupt and breach the midgut epithelium and eventually proliferate in the hemocoel, leading to deadly infections inside the host [72,73]. But bacteria can also interfere with the host as competitors of beneficial symbionts without directly harming the host, suggesting competitive exclusion as another important strategy for defensive symbionts to ensure homeostasis, which often occurs in the gut [74]. Defensive ectosymbionts fighting against bacteria have also been described in at least four insect orders (Coleoptera, Hemiptera, Hymenoptera, Lepidoptera), while evidence for antibacterial endosymbionts is limited to a hemipteran whitefly [75]. In *Bemisia tabaci*, *Rickettsia* symbionts, which are known to protect against fungi in pea aphids [71], are not exclusively defensive, but provide other benefits such as increased host fitness or tolerance against heat stress [76,77].

While the relevant antagonists of ectosymbionts seem to be bacterial and fungal pathogens, evidence for ectosymbionts acting against other natural enemies such as parasites or parasitoids is rather scarce (Figure 3). Contrary, parasites and parasitoids might be rather opposable by endosymbionts, as the currently available studies suggest (Figure 3). In fact, endosymbiotic microbes acting against parasites and parasitoids were found in Coleoptera [1], Diptera [78–80], and Hemiptera [65]. By contrast, ectosymbionts aiding in defense against parasites were shown for *Dysdercus fasciatus* (Hemiptera) [62] and *Bombus* (Hymenoptera) [81], and were so far not identified to act against parasitoids. Parasitoids infect insects by laying eggs on or inside their host, often suppressing the immune system so the offspring can develop inside the host [82]. While hosts themselves can fight against parasites through encapsulation or melanization [83], symbiont-mediated defense against parasitoid attacks can occur e.g. via bacteriophages [71] or other possible mechanisms [65,84]. By this, it can be advantageous to harbor defensive symbionts internally, which can induce toxins or support host defense mechanisms such as the immune system. Yet, studies involving mechanisms on symbiont defense against parasitoids and parasites are rare, and it remains therefore speculative if symbiont localization matters against these antagonists.

Similarly, evidence of symbiont-mediated protection against predators is very limited and was until recently only demonstrated for *Paederus* beetles [85]. In the last year, however, two more studies on Coleoptera confirmed the ability of microbial symbionts to tackle predatory attacks. Again, the endosymbiont of *O. surinamensis* was shown to provide defense against predatory wolf spiders, by improving the structural protection of the cuticle [8], while the

ectosymbiotic fungus protects the pupal stage of the leaf beetle *C. alternans* against ant predation by so far unknown mechanisms [20]. Interestingly this symbiont also seems to be a plant pathogen, similar to the ancestors of the *Lagria* symbionts [4]. Whether this trait is common among symbionts of herbivorous insects would be interesting to investigate.

For most of the natural enemies of insects, there is still not enough evidence to pinpoint whether a localization inside or outside the host is more advantageous than the other. Moreover, as discussed above, it is often difficult to identify relevant antagonists and to mimic ecologically meaningful scenarios experimentally. Yet, among insect pathogens, the localization on the host's surface might be effective regardless of their mode of action, also because anti-pathogenic ectosymbionts can be found among all insect orders that are described to harbor defensive symbionts (Figure 3).

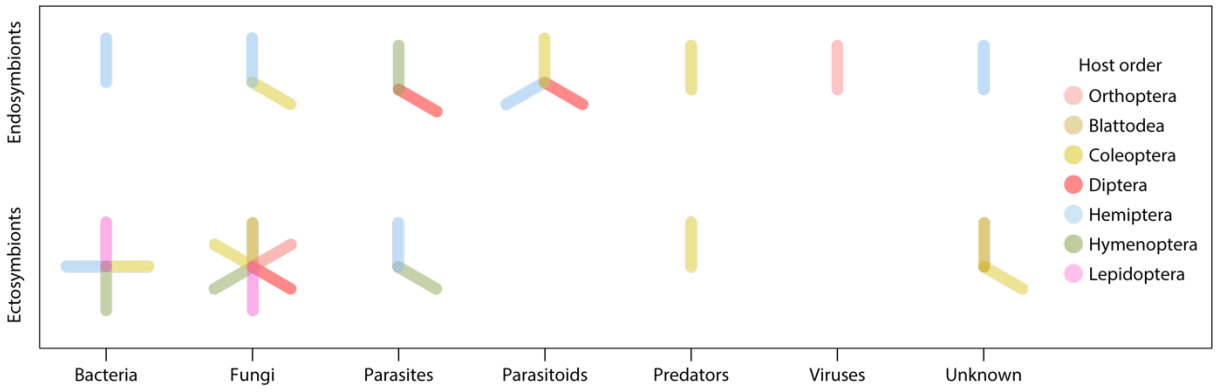

Figure 3: Correlation between symbiont localization and antagonist in defensive symbioses of different insect host orders. Each petal of the sunflower plot indicates if at least one incident was found among differently colored host orders for the different localizations (ectosymbionts, endosymbionts) and antagonists (bacteria, fungi, parasites, parasitoids, predators, viruses, unknown). This representation might not comprise a complete list of defensive symbioses.

2.2. Peculiarity and functionality of the symbiont-bearing organs of *Lagria* beetles

The ectosymbionts of *Lagria* beetles act effectively against a variety of fungal pathogens [4,21, Chapter I, Chapter IV], but their ability for fighting bacterial pathogens or outcompeting commensal bacteria is still unknown. Their efficiency of protecting their host against fungal infections might be facilitated by the host itself, through the special localization where the symbionts are accommodated. As in many symbioses, also in *Lagria* beetles it remains elusive what the host provides to support the growth of the symbiont. Here, I want to discuss potential advantages that both partners gain from each other and how the association is maintained through structural adaptations by the symbiont's localization in cuticular invaginations (Figure 4 a).

First, through the opening of the structure to the outer surface, symbionts can enter the organs, which is an important site for the colonization and establishment of a symbiont population (Figure 4 b). Thereby, vertical transmission is guaranteed from the egg stage, but since the structure remains open throughout larval development, horizontal acquisition is

also possible (Figure 4 c) [38]. Through that same opening, the symbionts can also be released, which might not only benefit the host for defense (Figure 4 b) [Chapter I], but could also allow the symbionts to leave the host (Figure 4 c). Since parts of the symbiont population are released during every molt onto the exuvia and larval surface, it might help symbionts to be dispersed into new environments or other hosts, including plants [38]. The narrow, but open channel could also aid in filtering specific symbionts and for creating a bottleneck during colonization [Chapter III]. Hosts require certain mechanisms to selectively incorporate, accommodate and maintain specific symbiotic partners, while nonsymbiotic microorganisms are excluded [86,87]. For this, structural modifications of the host can be useful and have been described e.g. in bean bugs [88], turtle ants [89], or bobtail squids [90]. Symbiotic organs can either respond to symbiont presence and are phenotypically plastic, or they are canalized , meaning they develop regardless of symbiotic stimuli [91]. The organs of *Lagria* beetles develop also in absence of the symbionts [38] and can be colonized by a community of symbionts [4, Chapter I, Chapter IV]. However, how the dominant strains are favored during early colonization of the larvae [Chapter III] and female adults [Chapter II] and if host factors or structural adaptations contribute to this, remains yet to be determined.

Moreover, the pouch-like structure of the larval organs offers a spacious environment for extracellular symbionts and might also leave room for spatial distributions within the organ between different symbionts, although this was not observed regularly *in situ* within *Lagria* beetles (Figure 4 d) [Chapter IV]. Since the invagination is sunken into the host's body at the region of the intersegmental membrane and partially covered by the outer cuticle, it also provides a sheltered environment for the symbionts, although the opening of the structure would allow the invasion of other microbes (Figure 4 e). For the host, having the symbionts in a restricted compartment, unwanted and detrimental microbial infections of the whole body by potential pathogens are possibly reduced, but at the same time, beneficial symbionts can be maintained and released. Thereby the chemical defense is available even directly after molting [Chapter I]. Other exocrine reservoirs are often completely expelled during attacks or are shed off during molting and their content needs to be replaced, leaving the host unprotected for a certain period [92]. In contrast, *L. villosa* beetles can maintain most of the symbiont population even throughout molting, and only parts are expelled and lost onto the exuvia, allowing for continuous protection and symbiont maintenance [Chapter I, Chapter III]. How exactly the symbiotic organs of larvae are kept intact during molting is yet not clear. In general, every part of the insect's cuticle is renewed during molting, which would theoretically mean that the symbiotic organs of *Lagria* beetles are either no longer cuticle lined, or the cuticle lining is disrupted and/or digested, shed off, or remain and accumulate layered one into another as described in a phasmid species [93]. We never observed the latter two [Chapter I], but histological comparisons of the symbiotic organs before and after molting suggest that the cuticle inside the organs is much thinner in comparison to the outermost cuticle (Figure 5). In addition, we see that a fine structure inside the organ detaches from the epithelium before molting when the new cuticle can be seen in other parts of the insect. This might

indicate that the cuticle inside the organ does indeed molt, but could be digested and reabsorbed, preventing the expulsion of the whole symbiont structure (Figure 4 f).

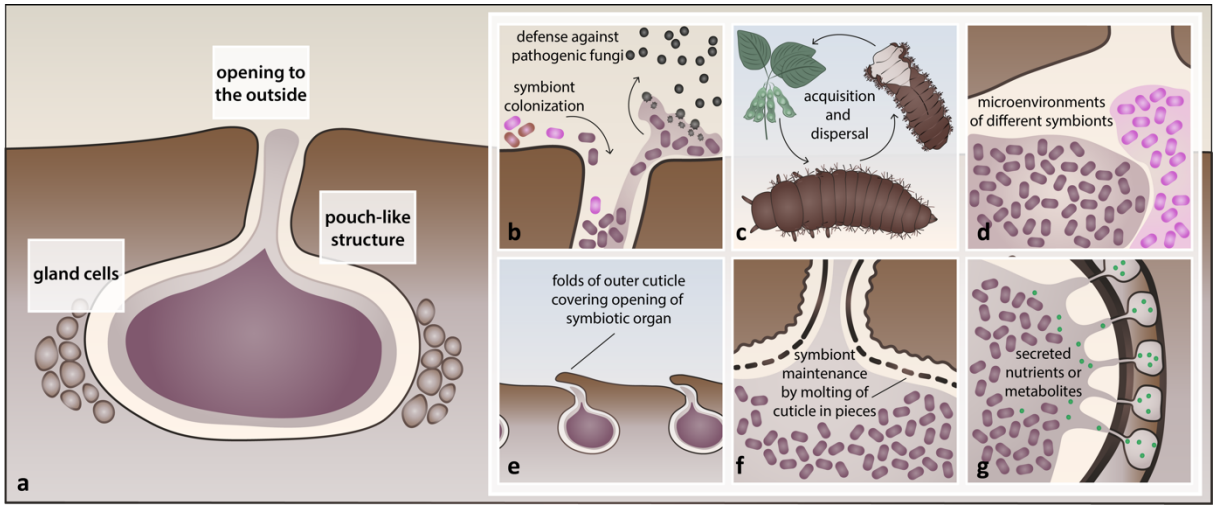

Figure 4: Morphological features of the dorsal symbiotic organ of *Lagria* larvae and their implications for host-symbiont interactions. a Simplified illustration of a transversal section through one dorsal symbiotic invagination with the symbiont population shown in purple. **b** The opening of the organ through a channel enables symbiont colonization and release for antifungal defense. **c** By this open system, horizontal acquisition and symbiont dispersal are likely possible. **d** Through the size and potential for symbiont acquisitions, the possibility for microenvironments inside the pouch is imaginable. **e** Sagittal sections elucidate that the organs are located between the segments and are sheltered under the outermost cuticle. **f** The cuticle inside the pouch likely does not shed as a whole, preventing complete symbiont loss during molting. **g** Gland cells flanking the organs putatively secret into the organ, possibly providing nutrients for the symbionts.

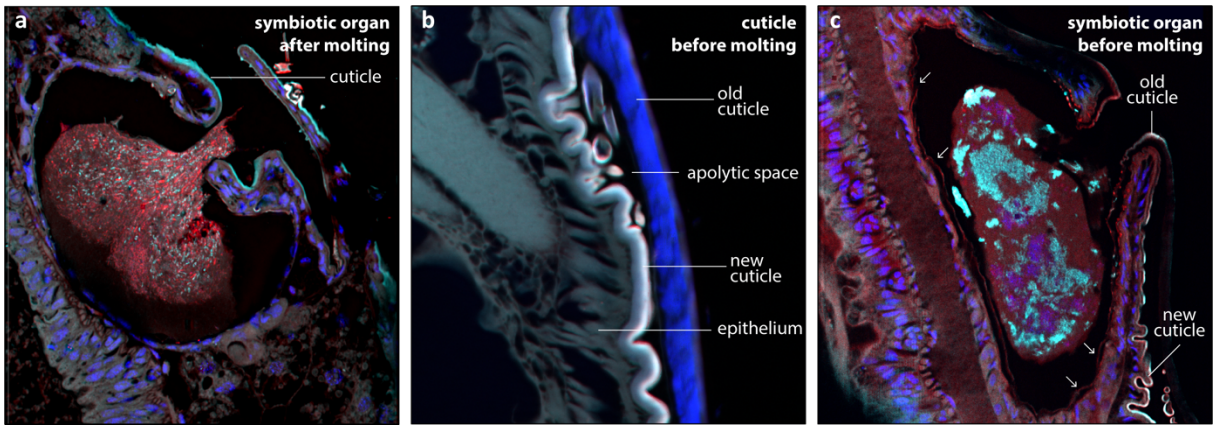

Figure 5: Cuticle formation inside the symbiotic organs after and before molting. a FISH on a symbiotic organ of a larva after molting. Autofluorescence of the cuticle is shown in cyan indicating a thicker cuticle outside of the organ compared to an almost invisible cuticular lining inside the organ. **b** Section through a non-symbiotic cuticular tissue of a larva before molting. The wrinkled new cuticle (white) is secreted from epithelial cells and is separated from the old cuticle (blue) through an apolytic space. **c** FISH on a symbiotic organ of a larva before molting. The old and new cuticles can be identified outside the symbiotic organ. Inside the symbiotic organ, the old cuticle appears thinner and partly separated from the epithelium and new cuticle (arrows).

Another advantage of the symbiotic organs might be a connection to gland cells and a potential glandular origin of the invagination (Figure 4 g). These glands are thought to secrete nutrients or metabolites into the organs and probably play a role in maintaining symbionts, protecting their inhabitants from desiccation, providing nourishment, and also in controlling the composition of symbiont species, and ensuring their specificity inside the organ [18,94–98]. Several systems where chemicals are used for antimicrobial protection consist of or are associated with gland cells, such as antennal gland reservoirs of beewolves [18,98],

crypts with associated exocrine glands in ants [51,99], or accessory nidamental glands of the bobtail squid [100]. Also, mycangia of the bark beetle *D. frontalis* contain glands that are thought to nurture inhabiting fungi through secreted substances [96,101]. For *L. hirta,* gland cells were described to be located on both sides of the organ underneath the epithelial cells, increasing in number while larvae develop [53]. They are connected with the symbiotic organ through little intracellular secretory channels [53]. Big gland cells can also be found laterally in the *L. villosa* organs (unpublished data), although their structure and connection to the symbiotic organ have not yet been examined. These secretory glands could be necessary to deliver the secretion by which the symbionts are nourished and maintained. By the secretion of certain nutrients or metabolites, the host can select and filter for specific bacteria that are allowed to colonize and remain in the structure, while others might be excluded (Figure 4 g). If the composition of the secretion is altered, e.g. through environmental influences such as restriction of the host diet under lab rearing conditions the host might not be able to provide or allocate certain nutrients for the symbionts any longer, leading to the loss of certain symbionts [23]. Whether and which nutrients the host might provide for the symbionts and if they are relevant for symbiont maintenance is however still unknown. Although very specific, generating beetles via RNAi knockdown of those particular gland cells and comparing their symbiont titer and composition to normal beetles could give valuable insights into their role in symbiont nourishment or symbiont selection.

Although the symbiotic invaginations are structurally similar between the two studied *Lagria* species, small morphological differences might result in functional differences. Preliminary examinations of the *L. hirta* larval organs did not show a clear opening of the structure to the outer surface (data not shown), in contrast to the wide channels and symbiont flux to the outside in *L. villosa* (Figure 5 a) Chapter I. It is imaginable that the larval organs of *L. hirta* do not release their content and serve a protective function, as already indicated by preliminary bioassays against the fungus *P. lilacinum* (Figure 1 d), but rather serve another function associated with diapause while overwintering as larvae. An enclosure of the symbionts during diapause might allow the host to maintain the symbionts, even when its metabolism is reduced, and it might not be able to allocate many resources for the symbionts. Whether both symbiont and host undergo a dormant stage during the colder months, and whether diapause or the closure has an effect on the microbial community composition or their provided functions [102] would be interesting to assess in this species since it might explain observed strain coexistence and varying symbiont abundances in different life stages Chapter V.

The larval organs of *L. hirta* were described to be lined by cuticle, whose thickness is reduced by every molt [53], while this was not studied in *L. villosa* yet. Although speculative, this could indicate that the organs of *L. hirta* are gradually more integrated into the host's body during larval development and are detached from the cuticle lining of the outer cuticle. Upon pupation, the organs might then dissociate inside the developing pupa, explaining the presence of symbiont cells in the male pupal gut of *L. hirta* [23]. However, we could not detect anything like that in our initial studies of male *L. hirta* pupae Chapter II. In female *L. hirta*

pupae, no symbionts could be found inside the gut, but in a dent of the dorsal thorax Chapter III, Chapter V, which would not speak for an integration of the organs, but rather for a complete discharge of the organ onto the surface before pupation. Although unlikely, the observed sexual dimorphism in the pupal stage Chapter III could already start earlier in larval development, leading to structural differences in female and male *L. hirta* larvae. Thorough histological analyses in combination with identifying sex-specific gene expression or presence [103] could resolve potential differences in the development of female and male symbiotic organs.

Another apparent difference between the larval organs of the two *Lagria* species is their size (Figure 6). While the larval organs of L1 in *L. villosa* have a width of ~ 100 µm, in *L. hirta* they are only one-fourth of the size with a width of ~ 25 µm, although *L. hirta* L1 larvae are equally sized or even slightly bigger. The size of the organs also reflects differences in titers of the defensive reduced-genome symbiont (LvStB titer in *L. villosa*: ~ 10^6 cells/L1 Chapter I, LhStH titer in *L. hirta*: ~ 10^5 cells/L1 Chapter V). This could suggest that *L. villosa* larvae are better protected by their symbionts than *L. hirta* larvae, disregarding possible differences in the amounts of produced defensive compounds by each symbiont, or their effectiveness and longevity. If this however could also indicate that risks of infection and the need for symbiont-mediated defense are more relevant in *L. villosa* would needs further examination. Moreover, whether morphological differences and symbiont titers in the symbiotic structures in larvae correlate with their role for defense in this particular stage would be another interesting point to analyze in a more extensive study including multiple *Lagria* species.

Overall, the peculiar symbiont-bearing organs of *Lagria* larvae deserve further attention to understand their origin and evolution. Although ambitious, it would be interesting to examine their presence and structure across several *Lagria* species and outgroups, comparing morphology, and their content by several omics techniques, to find out how widespread this adaptation for defense is, and if it is a specialty for *Lagria* beetles.

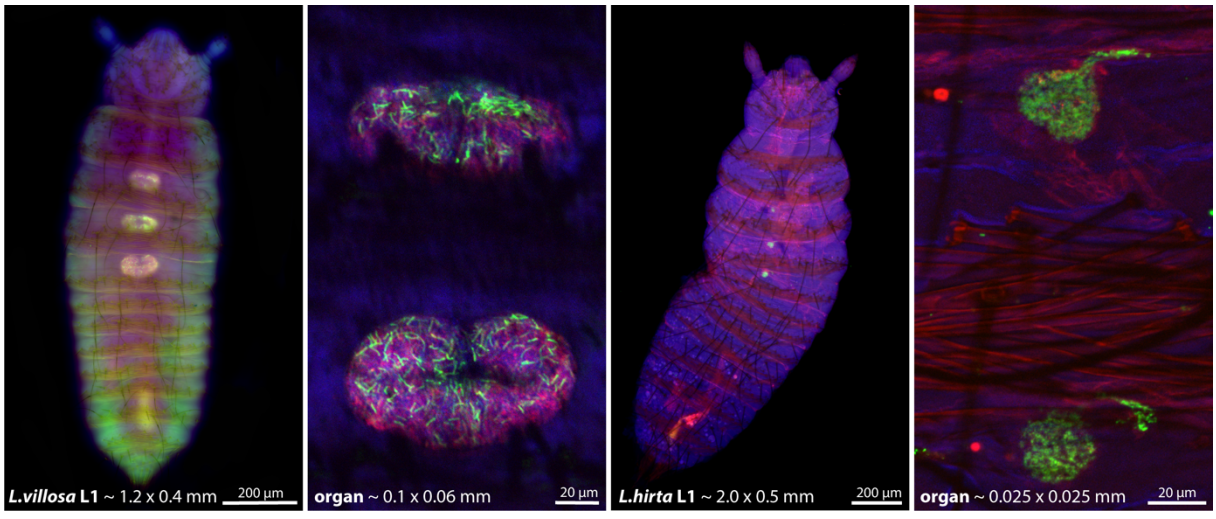

Figure 6: Size of symbiotic organs in comparison to body size in L1 larvae of *L. villosa* and *L. hirta*. FISH on whole L1 larvae hatched from field collected beetles. Sizes of the larval body and symbiotic organs were estimated by measuring the area from the coronal view using the scale bars. In the symbiotic organs, symbionts are shown in red and green, and host nuclei are shown in blue.

3. Conclusions

Defensive symbionts are widespread in insects [1,24,104,105] and their involvement in protection of immature life stages is increasingly acknowledged and investigated [2–7,20]. It is remarkable that the ectosymbionts of *Lagria* beetles are maintained across host development and are consistently associated and protective to the beetle, despite multiple molting events and metamorphosis, without the need of being translocated and stored in the environment [Chapter I, Chapter II]. This is facilitated by the peculiar pouch-like organs of the larvae and pupae, which are invaginations of the cuticle but are not shed off during molting. This enables extended protection for the host, which has not been observed in this manner for other defensive symbioses. Also notable is the presence and occasional coexistence of different defensive symbionts including multiple closely related symbiont strains, without clear signs of competition [Chapter I, Chapter IV, Chapter V]. The combination of high-abundance and tightly associated strains with low-abundance and lesser host dependence is likely a strategy of the host to retain flexibility in varying environments and antagonists, while at the same time yielding stability from consistent partners. Strikingly, these symbionts are also able to use different transmission modes [38] and colonization strategies [Chapter III], which might be reliant on their motility or host-dependence. Together, these points build a foundation for further questions, which are relevant also in other symbiotic systems, such as (i) which factors are necessary for individual symbiont establishment, (ii) how did the symbiont-bearing organs evolve, (iii) how is the diverse symbiont community established, maintained and controlled (iv), what is the ecological relevance of symbiont-mediated defense under natural conditions, (v) is the presence of certain symbionts or compounds phylogenetically congruent with their hosts? Answering these questions is essential to understand better the evolution and ecology of the multipartite defensive symbiosis in *Lagria* beetles and will also broaden the perspective of host-symbiont interactions in general. Especially the molecular machinery of host and symbiont underlying symbiont establishment and maintenance are only well-understood in a few systems but is essential to understand how symbionts but also pathogens can associate with a host. Unraveling the relevance of symbionts for host defense will advance the understanding of defensive symbioses in animals but could also give valuable insights into dynamics of ecosystems in general and might even be useful for controlling and stabilizing agricultural and nature preservative areas.

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“Die Welt ist es wert, um sie zu kämpfen”

Robert Marc Lehmann, Mission Erde

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CURRICULUM VITAE

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Education

2022 – now	**Postdoctoral researcher.** Max Planck Institute for Marine Microbiology, Bremen, Germany. In the lab of Prof. Dr. Nicole Dubilier, Symbiosis
2017 – 2022	**Doctoral student.** Johannes Gutenberg University of Mainz, Germany. In the lab of Prof. Dr. Martin Kaltenpoth, Evolutionary Ecology Supervision: Dr. Laura Flórez, Prof. Dr. Martin Kaltenpoth
2014 – 2017	**M. Sc. Biology.** Johannes Gutenberg University of Mainz, Germany. Master thesis in the lab of Prof. Dr. Martin Kaltenpoth, Evolutionary Ecology
2010 – 2014	**B. Sc. Biology.** Johannes Gutenberg University of Mainz, Germany. Bachelor thesis in the lab of Prof. Dr. Carsten Duch, Neurobiology

Teaching and Work Experience

2017 – 2022	Johannes Gutenberg University of Mainz, Germany. **Doctoral student**, co-supervising Bachelor and Master students, and theoretical and practical courses
	Supervision of Bachelor theses
2020	Basic, S. Interspecific interaction and relative abundance of bacterial symbionts of *Lagria* beetles
	Supervision of Master practical projects
2021	Moog, S. Symbiont abundance and morphology of symbiotic organs in male vs. female *Lagria* pupae
2020	Ackel, J. Localization and defensive potential of non-*Burkholderia* symbionts in *Lagria villosa*
2019	Fetzer, E. & Krämer, N. Symbiont-mediated defense of young *Lagria villosa* larvae against fungal growth
2018	Hinkfoth, F. Molecular characterization of symbionts from two European *Lagria* beetle species
	Supervision of Bachelor and Master practical courses
2018 – 2022	B. Ed and B. Sc practical course. Camouflage, mimicry and aposematism
2019	M. Sc theoretical and practical course. Introduction into FISH
2013 – 2014	Johannes Gutenberg University of Mainz, Germany. **Student assistant** in B.Sc. practical course. Animal Physiology

Complementary Education

2020	**Workshop.** Thinking Your Way Into Research Articles by Ian Patten. University of Mainz
2019	**Course.** Intercultural Communication. General Postgraduate Program, University of Mainz
2019	**Workshop.** Presenting in English. General Postgraduate Program, University of Mainz
2019	**Workshop.** What to Do with a PhD? Professional Orientation for PhD Students. General Postgraduate Program, University of Mainz
2019	**Workshop.** Wissenschaftliches Schreiben für Peer-Reviewed Journals. General Postgraduate Program, University of Mainz
2018	**Workshop.** CV-Workshop. General Postgraduate Program, University of Mainz
2018	**Workshop.** Project Management. General Postgraduate Program, University of Mainz
2018	**Workshop.** Academic Writing in English - Natural Sciences, Mathematics, Psychology. General Postgraduate Program, University of Mainz

Publications

Peer-reviewed

2022 Janke R.S., Kaftan F., Niehs S.P., Scherlach K., Rodrigues A., Svatoš A., Hertweck C., Kaltenpoth M., Flórez L.V. Bacterial ectosymbionts in cuticular organs chemically protect a beetle during molting stages. ISME J.

2022 Janke R.S., Moog S., Weiss B., Kaltenpoth M., Flórez L.V. Morphological adaptation for ectosymbionts maintenance and transmission during metamorphosis in *Lagria* beetles. Frontiers in Physiology.

Other

2022 Janke R.S., Flórez L.V. Guardians of the progeny: How ectosymbionts protect molting life stages of a beetle against fungal infection. Nature Portfolio Microbiology Community. Behind the Paper

2020 Flórez L.V., Bauer E., Janke R.S. Hide-Warn-Mock. Educational app to illustrate trait evolution for pupils and students. Euba.github.io/EvoGame/

Conference Contributions

Oral presentations

2022 Symbiont-mediated defense during host development in the *Lagria-Burkholderia* symbiosis. International Congress of Entomology. Helsinki, Finland

2021 Symbionts in a pocket: Antifungal defense of molting life stages by bacteria housed in dorsal cuticular invaginations of a beetle host. Entomology 2021, The Entomological Society of America (ESA). Online participation

2021 Guardians of the progeny: bacterial epibionts chemically protect an insect's molting stages from pathogens. ICE Symposium. Max Planck Institute for Chemical Ecology. Jena, Germany

Poster Presentations

2021 Relevance of bacterial symbiont strains during development in a defensive symbiosis. Entomology 2021, The Entomological Society of America (ESA). Online participation

2019 Symbiont-mediated defense during host development in the *Lagria-Burkholderia* symbiosis. Gordon Research Seminar & Gordon Research Conference on Animal-Microbe Symbioses. Mount Snow, United States of America

2018 Defensive potential and specificity of symbiotic strains in *Lagria* beetles. Day of the Faculty. Faculty of Biology, Johannes Gutenberg University of Mainz. Mainz, Germany

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2017, 2018, 2020 **Travel grants.** Field work and research in Brazil.
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ERKLÄRUNG

gemäß § 13 Abs. 4 der Promotionsordnung
des Fachbereiches Biologie der Johannes Gutenberg-Universität Mainz

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Bremen, den 05. Oktober 2022