Host adaptation protects a defensive symbiont during vertical transmission in beewolf wasps (Hymenoptera: Crabronidae)

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„Ein Gelehrter in seinem Laboratorium ist nicht nur ein Techniker; er steht auch vor den Naturgesetzen wie ein Kind vor der Märchenwelt.“

Marie Curie

SUMMARY

Microbial symbioses are ubiquitous in insects, and present key drivers of evolutionary innovation. To stabilize a symbiosis over long evolutionary timescales, the symbiont must be reliably transmitted to the next host generation. Vertically transmitted extracellular symbionts commonly face prolonged periods outside of the stable host environment during transmission, entailing exposure to diverse biotic and abiotic threats. These threats should be exacerbated in insect symbionts of evolutionary ancient associations, which generally possess eroded genomes with reduced genetic inventories. While external transmission is widespread among the Hemiptera, Hymenoptera, Coleoptera and Diptera, the mechanism protecting the symbionts from environmental threats during transmission remain poorly studied.

In this dissertation, I employ the defensive symbiosis of the European beewolf *Philanthus triangulum* (Hymneoptera: Crabronidae) and the Actinobacterium '*Candidatus* Streptomyces philanthi' (henceforth *S. philanthi*) to investigate how a symbiont with an eroded genome copes with a host-derived exogenous challenge during vertical transmission in an evolutionary ancient symbiosis. Female beewolves provide their brood cells with a symbiont-containing secretion, from which *S. philanthi* is later transferred to the cocoon to protect the offspring from microbial opportunists by producing antibiotics. In the brood cell, this secretion is exposed to extremely high concentrations of toxic nitric oxide (NO) emitted by the beewolf egg, which effectively kills antagonistic microbes. How *S. philanthi* withstands the lethal burst of NO remained unknown.

I show that the symbiont's global stress response to NO is not sufficient to survive NO concentrations mimicking brood cell-level concentrations *in vitro*. Instead, I demonstrate that the symbiont-containing secretion consisting of long-chain hydrocarbons (HCs) forms an effective NO diffusion barrier around *S. philanthi*, and additionally contains host-derived protective enzymes. While different functions of an insect's HC profile can exert conflicting selection pressures on its composition, *in vitro* assays with beewolf-derived and synthetic HCs reveal that the NO diffusion barrier function of HCs *in P. triangulum* does not constrain the insect's multifunctional HC profile. My comparisons of HC profiles across different beewolf hosts suggest their suitability for NO protection, and *in vitro* assays with their respective symbionts indicate a widespread NO sensitivity. Given the shared ecology among beewolves, as well as additional reports on NO defense in *P. gibbosus* and *P. basilaris*, NO fumigation and the concomitant HC-mediated protection of the symbiont from NO is likely crucial across beewolves.

My findings add a novel dimension to the plethora of functions of insect HCs, and constitute one of the few examples of a host adaptation protecting a symbiont from a lethal threat during external vertical transmission. I therefore illustrate a mechanism by which a symbiotic association can be stabilized over long evolutionary timescales, an aspect essential to our general understanding of microbial symbiosis.

ZUSAMMENFASSUNG

Mikrobielle Symbiosen sind allgegenwärtig in Insekten und stellen eine treibende Kraft für evolutionäre Innovationen dar. Um eine Symbiose über lange evolutionäre Zeiträume zu stabilisieren ist es erforderlich, dass der Symbiont zuverlässig auf die nächste Wirtsgeneration übertragen wird. Vertikal übertragene extrazelluläre Symbionten verbringen während der Transmission oftmals längere Zeiträume außerhalb des stabilen Wirtsmilieus, wobei sie mit diversen biotischen und abiotischen Bedrohungen konfrontiert sind. Insektensymbionten in evolutionär alten Assoziationen sind aufgrund ihrer erodierten Genome besonders anfällig gegenüber diesen Bedrohungen. Die externe Symbiontentransmission ist zwar innerhalb der Ordnungen Hemiptera, Hymenoptera, Coleoptera und Diptera weit verbreitet, dennoch sind die Mechanismen, die die Symbionten während dieser Transmission vor Bedrohungen schützen, weitgehend unerforscht.

In dieser Dissertation untersuche ich anhand der evolutionär alten Verteidigungssymbiose des Europäischen Bienenwolfes *Philanthus triangulum* (Hymenoptera: Crabronidae) mit dem Actinobakterium '*Candidatus* Streptomyces philanthi' (nachfolgend *S. philanthi*), wie ein Symbiont mit einem erodierten Genom mit einer vom Wirt ausgehenden externen Bedrohung während der vertikalen Transmission umgeht. Weibchen versehen ihre Brutzellen mit einem Sekret, das die Symbionten beinhaltet. Die Symbionten werden zu einem späteren Zeitpunkt in den Kokon integriert, wo sie Antibiotika zum Schutz der Brut vor opportunistischen Mikroben produzieren. Das Sekret ist in der Brutzelle sehr hohen Konzentrationen toxischen Stickstoffmonoxids (NO) ausgesetzt, welches vom Bienenwolf-Ei freigesetzt wird und antagonistische Mikroorganismen effektiv abtötet. Bisher war unbekannt, wie die Symbionten den lethalen NO-Konzentrationen in der Brutzelle standhalten.

Ich zeige, dass die eigene Stressreaktion des Symbionten auf NO nicht ausreicht, um die *in vitro* simulierten NO-Konzentrationen der Brutzelle zu überleben. Jedoch bildet das aus langkettigen Kohlenwasserstoffen (KWs) bestehende Sekret, das die Symbionten umgibt, eine effektive NO-Diffusionsbarriere um *S. philanthi*. Weiterhin enthält das Sekret vom Wirt produzierte Schutzenzyme. Verschiedene Funktionen eines KW-Profils können gegensätzliche Selektionsdrücke auf seine Komposition ausüben; allerdings zeigten *in vitro*-Experimente mit Bienenwolf- und synthetischen KWs, dass die Fähigkeit, eine NO-Diffusionbarriere zu bilden, die Komposition des multifunktionalen KW-Profils des Bienenwolfes nicht einschränkt. Vergleiche von KW-Profilen verschiedener Bienenwolf-Wirte legen deren Eignung für eine NO-Barrierefunktion nahe, und *in vitro*-Experimente mit den zugehörigen Symbionten weisen auf eine weit verbreitete NO-Sensitivität hin. Angesichts der vergleichbaren Ökologie aller Bienenwölfe, sowie zusätzlicher Berichte über die Freisetzung von NO in *P. gibbosus* und *P. basilaris*, ist der Einsatz von NO zu Verteidigungszwecken und der damit einhergehende KW-vermittelte Symbiontenschutz wahrscheinlich von großer Bedeutung für alle Bienenwölfe.

Meine Ergebnisse erweitern die Fülle an Funktionen von KWs in Insekten um eine neue Dimension, und beschreiben eine der wenigen bekannten Wirtsadaptationen zum Schutz eines Symbionten vor einer lethalen Bedrohung während der externen vertikalen Transmission. Sie illustrieren damit einen Mechanismus, mithilfe dessen eine symbiotische Beziehung über lange evolutionäre Zeiträume stabilisiert werden kann. Dieser Aspekt ist wesentlich für unser Verständnis von mikrobiellen Symbiosen.

LIST OF PUBLICATIONS

Chapter 2 is published in Proceedings of the National Academy of Sciences

Ingham CS*, [redacted]*, [redacted], [redacted], [redacted], [redacted], [redacted]:
Host hydrocarbons protect symbiont transmission from a radical host defense

*both authors contributed equally to this work

Chapter 3 is published in Biology Letters

Ingham CS, [redacted], [redacted]:
Protection of a defensive symbiont does not constrain the composition of the multifunctional hydrocarbon profile in digger wasps

Chapter 4 is published in Current Opinion in Insect Science

[redacted], [redacted], Ingham CS, [redacted]:
Transmission of mutualistic bacteria in social and gregarious insects

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CHAPTER 1: General introduction

1.1 Symbiosis

In 1879, Heinrich Anton de Bary first coined the term "symbiosis" as the "living together of two organisms of different species" (1). Originally, the term did not differentiate between parasitic, ammensal, commensal and mutualistic interactions, but comprised all interactions along the parasitism-mutualism continuum (2). Traditionally, researchers have focused on parasitic interactions due to their significance for human health and general welfare (3). In recent decades, however, mutualisms have experienced a surge of research attention, as their pivotal role in the ecology and evolution of the involved parties has become increasingly clear (3-5). In fact, virtually all organisms engage with one or several mutualistic partners at least once during their life cycle (6). Microorganisms are universal mutualistic associates of eukaryotes (7), since they are metabolically versatile and abundant in terrestrial and marine environments (3, 8-10).

Contrary to the original broad definition of the term symbiosis by de Bary, various researchers have used symbiosis in a stricter sense, referring exclusively to mutualistic associations between organisms of different species (2). Similarly, I will use the term "symbiosis" to primarily refer to the intimate physical association of two or more mutualistic partners throughout this thesis. The bigger, or biggest of all partners, will henceforth be termed "host", and the smaller partner(s) "symbiont(s)".

1.1.1 Symbiosis as a major contributor to the evolutionary success of insects

The invertebrate class 'Insecta' (phylum Arthropoda) constitutes the most speciose animal class on earth, and researchers anticipate the discovery of many more species as the exploration of previously untapped habitats progresses (11). Their evolutionary success is corroborated by estimations of arthropods constituting 50% of the world's animal biomass (12), and insects contributing 17% of this portion (13). Moreover, insects shape ecosystems in their fundamental roles in plant biomass degradation (14) and pollination (15), but can also act as crop pests (16) and disease vectors (17). Therefore, insects directly and indirectly affect human welfare.

An important factor enabling the impressive ecological and evolutionary success of insects is the symbiosis of insect hosts with microbial symbionts (4, 18). These symbionts confer a plethora of benefits to their hosts (19-21), enabling them to spread to previously inaccessible niches (19).

The symbioses of insects and microbes are remarkably diverse in structure and function. Associations range from ancient to more recent in age, and from a tight to a less tight level of integration (22-24). In an obligate symbiosis, both partners have become interdependent and can no longer exist as separate entities (3). In facultative associations, both partners are still capable of surviving and reproducing individually (3). Intracellular symbionts reside within host cells (22), while extracellular symbionts colonize the host's body surface (ectosymbionts) or body cavities such as the gut or crypts (endosymbionts) (25).

Functions of insect symbionts in host nutrition have been well-documented. Supplementation of essential nutrients lacking in the host's diet, for instance B vitamins and essential amino acids in sap-sucking insects, allows insect hosts to thrive on nutrient-deficient food sources (21). Microbial symbionts also aid in the digestion of complex macropolymers like poylsaccharides of the plant cell well, and can detoxify otherwise harmful compounds in the host's diet (21). Therefore, nutritional symbionts often enable their hosts to exploit new food sources.

Other functions of microbial symbionts in insects include, but are not limited to, adaptive coloration (19), thermotolerance (19), and protection from pathogens, parasites and parasitoids (20). The latter can be achieved by various mechanisms. On the one hand, microbial symbionts can competitively exclude antagonistic microorganisms by occupying a niche that would otherwise be claimed by the antagonist (26). On the other hand, microbial symbionts can prime the host immune system to elicit an increased immune response when the host encounters the antagonist (27). Lastly, microbial symbionts can produce deterrents and antimicrobial compounds to fend off antagonists (28).

1.1.2 Challenges associated with different phases of a symbiotic lifestyle

To initiate a symbiotic association with the insect host, the microbial symbiont must first get in contact with the host before translocating to its niche within the host (29). Upon establishing a persistent symbiotic association, the microbial symbiont must further adjust to the challenges imposed by the host environment (30, 31). Host physiology-related stressors include, but are not limited to, host innate immunity and exposure to reactive oxygen and nitrogen species (32, 33). Finally, a symbiotic association is stabilized by symbiont transmission to the next generation. Once they have adapted to the host environment, symbionts may be challenged by adverse conditions outside the host environment in the event of external symbiont transmission (32-34).

1.1.2.1 Mediators of symbiosis initiation

To first initiate an association, a symbiont needs to be able to successfully colonize the host. Symbiont motility and chemotaxis can be essential to locate and enter a host, but the prospective symbiont can also take advantage of the host's biology and behaviour to do so (29). Translocation from the point of entry to the symbiont niche can either be mediated by the symbiont or by the host (29). Host-symbiont interaction is subsequently facilitated by symbiont surface structures, adhesion factors, biofilm formation and secretion systems (29). Such communication can additionally induce the formation of symbiont-specific organ(s) in several systems (29). Host colonization is furthermore affected by competition among multiple prospective symbiont strains, as well as the timing and order of arrival of these strains during the colonization process (29).

1.1.2.2 Challenges imposed by the host immune system during the establishment of a persistent association

After the successful colonization of the host, the symbiont needs to overcome the host immune system to persistently establish in the host. The mechanisms of the insect immune system are multifaceted and complex, and can be divided into cellular and humoral immune responses (reviewed e.g. in (32, 35)). Humoral immune responses include the production of reactive oxygen and nitrogen species (35), the production of antimicrobial peptides (AMPs) (36), and melanization (37). The cellular immune response comprises the phagocytosis or encapsulation of pathogens by hemocytes (32). More recently, it has been suggested to expand the concept of insect immunity by including external immune defense mechanisms (38), such as behavioural adaptations (e.g. grooming) and antimicrobial volatile and non-volatile secretions (38-41).

Symbionts have evolved various adaptations to internal host immune effectors, and they can be adapted to the point of being insensitive, or even resistant (42-44). Other bacterial symbionts are thought to avoid triggering, or actively suppress a host immune response (45-50).

In contrast, knowledge on host adaptations preventing exposure or protecting the symbionts from the host's own immune effectors has remained scarce. However, confining symbionts to specialized

organs, such as bacteriocytes or extracellular reservoirs, is likely a key host adaptation for maintenance of symbioses (51, 52). In bacteriocytes and in the gut, the expression of host immune effectors can be modulated to accommodate symbionts (45, 53-58). In aphids, the loss of numerous key genes of the IMD pathway plays a role in its tolerance of various symbionts (59-63).

1.1.2.2.1 Challenges imposed by reactive oxygen and nitrogen species

Reactive oxygen and nitrogen species (ROS and RNS, respectively) constitute common stressors to which microbial symbionts are exposed within and outside the host environment (30, 33). Among them, nitric oxide (NO) is a key effector molecule in both mutualistic and pathogenic interactions (64, 65). In low concentrations, NO acts as a signaling molecule, for instance in the formation of the long-term memory and in sensory information processes (reviewed e.g. in (66, 67). Additionally, NO is known to play a role in regulating the establishment of mutualism with microbes. For instance, NO is vital for the establishment of the squid-*Aliivibrio* symbiosis, since it mediates host-symbiont specificity (68, 69). Furthermore, it contributes to the regulation of nodule formation in the legume-rhizobium interaction (70), and high levels of host-produced NO result in the breakdown of the coral-dinoflagellate symbiosis under climatic stressors (71).

Apart from its involvement in signaling and regulatory processes, NO is also employed in a defensive context (64, 67). In insects, NO is produced in hemocytes, midgut cells and the fat body to eliminate pathogens (67). As a small radical, it is thought to freely diffuse through cell membranes (72) and react with oxygen, super- and peroxides to form highly reactive radicals, such as nitrogen dioxide, peroxynitrite and hydroxyl radicals (73). These ROS and RNS harm proteins, nucleic acids, lipids and prosthetic groups (73, 74).

Bacteria possess a variety of mechanisms to attenuate the nitrosative and oxidative stress-inflicted imbalance of cellular redox homeostasis and its deleterious consequences (reviewed in (75)). NO can be directly detoxified by flavohemoprotein (Hmp) (76). Superoxide dismutases convert superoxide anions to hydrogen peroxide (77, 78), which is subsequently converted to water by catalases (79, 80) to avoid the formation of even more reactive reaction products. Free ferrous iron is kept at low cellular levels to prevent the formation of reactive oxygen species caused by ferrous iron via Fenton chemistry in the first place (81, 82). Low-molecular-weight thiols, e.g. mycothiol and cysteine, act as antioxidants (75), while small oxidoreductases such as thioredoxin perform the reduction of protein thiols (83). Protein repair and turnover can be increased while chaperones restore the native conformation of oxidized proteins (84), and DNA-binding proteins deflect oxidative damage from the DNA (85).

Bacteria constitutively express the genes involved in these mechanisms due to endogenous ROS and reactive nitrogen species (RNS). Additionally, they possess a variety of transcriptional regulators sensing elevated levels of reactive nitrogen and oxygen species caused exogenously, which subsequently induce increased expression of the defense systems (reviewed in (75)). Among them, NorR and NsrR directly sense NO via nitrosylation of coordinated iron and iron-sulfur clusters, respectively (86, 87). Genes under their control include the flavohemoprotein Hmp and flavorubredoxin NorV (88). OxyR, a sensor in control of e.g. catalase and thioredoxin (89), detects hydrogen peroxide via cysteine oxidation (90).

1.1.2.3 Challenges imposed by the external transmission of extracellular symbionts during the long-term stabilization of a symbiosis

Maintaining a beneficial association in the long-term requires the insect host to transmit the symbionts to the next generation, and there are various ways for an insect host to achieve this. Vertical

transmission refers to the direct transmission of symbionts from the mother and – in a few rare cases – the father, to the offspring (91). The transmission of obligate intracellular symbionts commonly occurs within the body of the mother (92), and can be achieved e.g. transovarially or via milk gland secretions *in utero* (93, 94). In addition to maternal transmission, there is a report of paternal transmission via an intrasperm passage (95).

The mechanisms of vertical transmission of extracellular symbionts are manifold, and comprise egg smearing, oviposition site inoculation, and capsule and jelly transmission (34). They are widespread across hemipteran, coleopteran, hymenopteran and dipteran insects, with egg smearing being the most commonly observed mode (34).

For the host, vertical transmission translates to control over the symbionts, and ensures their reliable passage to the next generation of host individuals (29). Since vertical transmission aligns host and symbiont interests, selection should favor the most beneficial symbionts, creating another advantage for the host (29). As for the downsides, vertical transmission entails the need for putatively costly specialized structures for this purpose (29). Symbiont genome erosion - a result of relaxed selection pressure on genes not needed in a host-associated lifestyle, combined with severe population bottlenecks and genetic drift during vertical transmission (96) - may decrease the quality of symbiont-provided benefits to the host over evolutionary timescales (29). Additionally, vertical transmission renders the host-symbiont-system less flexible when faced with environmental change, as an increased level of specialization allows for less opportunities to acquire new symbionts (29).

Regarding the symbiont, host-provided nutrients and reduced competition in the stable host environment can be considered benefits (29). In contrast, vertical transmission should promote greater dependence on the host and hence less possibilities to access new hosts or habitats (29). This is probably exacerbated by increased host specialization due to symbiont genome erosion (29).

Extracellular symbionts of insects can also be horizontally transmitted, for example by picking up their symbionts from the environment in every generation (34, 91). This mode of transmission enables hosts to flexibly acquire new symbionts if it needs to adapt to a changing environment, but bears the risk of uninfected progeny in case symbionts are not invariably available or fail to establish in the offspring (29). Besides, hosts need to implement putatively costly control and filtering mechanisms to specifically acquire the desired symbiont (29). As host and symbiont interests are not necessarily aligned, the association may shift from mutualism to commensalism or parasitism (29). Finally, competition among the available symbionts is likely to decrease symbiont-derived benefits to the host (29).

Concerning the symbiont, horizontal transmission should provide opportunities for horizontal gene transfer from other bacteria, which would allow them to broaden their functional repertoire (29). Similarly, a biphasic lifestyle with prolonged periods outside the host would prevent or decelerate symbiont genome erosion (29). These periods would also allow the symbionts access to other habitats or hosts; however, the latter may not always be present (29). Other downsides of horizontal transmission for the symbiont include facing higher competition for successful colonization of the host, requiring costly adaptations to various niches, and the transition between niches being challenging (29).

Many systems display a combination of both vertical and horizontal transmission, referred to as mixed-mode transmission (29). In these cases, host and symbiont trade-offs should entail a mix of benefits and downsides from each transmission mode.

Vertically transmitted extracellular symbionts also often experience extended periods outside the host during transmission, especially in insects that do not provide any parental care (e.g. (97, 98)). These

periods entail exposure to diverse, and potentially fluctuating environmental stressors, such as UV radiation, heat and cold stress, the risk of desiccation, oxidative stress, microbial competition and nutrient scarcity (33, 99-101). The threat of these stressors should be exacerbated by the fact that many vertically transmitted symbionts of insects possess eroded genomes with reduced metabolic versatility (102-104). Due to their limited genetic inventories, these symbionts may not be able to raise self-protective measures to counteract environmental stressors during external transmission. Instead, hosts adaptations have been hypothesized to protect the symbionts during this vulnerable time and ensure their successful transmission to the next generation (105). However, experimental evidence of such a host adaptation has to date only been reported in the stinkbug *Megacopta punctatissima* (Hemiptera: Plataspidae): This stinkbug stabilizes its nutritional symbiont '*Candidatus* Ishikawaella capsulata' by embedding it in the intestinal secretion protein PMDP, which is indispensable for a successful transmission (97). Nevertheless, host adaptations protecting symbionts during external transmission remain understudied, despite the notion that they are likely widespread among insects in the orders Hemiptera, Coleoptera, Hymenoptera and Diptera.

1.2 The biology of beewolves

1.2.1 Phylogenetic classification and geographic distribution

Beewolves (Hymenoptera: Crabronidae; Fig. 1) are a group of solitary digger wasps comprising the genera *Philanthus*, *Trachypus* and *Philanthinus*. Beewolves of the genus *Philanthus*, which consists of ~137 species, are found on all continents but Australia, Antarctica, and South America (106-116). Around 30 known species of the genus *Trachypus* occur in Central and South America (107, 117). The rare genus *Philanthinus* comprises four species (118). Within the Philanthinae subfamily, *Philanthus*, *Trachypus* and *Philanthinus* form the tribe Philanthini, representing a sister clade to the tribes Cercerini and Aphilanthopsini (117).

Fig. 1: (A) Female and (B) male individual of the European beewolf *Philanthus triangulum* on *Eryngium campestre* (Apiales: Asteraceae). Photos by Martin Kaltenpoth.

1.2.2 Life history of beewolves

Female European beewolves (*Philanthus triangulum*) exclusively hunt honeybee workers (*Apis mellifera*) as prey for their offspring, which they recognize on flowers or in front of the beehive utilizing olfactory and visual cues (119-123). Upon capture, the female paralyzes the bee by injecting a venom into its thorax and carries it back to the nest in flight (124-127). The subterranean beewolf nest –

typically located in sandy soil – comprises a main burrow and various side burrows (120, 128-130). The latter end in one terminal brood cell each (120, 128-130). The beewolf female provisions each brood cell with one to several paralyzed bees, depending on prey availability, the sex of the offspring, the temperature, and the individual female (130-136). The beewolf egg is then laid on the topmost prey item (120). After oviposition, the female blocks the side burrow with sand, presumably to protect the offspring from parasitoid sarcophagic flies and chrysidid wasps (120), and does not provide any further brood care (120). Approximately two days after oviposition, the egg hatches (120), and the larva feeds on the provision for around one week (120). After feeding, the larva starts spinning the cocoons, whose basal part adheres to the distal end of the brood cell (130, 137). The immature larvae commonly hibernate in the cocoon during winter before they emerge in the following spring. In warmer climates, however, beewolf populations may be bivoltine (120).

Since the brood cell has been disconnected from the main burrow, the newly emerged beewolf faces the challenge of leaving the nest upon eclosion (120, 128, 130, 137). To solve this problem, females of *P. triangulum* apply an antennal gland secretion (AGS) to the distal end of their brood cells, serving as a directional cue to the larva during cocoon spinning (130, 137): The larva detects the AGS and orients the anterior part of the cocoon towards the main burrow (137). How exactly the larva perceives this directional cue is unknown to date, but it has been suggested that hydrocarbons in the AGS may act as an olfactory cue (138). Upon eclosion, the mature adult starts digging forward, reaches the main burrow and leaves the nest (137). Given their highly similar ecologies, this strategy is likely utilized by other beewolves as well (139). A graphical overview of the beewolf life cycle is given in Fig. 1 of Chapter 2.

During the vulnerable life stages in the warm and humid brood cell, the developing beewolf is prone to infestation by a wide community of opportunistic microbes (140). To circumvent this threat, beewolves have evolved various adaptations. First, paralyzing the prey instead of killing it preserves its immunological activity and therefore reduces the risk of microbial infestation (124, 125, 141). Second, beewolf females embalm the prey with a secretion from their postpharyngeal glands rich in unsaturated linear hydrocarbons (142-145). This treatment effectively decreases fungal germination and delays fungal growth by reducing water condensation (142, 143). Third, the eggs of *P. triangulum* sanitize the brood cell with extremely high concentrations of NO (1690 ± 680 ppm) produced by NO-synthase (NOS), effectively killing microbial opportunists (146). Interestingly, the production of such high NO concentrations is achieved neither by several gene copies of NOS nor by major evolutionary changes in gene sequence, but has rather been suggested to be the result of alternative splicing (146). Fourth, beewolves engage in a defensive symbiosis with '*Candidatus* Streptomyces philanthi', a member of the phylum Actinobacteria (147). The symbionts produce a cocktail of antibiotics on the beewolf cocoon surface and thereby reliably protect the immature beewolf from a wide array of microbial threats (147-149).

1.2.3 The beewolf-*Streptomyces* symbiosis

Beewolves engage in a defensive symbiosis with '*Candidatus* Streptomyces philanthi' (henceforth *S. philanthi*), first described in the European beewolf *P. triangulum* (147). The females harbor the symbionts in specialized antennal gland reservoirs of unique structure (150), and transmit them into the brood cell within the AGS (see above), where the symbionts are embedded in a hydrocarbon matrix (138). This matrix primarily consists of long-chain linear unsaturated hydrocarbons, with smaller proportions of long-chain linear saturated hydrocarbons and traces of ketones and methyl-alkanes (138). The hydrocarbon matrix – or at least some of its components – have been hypothesized to either

serve as nutrition for *S. philanthi* in the presumably nutrient-limited environment outside the host, or as volatile directional cue for the larva (see above) (138), but empirical proof for its function is lacking to date. The larva localizes the symbiont-containing AGS to take up *S. philanthi*, and subsequently incorporates the symbionts into the cocoon silk during spinning (101, 147). On the cocoon, the symbionts produce up to 49 different antibiotics, including 8 piericidins and streptochlorin (148, 149). These antibiotics are found in high concentrations and uniformly distributed on the outside of the cocoon (148), offering efficient long-term protection of the progeny from opportunistic soil-dwelling microbial pathogens (147). In contrast, the inside of the cocoon only harbors low amounts of antibiotics, presumably to reduce detrimental effects of the insecticidal piericidins to the immature larva (148). The expression of symbiont genes involved in piericidin and streptochlorin biosynthesis reaches a peak within 1-2 weeks after cocoon spinning (151). Concordantly, the amount of antibiotics on the cocoon increases within this time period and remains stable after that (151). While antibiotic titers appear to be affected by symbiont titer on (152) and the presence of the larva in the cocoon (153), humidity, temperature and the presence of fungal spores on the cocoon do not have an impact (152). After antibiotic production, *S. philanthi* likely becomes metabolically inactive to withstand the unfavorable conditions in the brood cell during host hibernation (151). Before or during eclosion, the new female beewolves probe the cocoon with their antennae to pick up *S. philanthi*. Emerging males do not acquire *S. philanthi* due to their lack of antennal gland reservoirs (139). A graphical overview of the symbiosis within the beewolf life cycle is given in Fig. 1 of Chapter 2.

Over the course of vertical transmission from mother to offspring, *S. philanthi* experiences several population bottlenecks (101). Of the approximately 1.5×10^7 symbionts harbored in the antennal gland reservoirs, merely around 2.7×10^5 symbionts are secreted into the brood cell in the AGS, which translates to a proportion of 1.9% (101). Upon incorporation into the cocoon, the symbiont population is on average halved (~1.4×10^5 symbionts) (101). The final reduction takes place when the emerging female takes up the symbiont from the cocoon, and initially harbors around $9.7*10^2$ symbiont cells in the antennal gland reservoirs (101). The overall decrease in symbiont numbers by $6.7*10^{-5}$ throughout the beewolf life cycle represents one of the biggest population bottleneck of an insect symbiont reported to date, with *Spiroplasma* symbionts of the fruit fly *Drosophila melanogaster* being the only symbiont with a comparable reduction (101, 154).

Such a reduction of effective population sizes during vertical transmission, together with relaxed selective pressure on genes not needed in a host-associated lifestyle, has been described to result in symbiont genome erosion in asexual populations of insect symbionts (96). Indeed, in spite of its relatively large size (7.3 Mb), the genome of *S. philanthi* bears signatures of incipient genome erosion, most notably pseudogenization of >30% of all protein-coding genes, which not only affects regulators, transporters and accessory genes, but also certain central metabolic pathways (155). Nevertheless, the genome of *S. philanthi* is considerably less eroded than the highly degraded genomes of obligate intracellular symbionts from systems of similar evolutionary age (156, 157).

The symbiosis with *S. philanthi* is not restricted to *P. triangulum*, but rather extends to all 28 investigated species in the genus *Philanthus* (158), the South American genus *Trachypus* (159), and the rare genus *Philanthinus* (160). In contrast, no symbiotic bacteria were found in the genera *Aphilanthops*, *Clypeadon* and *Cerceris*, thereby limiting the symbiosis to the tribe Philanthini (158). Age calibration with the fossil record revealed that the symbiosis evolved approximately 68 my ago (161). This estimate likely renders the beewolf-*Streptomyces* symbiosis more ancient than the functionally similar symbiosis between fungus-farming ants and their defensive *Pseudonocardia* symbionts (139, 162), since fungus-farming evolved around 50 my ago (163). In contrast, it originated substantially later

than symbioses with obligate intracellular nutritional symbionts (139), e.g in the Auchenorrhyncha (260-280 my ago) (164), aphids (160-280 my ago) (24), cockroaches (135-250 my ago) (23) and planthoppers (>130 my ago) (165).
Members of the Philanthini associated with *S. philanthi* form a monophyletic clade, as do the symbionts of the different hosts, implying that the symbiont was acquired once before host and symbionts subsequently co-diversified (161). In line with vertical symbiont transmission, co-phylogenetic analyses provided evidence for co-cladogenesis (161). However, these analyses also found several divergences, suggesting that symbionts have additionally been horizontally transferred among host species (161). Interestingly, several *S. philanthi* symbionts from other beewolf hosts, e.g. of the North American beewolf *P. gibbosus*, retain a larger genome (~9 Mb) without signatures of incipient genome erosion (Sandoval-Calderón et al., unpublished data).

1.3 Thesis outline

As mentioned above, the eggs of the European beewolf *P. triangulum* fumigate their brood cells with NO. Under the assumption that NO accumulates in the brood cell without diffusion through the soil and reacting with other molecules, the NO concentration has been estimated to reach 1690 ± 680 ppm (146). This value by far exceeds NO concentrations employed to treat skin infections with multiple drug resistant pathogens (200 ppm), and to sanitize fruit and vegetables (50-500 ppm) (166, 167). It is also at least twice as high as the lethal dose (LD_{50}) of NO in mammals, reported as 797 ppm when exposed for 1 h (but concentrations as low as 315 ppm already present a severe health hazard) (168).
Strikingly, *S. philanthi* – embedded in the antennal gland secretion – is present in the brood cell during the NO release by the beewolf egg. The ensuing extraordinarily high concentration of NO in the brood cell raises the question how *S. philanthi* withstands NO fumigation while other microbial opportunists are effectively killed. The beewolf-*Streptomyces* defensive association therefore presents an excellent model system to study how a symbiont with an eroded genome copes with host-derived exogenous challenges during vertical transmission in an evolutionary ancient symbiosis.
In Chapter 2, I investigated whether host and/or symbiont adaptations enable *S. philanthi* survival upon exposure to the extremely high NO concentrations released by the beewolf egg into the brood cell. To analyze the potential for symbiont adaptation, I combined fluorescence survival assays (including the free-living *Streptomyces coelicolor*) with RNAseq and proteomics. I demonstrate that the upregulation of symbiont enzymes with protective functions against NO is insufficient for symbiont survival upon confrontation with NO in the brood cell. To analyze the potential for host adaptation, I searched the proteome of the AGS for host-derived protective proteins, and explored whether the AGS can provide a barrier against NO. I provide evidence for sufficient protection from NO mediated by the AGS, and reconstitute this finding *in vitro* using a beewolf hydrocarbon extract and synthetic (Z)-9-tricosene. These findings unravel a physicochemical mechanism by which a host protects its symbiont from the host's own external immune effector during external transmission, which enables the maintenance of the symbiosis and guarantees benefits provided by the symbiont during hibernation and metamorphosis.
In Chapter 3, I examined the NO-blocking function of hydrocarbons in the AGS discovered in Chapter 2 in more detail. To this end, I evaluated the capacity of single and binary mixtures of synthetic linear saturated and unsaturated hydrocarbons, corresponding to those found in the AGS, to block NO. I show that various individual hydrocarbons, and hydrocarbon mixtures imitating the composition in the AGS, form effective NO barriers. By screening symbiont biovariations from different beewolf hosts for their NO susceptibility, and comparing hydrocarbon profiles among the respective hosts, I provide

indications for NO fumigation, as well as hydrocarbon-mediated symbiont protection, being widespread among digger wasps in the tribe Philanthini.

In Chapter 4, I complement the current knowledge and our new insights on how the challenges of external symbiont transmission are tackled in a solitary insect host, by reviewing the transmission routes and the associated challenges in social and gregarious insects. I highlight that in contrast to solitary insects, transmission of extracellular symbionts in social insects predominantly harnesses the close proximity of individuals concomitant with sociality. I discuss a reduced risk of symbiont loss and a possible co-transmission of pathogens and parasites as respective benefit and risk of social transmission, and comment on a putative role of social transmission in reinforcing the evolution of sociality.

In a general discussion (Chapter 5), I connect the host adaptation in the beewolf-*Streptomyces* symbiosis to putative host adaptations in other systems, and emphasize that they are most likely widespread across insect orders.

This thesis demonstrates that an insect host can evolve to protect its symbiont during vertical transmission, which is essential in stabilizing symbioses over long evolutionary timescales.

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CHAPTER 2: Host hydrocarbons protect symbiont transmission from a radical host defense

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Authors' contributions

[redacted] and [redacted] designed research; C.S.I., [redacted], [redacted], [redacted], [redacted], and [redacted] performed research; [redacted] contributed new reagents/analytic tools; C.S.I. and [redacted] analyzed data; and C.S.I., [redacted], and [redacted] wrote the paper.

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2.1 Abstract

Symbioses with microbes play a pivotal role in the evolutionary success of insects, and can lead to intimate host–symbiont associations. However, how the host maintains a stable symbiosis with its beneficial partners while keeping antagonistic microbes in check remains incompletely understood. Here, we uncover a mechanism by which a host protects its symbiont from the host's own broad-range antimicrobial defense during transmission. Beewolves, a group of solitary digger wasps (Hymenoptera: Crabronidae), provide their brood cells with symbiotic *Streptomyces* bacteria that are later transferred to the cocoon and protect the offspring from opportunistic pathogens by producing antibiotics. In the brood cell, however, the symbiont-containing secretion is exposed to a toxic burst of nitric oxide (NO) released by the beewolf egg, which effectively kills antagonistic microorganisms. How the symbiont survives this lethal NO burst remained unknown. Here, we report that upon NO exposure *in vitro*, the symbionts mount a global stress response, but this is insufficient to ensure survival at brood cell-level NO concentrations. Instead, *in vivo* bioassays demonstrate that the host's

antennal gland secretion (AGS) surrounding the symbionts in the brood cell provides an effective diffusion barrier against NO. This physicochemical protection can be reconstituted *in vitro* by beewolf hydrocarbon extracts and synthetic hydrocarbons, indicating that the host-derived long-chain alkenes and alkanes in the AGS are responsible for shielding the symbionts from NO. Our results reveal how host adaptations can protect a symbiont from host-generated oxidative and nitrosative stress during transmission, thereby efficiently balancing pathogen defense and mutualism maintenance.

2.2 Significance

Periods of exposure to environmental pressures during transmission across host generations are universal to extracellular symbionts of many Hemiptera, Coleoptera, some Hymenoptera and Diptera, as well as other insect orders. Given that vertically transmitted symbionts generally exhibit signatures of genome erosion and metabolic streamlining, host adaptations to protect the symbiont from abiotic and biotic challenges during transmission should be widespread, but remain poorly studied to date. Here, we show that a beewolf host embalms its antibiotic-producing "*Streptomyces philanthi*" symbionts in a secretion containing long-chain hydrocarbons, which effectively blocks diffusion of toxic nitric oxide produced by the beewolf egg. This host adaptation protects the symbionts from nitrosative and oxidative stress during the vulnerable period of extracellular transmission.

2.3 Introduction

Microbial symbioses are ubiquitous in nature, and they constitute important drivers of evolutionary innovation (1, 2). In insects, symbionts confer a wide range of benefits to their hosts, such as nutrient supplementation, digestion, detoxification, and protection from predators, pathogens, and parasites (3-5), enabling their hosts to colonize otherwise inaccessible niches (3). As a symbiont establishes a persistent beneficial association with the host insect, it must overcome the challenges posed by the host-associated lifestyle (6). This includes, but is not limited to, coping with host physiology–related stressors, such as host immune responses (7-13), high levels of reactive oxygen species (14, 15), as well as dealing with unfavorable conditions outside the host in case of external transmission (16). While symbiont factors to cope with host immune effectors have been intensively studied (7, 17-24), much less is known about host adaptations that prevent exposure or protect the symbionts from the host's own immune effectors as well as environmental stressors (but see refs. (7, 13, 14) and (25-28)). A common stressor for symbionts is the exposure to reactive oxygen or nitrogen species (ROS and RNS, respectively). In particular, nitric oxide (NO) is an important effector molecule in both mutualistic and pathogenic interactions (29, 30), but also serves as a signaling molecule at low concentrations (reviewed e.g. in refs. (31) and (32)). A regulatory function in establishing mutualistic interactions with microorganisms has been recognized in the squid–*Aliivibrio* symbiosis, where NO is essential in symbiosis establishment and mediates host-symbiont specificity (33, 34). In the legume–rhizobium interaction, it contributes to the regulation of nodule formation (35), whereas in the coral–dinoflagellate symbiosis stressful climatic conditions lead to high levels of host-produced NO, resulting in the breakdown of symbiosis (36). As a constituent of the immune system (reviewed e.g. in refs. (29) and (32)), NO is produced by vertebrate macrophages (29) and by insect hemocytes, fat body and midgut cells to combat pathogens (32). Its cytotoxic effects stem from its diffusion through cell membranes (32) and the formation of highly reactive radicals upon reaction with oxygen or super- and peroxides (37), which in turn inflict oxidative damage upon macromolecules, such as DNA, RNA, proteins, lipids, and prosthetic groups (37, 38).

Recently, an intriguing case of NO as an external immune defense has been described in beewolf wasps (Hymenoptera: Crabronidae) (39). These solitary digger wasps construct subterranean brood cells (Fig. 1), where the offspring is confronted with a diverse community of opportunistic mold fungi during development (40). In response, beewolves have evolved multiple adaptations to prevent infections. First, the eggs of the European beewolf (*Philanthus triangulum*) fumigate the brood cell with high concentrations of NO (1,690 ± 680 ppm), effectively killing antagonistic soil-dwelling microbes (Fig. 1) (39). Second, female beewolves embalm the larval provisions in a secretion from their postpharyngeal gland containing high amounts of long-chain saturated and unsaturated hydrocarbons that prevent infection of the provisions by reducing water condensation and thereby impairing germination of mold fungi (41-44). Last, female beewolves harbor symbiotic "*Streptomyces philanthi*" bacteria in specialized antennal reservoirs (45) and secrete them into the brood cell (46). Later, the larva incorporates the deposited symbionts into its cocoon during spinning (46). On the cocoon, the symbionts produce an antibiotic cocktail containing several piericidin and streptochlorin derivatives (47) that effectively protect the offspring from pathogen infestation during the vulnerable phases of hibernation and metamorphosis (46). The symbiosis between beewolves and *Streptomyces* originated about 68 Mya (48), with the symbionts providing a long-term stable combination prophylaxis (49) despite signatures of ongoing erosion in the *P. triangulum* symbiont genome (50). Interestingly, because female beewolves secrete *S. philanthi* into the brood cell prior to NO fumigation, the symbionts are exposed to the high concentrations of NO while they are embedded in the antennal gland secretion (AGS). However, the mechanisms allowing the symbionts to survive NO exposure remain enigmatic.

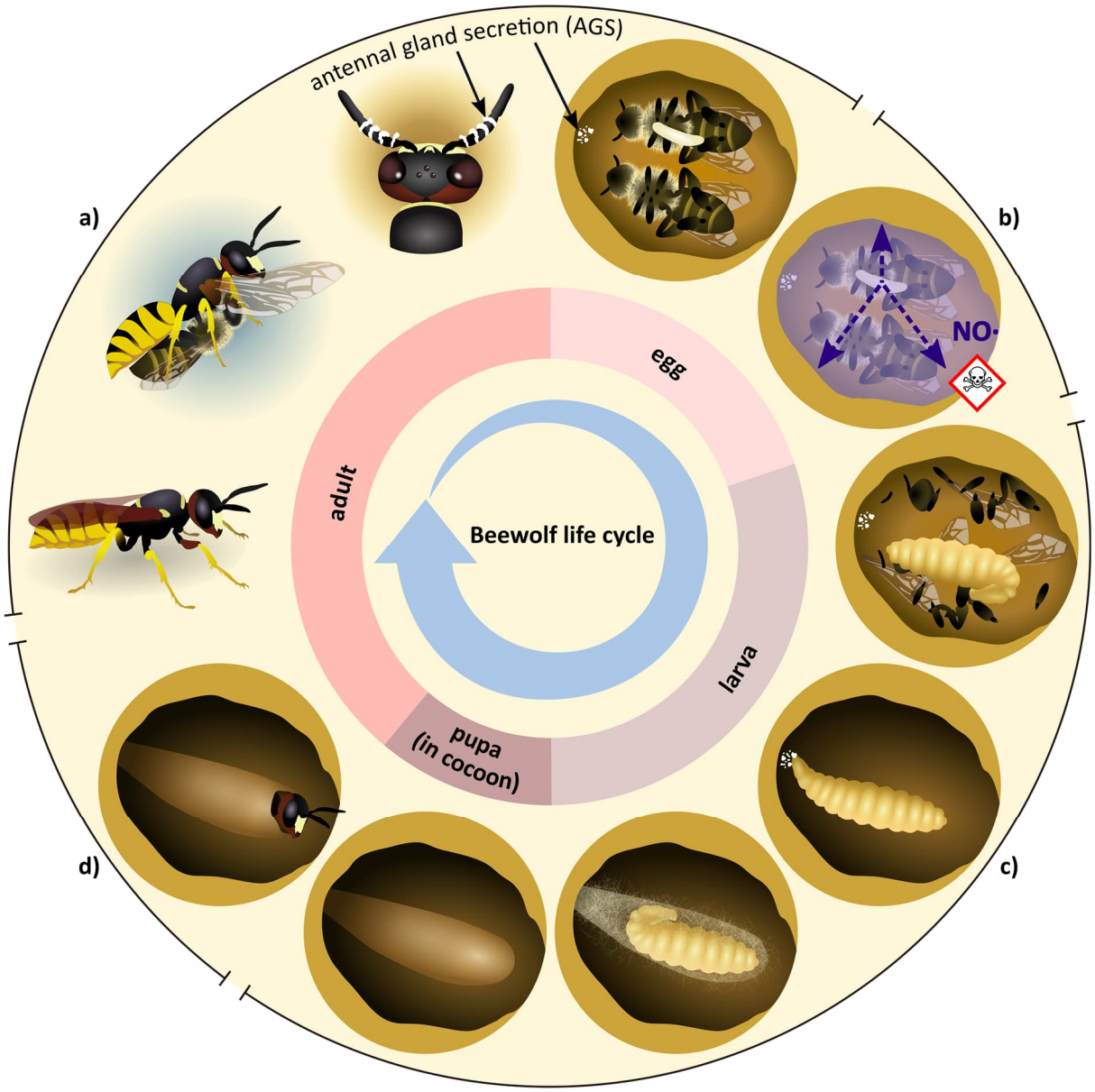


Fig. 1: Life cycle of the European beewolf (*P. triangulum*). (A) Beewolf females hunt *Apis mellifera* workers and paralyze them by injecting their venom into the bee's thorax (51). They provide their subterranean brood cells with one to several bees, embalmed in a secretion from their postpharyngeal gland (41). This secretion contains high amounts of long-chain saturated and unsaturated hydrocarbons that prevent infection of the provisions by reducing water condensation (41-44). The female deposits an egg on top of the provisions (52). Before sealing the brood cell, the female applies an AGS rich in linear unsaturated and saturated hydrocarbons (53) and containing the defensive symbiont *Streptomyces philanthi* to the brood cell ceiling (46, 54). (B) The beewolf egg sanitizes the brood cell by releasing high amounts of toxic nitric oxide (NO), with NO emission peaking at ~14 to 16 h after oviposition (39). While NO effectively kills microbial opportunists in the brood cell (39), *S. philanthi* withstands NO via a previously unknown mechanism. (C) After hatching and feeding on the provisioned bees for several days, the larva integrates *S. philanthi* into its cocoon (46). On the cocoon surface, the symbionts produce an antibiotic cocktail (47, 55) that provides protection from microbial infestation (46, 47). (D) After 4 to 6 wk or in the following summer, the larva undergoes metamorphosis, and the adult ecloses from the cocoon (52, 56).

Here, we set out to elucidate whether host or symbiont adaptations are responsible for protecting the symbionts from recurrent NO exposure in the beewolf brood cell. We first assessed *S. philanthi* survival upon ecologically relevant NO concentrations *in vitro*, in comparison to the free-living relative *Streptomyces coelicolor*. We then used RNAseq and proteomics to characterize the symbiont's response to NO exposure *in vitro* and *in vivo*. Additionally, we examined the potential for host-

mediated protection of the symbionts by screening the AGS proteome for proteins with putative protective functions against NO, and by testing the capacity of the AGS to act as an NO diffusion barrier. Our results reveal that the symbionts up-regulate protective enzymes upon NO exposure, but these are insufficient to protect the bacteria from the high NO concentration in the brood cell. However, the AGS provides efficient protection from NO, an effect that can be reconstituted *in vitro* by a beewolf hydrocarbon extract and by synthetic (Z)-9-tricosene. Our findings uncover a physicochemical mechanism that a host uses to protect its symbiont from oxidative and nitrosative stress during the vulnerable period outside of the host environment, thereby stabilizing the symbiotic association and ensuring symbiotic benefits during hibernation and metamorphosis.

2.4 Results

2.4.1 Symbionts mount a global stress response but are susceptible to NO *in vitro*

We first sought to find out whether symbionts show resistance to NO *in vitro*. We investigated the effect of NO at brood cell-level concentration (10× injection of 1 mL 1% NO in N_2 vs. pure N_2 into the headspace of the culture containers) on "*S. philanthi biovar triangulum*" strain 23Af2 *of P. triangulum* (henceforth *S. philanthi*) and the free-living *Streptomyces coelicolor* M145 A2(3) (henceforth *S. coelicolor*). A qualitative survival assessment with a fluorescent live-dead stain indicated that NO drastically reduced the survival of both species at all timepoints (Suppl. Fig. 1).

We then set out to characterize the molecular response of both species to NO. To this end, we exposed *S. philanthi* and *S. coelicolor* to 1% NO in N_2 again, but this time analyzed the respective changes in gene expression 2 h and 6 h post exposure in comparison to an N_2 control treatment. Additionally, we examined the NO response of *S. philanthi in vivo* by analyzing the gene expression of symbionts in the AGS in the presence and absence of the NO-emitting egg. Genes with at least twofold and significant expression change were considered differentially expressed in all datasets.

For both symbiotic *S. philanthi* and free-living *S. coelicolor*, most of the genes encoded in their genomes were found to be expressed across all conditions, with the replicates showing consistent gene expression profiles (Suppl. Tab. 1 and 2, Suppl. Fig. 2). *S. coelicolor* showed a moderate response to NO, with 353 differentially expressed genes after 2 h, and 413 after 6 h (Fig. 2A, Suppl. Fig. 3A). Similarly, *S. philanthi* gene expression was not profoundly affected by NO after 2 h. At this timepoint, 218 up- and 37 down-regulated genes accounted for a total of 255 differentially expressed genes (Suppl. Fig. 3B and 4). However, we found a drastic change in *S. philanthi* gene expression 6 h after NO exposure (Fig. 2B). 895 up- and 460 down-regulated genes accounted for a total of 1,355 differentially expressed genes (Suppl. Fig. 4). Strikingly, *S. philanthi* exposed to NO released by the beewolf egg *in vivo* did not show a strong response to NO, with only 50 genes being differentially expressed, all of them up-regulated (Fig. 2C and Suppl. Fig. 4A).

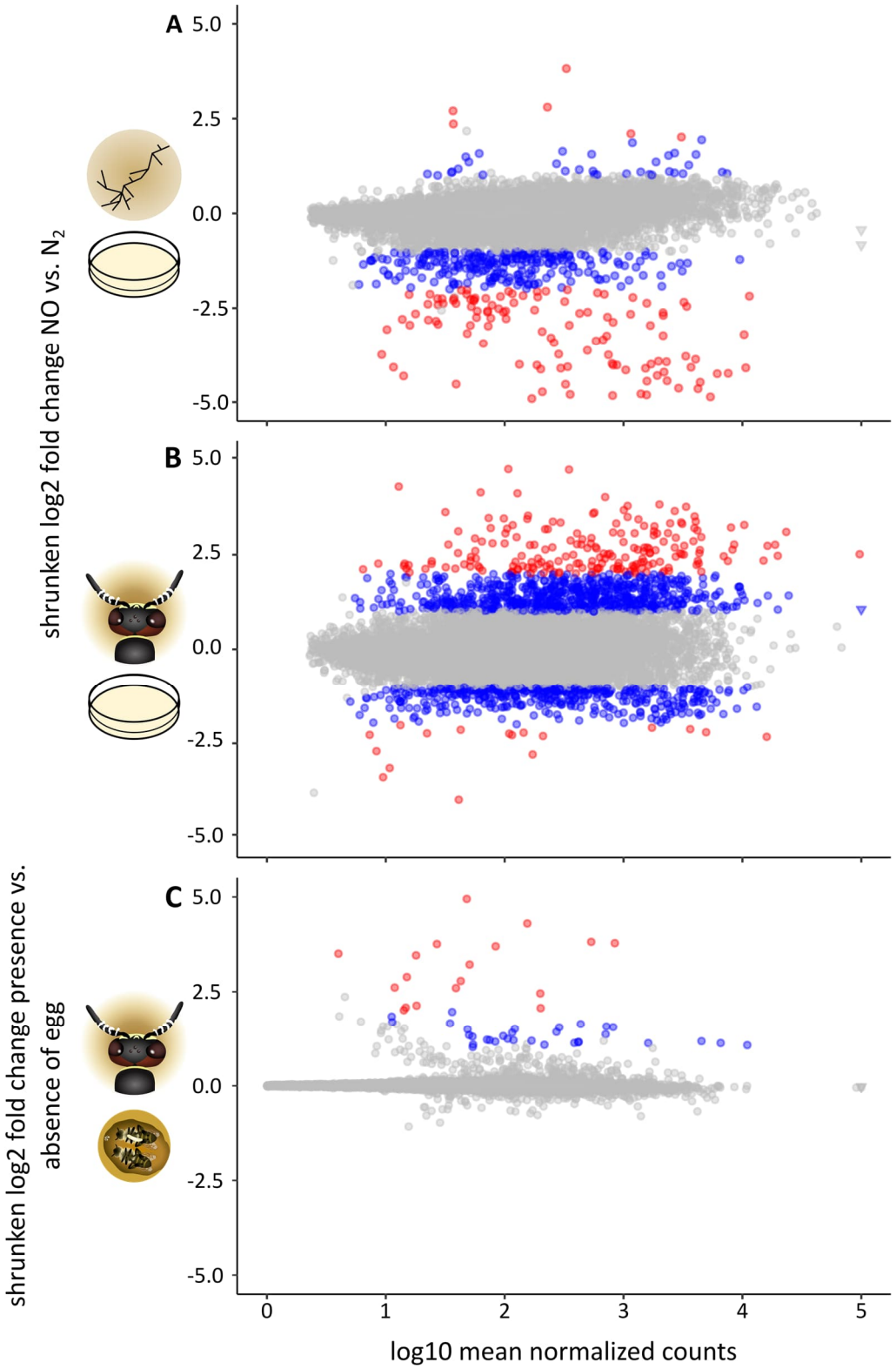


Fig. 2: Changes in gene expression in (A) free-living *S. coelicolor* (indicated by the symbol of filamentous bacteria) and (B) symbiotic *S. philanthi* (indicated by the beewolf head) 6 h after *in vitro* exposure to NO in comparison to exposure to N_2 (indicated by the petri dish), and in (C) symbiotic *S. philanthi* in the AGS within beewolf brood cells in the presence vs. the absence of a beewolf egg emitting NO (indicated by the beewolf brood cell). Significant gene expression differences are highlighted in color (adjusted $p < 0.05$ and twofold to fourfold differential expression in blue, and more than fourfold change in red). Log-transformed values of some extremely highly expressed genes were set to 5 to improve readability and are indicated with triangles instead of circles.

In addition to the global changes in gene expression profiles, we also assessed the expression of genes known to be involved in bacterial responses to NO (reviewed in ref. (57) and Suppl. Fig. 5). The flavohemoprotein Hmp converts NO to nitrate (58), and superoxide dismutases convert superoxide anions to hydrogen peroxide (59, 60). The latter is subsequently converted to water by catalases (61, 62). Low-molecular-weight thiols, such as mycothiol and cysteine, serve as antioxidants (57), and small oxidoreductases such as thioredoxin reduce protein thiols (63). Protein chaperones reconstitute the native conformation of oxidized proteins (64), and DNA-binding proteins deflect oxidative damage from the DNA (65). Cellular levels of free ferrous iron are kept low to avert additional oxidative stress caused by ferrous iron via Fenton chemistry (66, 67). At the same time, iron–sulfur clusters, which are essential components of a multitude of proteins (68) but damaged by RNS and ROS, are replenished (69).

We identified 69 genes in the *S. philanthi* genome involved in these pathways, as well as their homologues in *S. coelicolor* (Fig. 3). In *S. coelicolor*, we found a relatively high constitutive expression of flavohemoprotein, superoxide dismutases, enzymes of the mycothiol biosynthesis pathway, cold shock proteins, oxidoreductases, and genes related to iron homeostasis, such as ferredoxins (Fig. 3). However, only a few genes belonging to redox and iron homeostasis pathways were differentially expressed after NO exposure *in vitro* (Fig. 3). By contrast, in *S. philanthi*, most NO stress–related genes were up-regulated after NO exposure, especially those involved in redox and iron homeostasis (Fig. 3). Additionally, many nondifferentially expressed genes exhibited high constitutive expression levels. In stark contrast, only a small number of genes, mainly encoding chaperones, were up-regulated in *S. philanthi* after *in vivo* exposure to the beewolf egg (Fig. 3). In addition, 20 genes without a corresponding homologue (18 for *S. philanthi*, 2 for *S. coelicolor*) exhibited differential expression after NO exposure (Suppl. Fig. 6), including genes coding for proteins involved in iron uptake in *S. philanthi*.

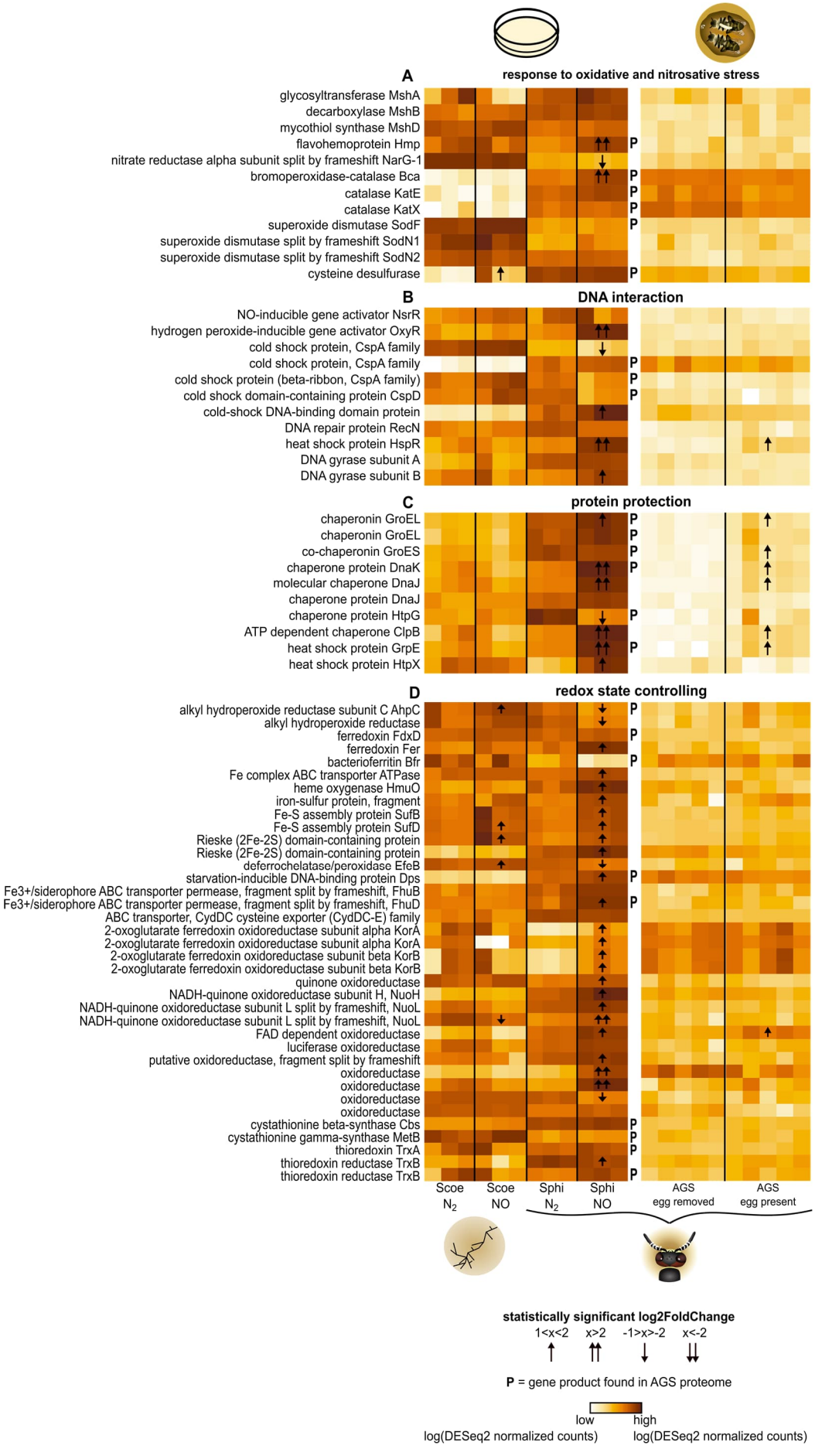

A
response to oxidative and nitrosative stress
glycosyltransferase MshA
decarboxylase MshB
mycothiol synthase MshD
flavohemoprotein Hmp
nitrate reductase alpha subunit split by frameshift NarG-1
bromoperoxidase-catalase Bca
catalase KatE
catalase KatX
superoxide dismutase SodF
superoxide dismutase split by frameshift SodN1
superoxide dismutase split by frameshift SodN2
cysteine desulfurase
B
DNA interaction
NO-inducible gene activator NsrR
hydrogen peroxide-inducible gene activator OxyR
cold shock protein, CspA family
cold shock protein, CspA family
cold shock protein (beta-ribbon, CspA family)
cold shock domain-containing protein CspD
cold-shock DNA-binding domain protein
DNA repair protein RecN
heat shock protein HspR
DNA gyrase subunit A
DNA gyrase subunit B
C
protein protection
chaperonin GroEL
chaperonin GroEL
co-chaperonin GroES
chaperone protein DnaK
molecular chaperone DnaJ
chaperone protein DnaJ
chaperone protein HtpG
ATP dependent chaperone ClpB
heat shock protein GrpE
heat shock protein HtpX
D
redox state controlling
alkyl hydroperoxide reductase subunit C AhpC
alkyl hydroperoxide reductase
ferredoxin FdxD
ferredoxin Fer
bacterioferritin Bfr
Fe complex ABC transporter ATPase
heme oxygenase HmuO
iron-sulfur protein, fragment
Fe-S assembly protein SufB
Fe-S assembly protein SufD
Rieske (2Fe-2S) domain-containing protein
Rieske (2Fe-2S) domain-containing protein
deferrochelatase/peroxidase EfeB
starvation-inducible DNA-binding protein Dps
Fe3+/siderophore ABC transporter permease, fragment split by frameshift, FhuB
Fe3+/siderophore ABC transporter permease, fragment split by frameshift, FhuD
ABC transporter, CydDC cysteine exporter (CydDC-E) family
2-oxoglutarate ferredoxin oxidoreductase subunit alpha KorA
2-oxoglutarate ferredoxin oxidoreductase subunit alpha KorA
2-oxoglutarate ferredoxin oxidoreductase subunit beta KorB
2-oxoglutarate ferredoxin oxidoreductase subunit beta KorB
quinone oxidoreductase
NADH-quinone oxidoreductase subunit H, NuoH
NADH-quinone oxidoreductase subunit L split by frameshift, NuoL
NADH-quinone oxidoreductase subunit L split by frameshift, NuoL
FAD dependent oxidoreductase
luciferase oxidoreductase
putative oxidoreductase, fragment split by frameshift
oxidoreductase
oxidoreductase
oxidoreductase
oxidoreductase
cystathionine beta-synthase Cbs
cystathionine gamma-synthase MetB
thioredoxin TrxA
thioredoxin reductase TrxB
thioredoxin reductase TrxB
P
Scoe N2
Scoe NO
Sphi N2
Sphi NO
AGS egg removed
AGS egg present
statistically significant log2FoldChange
1<x<2
x>2
-1>x>-2
x<-2
P = gene product found in AGS proteome
low
high
log(DESeq2 normalized counts)
log(DESeq2 normalized counts)

Fig. 3: Symbionts display a drastic change in the expression of general stress response genes, as well as oxidative and nitrosative stress-specific responses *in vitro*, but not *in vivo* (i.e., within the beewolf brood cell). Treatment-specific expression of genes associated with (A) the response to nitrosative and oxidative stress, (B) DNA interaction, (C) protein protection, and (D) redox state controlling. Scoe = free-living *S. coelicolor* (also indicated by the symbol of filamentous bacteria), Sphi = symbiotic *S. philanthi* (also indicated by the beewolf head), AGS = antennal gland secretion. The petri dish indicates gene expression *in vitro*, the beewolf brood cell symbolizes gene expression within the AGS. The *P* denotes genes for which the respective protein was detected in the proteomic analysis of the AGS.

2.4.2 The AGS contains host- and symbiont-derived proteins with protective properties against NO

Many of the proteins involved in protection against oxidative and nitrosative damage were also detected in our proteomic analysis of the AGS, including several chaperones, catalases, superoxide dismutase, flavohemoprotein, and many genes involved in redox state controlling (Fig. 3, Suppl. Fig. 7, and Dataset S1 (70)). In addition to these symbiont-produced proteins, the proteome analysis also revealed several host factors antagonizing oxidative and nitrosative stress. Apart from a superoxide dismutase and a heat shock protein of insect origin, we also identified a glutathione synthase, a glutathione S-transferase and a glutathione peroxidase (71).

2.4.3 NO exposure does not influence antibiotic production by the symbionts *in vivo*

We tested whether the NO release by the beewolf egg influenced the symbiont-mediated defense of the larva, as NO serves as a signal affecting morphological differentiations and antibiotic production in free-living *Streptomyces* (72-74). Specifically, we examined the impact of NO on symbiont titer and antibiotic production on the cocoon in a semi-natural setting (Suppl. Fig. 8). However, neither the symbiont titer nor the amount of the three major antibiotics, i.e. piericidin A1 and B1 and streptochlorin on the cocoon was influenced by prior NO exposure of the symbionts in the brood cell (Suppl. Fig. 8 and Suppl. Tab. 3-5; symbiont titer: paired *t* test, $t = -0.835$, $df = 8$, $p = 0.428$, $N = 9$; antibiotic production: paired *t* tests, $p > 0.05$, $N = 5$).

2.4.4 AGS provides protection against NO *in vivo*

Considering that the symbionts do not survive ecologically relevant concentrations of NO *in vitro*, we examined whether the host-secreted AGS has the potential to protect *S. philanthi* from NO exposure in the beewolf brood cell (Fig. 4A and B). We used the change in coloration of a potassium–iodide–starch oxidation indicator to visualize NO exposure *in situ*. We transferred the AGS on a filter paper impregnated with the indicator solution, and applied it across the ceiling of the brood cell during NO production by the beewolf egg. We found that the presence of the AGS prevented the color change of the filter paper upon NO release by the beewolf egg (Fig. 4C and D). We made use of AGS autofluorescence to confirm its location on the specific noncolored zone of the indicator filter paper (Fig. 4E and F). A comparison of the mean gray values, i.e. the mean darkness of the respective pixels, confirmed that the change in coloration of this noncolored, AGS-bearing zone was strongly and significantly reduced compared to the surrounding area that was not covered by AGS (Fig. 4G; Tukey's HSD, $p < 0.05$, $N = 9$). However, the AGS-bearing zone still displayed a slightly darker hue than a control filter paper without NO exposure (Fig. 4C and G; Suppl. Tab. 6 and Suppl. Fig. 9; Tukey's HSD, $p < 0.05$, $N = 9$), indicating that low amounts of NO can either penetrate the AGS or diffuse through the filter paper from the sides.

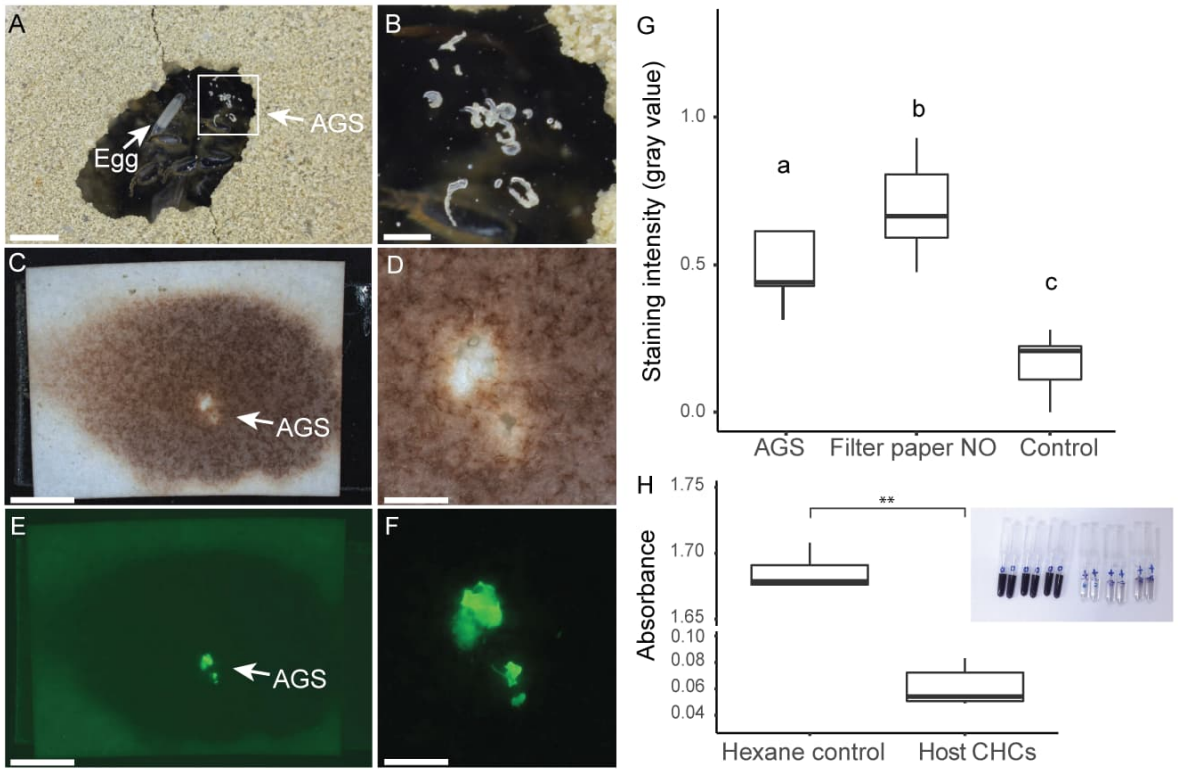


Fig. 4: The beewolf female's AGS blocks diffusion of NO both *in vivo* and *in vitro*. (A) Beewolf brood cell in an observation cage, with paralyzed bees at the bottom of the brood cell and the beewolf egg on top of one of the bees. The AGS is visible as small white specks at the ceiling of the brood cell, in this case a transparent plastic sheet. (B) Enlarged region of the brood cell from *A*, showing the AGS on the brood cell ceiling. (C) Representative filter paper prepared with NO indicator that turned brown after NO exposure in the brood cell, with a clear zone where the AGS is localized, enlarged in (D). (E and F) Same areas as in (C) and (D) under fluorescent light, corroborating the position of the AGS by its autofluorescence. [Scale bars: (A, C, and E): 4 mm; (B, D, and F): 1 mm.] (G) Quantification of indicator reaction upon NO exposure in beewolf brood cells. Comparison of normalized mean gray values between zones of filter paper treated with AGS vs. the surrounding area exposed to NO in the brood cell, and a control without NO exposure (artificial brood cell without beewolf egg that was excavated next to the target brood cell). Different letters above the boxes indicate significant differences (Tukey's HSD, $p < 0.05$). (H) Quantification of indicator reaction upon NO exposure in solutions with a cover of beewolf CHCs vs. a control of hexane (Wilcoxon rank sum exact test, $W = 0$, $p = 0.002$, $N = 6$). The *Inset* shows the dark blue indicator solution in control tubes after NO exposure (left) as compared to the transparent solutions covered by beewolf CHCs that prevent NO diffusion (right).

2.4.5 Beewolf hydrocarbon extracts provide an NO diffusion barrier *in vitro*

The AGS is mainly composed of unsaturated and saturated hydrocarbons (53), hence we considered hydrocarbons the primary candidates responsible for the observed protection from NO exposure. To test this, we extracted beewolf cuticular hydrocarbons (CHCs) from two adult males per extract in hexane. The beewolf CHC composition was previously shown to correspond to that of the AGS in both composition and relative amounts of hydrocarbons (53, 75, 76). As beewolf males produce copious amounts of a CHC-derived sex pheromone in specialized head glands, we removed the head during extraction, resulting in an extract of CHCs from thorax and abdomen. We applied the CHCs as a layer on top of 40 µL of an NO indicator iodide–starch solution in qPCR tubes, and exposed the tubes to synthetic NO (0.01% in N_2). After 1 h, the hexane-treated control solutions had turned dark blue due to the exposure to NO, while the solutions covered with beewolf CHCs remained transparent, similar to control solutions that were not exposed to NO. Concordantly, spectrophotometric analysis revealed a clear inhibition of oxidation (i.e. less change in coloration) of the indicator iodide–starch solution in

the samples covered with beewolf CHCs as compared to hexane-treated controls (Fig. 4E and Suppl. Tab. 7, Wilcoxon rank sum exact test, W = 0, p = 0.002, N = 6).

2.4.6 Synthetic (Z)-9-tricosene rescues *S. philanthi* survival upon NO exposure *in vitro*

To directly confirm the activity of hydrocarbons to protect the symbionts from NO, we covered *S. philanthi* with (Z)-9-tricosene and exposed it to a lethal NO concentration. (Z)-9-tricosene is a constituent of the AGS. It is liquid at room temperature and applicable to the symbionts without a harmful solvent. We monitored macroscopically visible growth for 8 d. (Z)-9-tricosene rescued *S. philanthi* growth in all five replicates, while control inoculants without (Z)-9-tricosene did not visibly grow (Suppl. Fig. 10).

2.4.7 *S. philanthi* cells are embedded within the AGS matrix *in vivo*

We performed scanning electron microscopy on the AGS to assess whether the symbiont cells are indeed embedded within the AGS matrix. While symbiont cells growing *in vitro* showed the typical mycelial structure (Suppl. Fig. 11A and B), the AGS exhibited a smooth surface, with symbiont cells being either not visible at all or appearing covered by a film of AGS (Suppl. Fig. 11C–F). Thus, most or all of the symbionts are likely shielded by the hydrocarbon-rich AGS from the external environment.

2.5 Discussion

During transmission, the beewolf's defensive symbiont-bearing secretion experiences toxic nitric oxide (NO) concentrations released by the beewolf egg for brood cell sanitation. The mechanisms ensuring *S. philanthi* survival and therefore the long-term stability of the symbiosis remained enigmatic. Here, we show that *S. philanthi* mounts a profound stress response upon NO exposure *in vitro*, but the majority of cells do not withstand NO concentrations resembling those in the brood cell. However, our *in vivo* assays revealed that the host's symbiont-bearing AGS serves as an efficient diffusion barrier against NO, an effect that we could reconstitute *in vitro* using beewolf cuticular hydrocarbon (CHC) extracts with a composition representing the hydrocarbon profile in the AGS, as well as using a single synthetic hydrocarbon to rescue *S. philanthi* survival upon otherwise lethal NO stress.

2.5.1 Symbiont response to NO exposure

Surprisingly, *in vitro* exposure to NO in ecologically relevant concentrations was not only lethal to the free-living soil bacterium *S. coelicolor*, but also killed most cells of the beewolf symbiont *S. philanthi*. As opposed to *S. coelicolor,* however, *S. philanthi* displayed major gene expression changes upon NO exposure, particularly characterized by the upregulation of genes involved in scavenging NO and its reaction products as well as those preventing or alleviating cellular damage inflicted by RNS and ROS. Microbial responses to protect from NO are observed across several other host–symbiont and host–pathogen interactions, where NO serves as an important signaling and effector molecule (29, 30). For example, *Aliivibrio fischeri*, the symbiont of the Hawaiian bobtail squid *Euprymna scolopes* (reviewed in ref. (77)) is exposed to host-derived NO (34), which contributes to ensuring host-symbiont specificity (33, 34). NO resistance in *A. fischeri* is mediated by upregulation of flavohemoprotein (*hmp*) (78), which is also up-regulated in *S. philanthi* upon NO exposure, and detoxifies NO by converting it to nitrate (58). Flavohemoprotein is also essential for NO resistance of nitrogen-fixing symbionts in the legume-rhizobium symbiosis, where NO constitutes an important signaling molecule (79, 80).

In the intracellular pathogen *Mycobacterium tuberculosis,* the causative organism of tuberculosis (81), NO exposure *in vitro* triggers responses to ROS and reactive radicals in general. This includes the upregulation of thioredoxin- and thioredoxin reductase genes (*thiX*, *trxB1*, *trxC*; *trxB2*) (69), while genes implicated in the protection from nitrosative and oxidative stress show high constitutive expression, particularly superoxide dismutase (*sodA*, *sodC*) and alkyl hydroperoxide reductase (*ahpD*, *ahpE*, *ahpC*) (69). Similarly, we observed high constitutive expression of genes with superoxide dismutase function (*sodF*, *sodN1*, *sodN2*) in *S. philanthi in vitro*, whereas genes with alkyl peroxidase function (e.g., *ahpC*) were down-regulated. Furthermore, genes for iron–sulfur and cysteine biosynthesis were up-regulated in *M. tuberculosis* in an effort to replace degraded iron–sulfur clusters (69). We also observed an upregulation of these genes in *S. philanthi in vitro*.
In the natural environment of beewolf brood cells, *S. philanthi* does not appear to suffer high mortality upon exposure to NO released by the beewolf egg, as previous studies indicate that a large proportion of the symbiont population in the brood cell is later transferred to the cocoon (82). Concordantly, within beewolf brood cells, *S. philanthi* did not exhibit the profound gene expression changes upon NO exposure that we observed *in vitro*. Nevertheless, multiple chaperones were up-regulated, and several catalases, oxidoreductases, bacterioferritin, and a cold shock protein exhibited high constitutive expression levels. Furthermore, the AGS proteome contained many symbiont-encoded proteins that are putatively involved in protection from nitrosative and oxidative stress, including flavohemoprotein, ferredoxin, thioredoxin and thioredoxin reductase, various catalases, and molecular chaperones, as well as superoxide dismutase and enzymes connected to iron uptake and homeostasis. Thus, the biosynthesis of proteins that scavenge NO or alleviate its detrimental effects is likely important for enhancing symbiont survival in the natural setting. However, the high mortality upon exposure to ecologically relevant NO concentrations *in vitro* indicates that host factors are necessary to protect the symbiont from the host-emitted NO in the brood cell.

2.5.2 Host-mediated protection from NO

To assess whether *P. triangulum* may protect *S. philanthi* from NO-inflicted cellular damage, we first screened the AGS proteome for proteins with putative protective functions against NO. Indeed, our proteome analysis revealed the presence of a host-derived superoxide dismutase, a heat shock protein of insect origin, as well as a glutathione synthase, a glutathione S-transferase, and a glutathione peroxidase. Glutathione is a low-molecular-weight thiol with antitoxic, antioxidant and modulator properties, and glutathione-mediated reactions protect bacteria from RNS and ROS (57, 83, 84). While glutathione synthase converts the precursor glutamyl-cysteine to glutathione, glutathione S-transferase catalyzes glutathione conjugation to endogenous metabolites or xenobiotic substances for detoxification purposes, and glutathione peroxidase performs the glutathione-dependent reduction of hydroperoxide (84). These findings suggest that *P. triangulum* secretes proteins that help to protect its symbiont from the host's own NO defense.
Considering the high mortality of *S. philanthi* upon NO exposure *in vitro*, we hypothesized that the AGS might additionally shield *S. philanthi* from NO by acting as a diffusion barrier. In line with this hypothesis, our *in vivo* assays revealed that the application of the AGS to an iodide-starch indicator filter paper in a beewolf brood cell protected the covered region from oxidation. Our findings provide evidence for *P. triangulum* adaptations to protect *S. philanthi* from the host's NO defense mechanism through the physicochemical prevention of NO diffusion into the aqueous milieu of the symbiont cells in the AGS. The AGS has previously been described to serve a dual function: First, it provides the larva with directional information for proper cocoon spinning and later emergence (54). Second, it mediates

transmission of the symbiont to the larva to incorporate into the cocoon for protection against infection (46). Our study indicates that the latter function is supported by an additional trait of the AGS, i.e. the physicochemical protection of the symbiont from host-emitted NO.

The AGS contains high amounts of long-chain saturated and unsaturated hydrocarbons (53). Thus, we hypothesized that these compounds might be responsible for preventing NO diffusion. Concordantly, beewolf CHC hexane extracts with a composition comparable to the AGS, but without the background of symbiont- and host-encoded proteins of the AGS, prevented diffusion of NO into a liquid indicator solution *in vitro*. Furthermore, synthetic (Z)-9-tricosene, a constituent of the AGS, rescued *S. philanthi* survival upon exposure to otherwise lethal NO concentrations *in vitro*, albeit in higher concentrations than the approximated HC concentrations in the AGS, due to the need to apply the HC without a harmful solvent.

While the AGS hydrocarbons have been hypothesized to serve as an olfactory cue to help the larva localize the AGS, and/or as a nutritional resource for *S. philanthi* (53), our results indicate that they play an important role in protecting the symbionts from the detrimental effects of the host-produced NO. Considering that the hydrocarbon composition of NO-exposed AGS (53) closely resembles that of the postpharyngeal gland (PPG), the hemolymph, and the cuticle of *P. triangulum* (53, 75, 76, 85), the protective effect is likely due to the physical properties of forming a hydrophobic barrier impenetrable to the water-soluble NO, rather than a chemical mechanism of radical scavenging. Consistent with this hypothesis, SEM images of the AGS revealed that the secretion entirely covers the symbiont cells. CHCs have long been known to protect insects from environmental stress, including desiccation and pathogen attack (reviewed in refs. (86) and (87)). Secondarily, CHCs have often evolved to serve as signals in chemical intraspecific and interspecific communication, particularly in social insects (reviewed in refs. (86) and (87)), or as a physicochemical mechanism to reduce water condensation and thereby delay fungal growth (43). Our description of hydrocarbons protecting an insect symbiont from the host's own defenses adds another facet to the diverse array of functions fulfilled by insect CHCs.

Most extracellular symbionts in insects experience periods of exposure to the environment during transmission across host generations (16). Especially considering the commonly observed patterns of genome erosion and metabolic streamlining in vertically transmitted symbionts (88-90), host adaptations for symbiont protection from abiotic and biotic stressors are likely common, but remain poorly studied to date. Recently, however, a host adaptation to protect its symbiont from the harsh conditions during the vulnerable period outside of the host environment has been described in plataspid stinkbugs (91). These bugs are obligately associated with *Ishikawaella*, a gut symbiont with a severely reduced genome (92, 93). During its vertical transmission via specialized symbiont capsules, *Ishikawaella* is subjected to intense sunlight for seven to 10 days (94). Within the capsule, *Ishikawaella* cells are embedded in a special intestinal protein provided by the females, which ensures survival during transmission and successful establishment in the offspring, but the mechanism of protection remains elusive (91). The widespread occurrence of extracellular transmission routes on the surface or in the vicinity of eggs across many Hemiptera, Coleoptera, some Hymenoptera and Diptera, as well as other insect orders (16) highlights the need for protective adaptations from the symbiont or host side to buffer environmental challenges and sustain a long-term stable symbiotic association.

2.6 Conclusion

During transmission, the beewolf's AGS containing the defensive symbiont *S. philanthi* is exposed to toxic nitric oxide (NO) that the host produces to reduce the growth of harmful fungi in the brood cell.

In vitro assays show that the symbiont mounts a global stress response upon NO exposure, but is unable to survive concentrations of the gaseous radical that mimic those within the beewolf brood cell. Instead, our results reveal that the beewolf protects *S. philanthi* from oxidative and nitrosative damage by producing protective enzymes and embalming the symbiont in a secretion containing long-chain hydrocarbons. These hydrocarbons block diffusion of the toxic NO, adding yet another dimension to the diverse array of functions fulfilled by insect hydrocarbons. In light of our experiments, the beewolf–*Streptomyces* symbiosis presents an interesting example of a host adaptation to protect its symbiont from the host's own immune defense, thereby ensuring reliable vertical transmission and long-term stability of the symbiosis.

2.7 Material & methods

2.7.1 *Streptomyces* cultivation

S. philanthi 23Af2 was cultivated *in vitro* following a previously published protocol (95). An inoculum of *S. philanthi* from a glycerol stock was grown without shaking in liquid Grace medium (ThermoScientific, Germany). Grace medium base was dissolved in 1 L distilled water and supplemented with 50 µL phenol red, tryptose-phosphate broth (final concentration of 0.2 g/L tryptose, 0.02 g/L dextrose, 0.05 g/L NaCl, 0.025 g/L disodium phosphate; all from Carl-Roth, Germany) and the pH was adjusted to pH 6.5 with 1 M NaOH (Carl-Roth, Germany). *Streptomyces coelicolor* was cultured in YEME broth (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone; all from Carl-Roth, Germany). To obtain spores, an inoculum was cultivated on YEME agar (same as YEME broth with the addition of 15 g/L agar; Carl-Roth, Germany) for 14 d at 25 °C. After sporulation occurred, spores were harvested in 0.05% Triton X-100 and stored in glycerol stocks (0.025% Triton X-100, 50% Glycerol; all from Carl-Roth, Germany) at −80 °C.

2.7.2 Symbiont survival upon NO exposure *in vitro*

We used 7-d-old *S. philanthi* cultures grown at 25 °C as described above, with the addition of chicken egg lysozyme (7.5 µg/mL) to the cultivation medium to obtain short filamentous single cells. Similar growth stages occur *in vivo* in beewolf female antennae before secretion and NO exposure in natural brood cells (46). For *S. coelicolor* experiments, spores from a glycerol stock (see above) were washed twice with YEME broth.

Three technical replicates of each species were inoculated on solidified Grace agar (same composition as liquid grace medium described above, with addition of 10 g/L agar; Carl-Roth, Germany) and YEME agar, respectively. Two petri dishes were prepared for each species. The petri dishes were filled with 30 mL agar and had 60 mL air volume left. Both species were incubated at 25 °C for one more day.

In the brood cell, initial concentrations of NO are low. However, the egg emits a concentrated NO burst within 24 h after oviposition at temperatures above 24 °C, with the majority of NO being released within a 2 h period (39). In order to mimic the conditions in the beewolf brood cell, we assessed at which NO concentration we achieved antifungal effects that were equivalent to those obtained with an exposure to a beewolf egg (39). We found that replacing half of the remaining headspace of a petri dish with 1% NO in N_2 10 times over the course of 1 h reproduced the antifungal effect of a beewolf egg on inoculated fungal spores *in vitro*. We used this activity-guided approach henceforward for bacterial exposure to NO *in vitro*. While this procedure theoretically results in a concentration of 5,000 to 9,990 ppm in the petri dish, losses due to the reaction of NO with the plastic and diffusion into the

agar medium likely reduce the concentrations to levels that have been recorded for beewolf brood cells *in vivo* (1,690 ± 680 ppm, maximum 4,448 ppm) (39).
Colonies were exposed to either 1% NO in N_2 (purity NO: 2.0; purity N_2: 5.0; 2% error tolerance; Westfalen AG, Germany) or pure N_2 (purity 5.0, Westfalen AG, Germany). Prior to gas exposure, petri dishes were sealed with parafilm (Bemis, USA), pierced in the center with a hot needle to allow for gas application and resealed with adhesive tape (Tesa, Germany). The 60 mL air volume of the petri dishes was displaced 10 times with 30 mL fresh NO/N_2 or N_2 every 6 min. Gas-tight glass syringes with Teflon stoppers (Hamilton, USA) were used to apply NO/N_2 or N_2.
The three replicates of one dish were assessed for bacterial survival consecutively at 0 h, 6 h, and 24 h after the exposure with random assignment of the single colonies to the three time points. We used the LIVE/DEAD™ BacLight™ Bacterial Viability Kit (ThermoScientific, Germany) that consists of propidium iodide to stain dead or dying bacteria with compromised cell membranes, and SYTO9 to stain cells with intact cell membranes. 5 µL of a solution of 1% of each dye in distilled water was added on top of a colony 15 min before imaging. Imaging was conducted with a Thunder Imaging System (Leica, Germany) using a 20×/0.80 HC PL APO objective and a monochrome CMOS camera (DFC9000GT-VSC13735). The propidium iodide signal was acquired with the 475-nm LED at 50% power, the DFT51010 filter cube, and a 535-nm fast emission filter. The SYTO9 signal was acquired with the 555-nm LED at 50% power, the DFT51010 filter cube and a 590-nm fast emission filter. As background fluorescence varied between species, treatments and time points, we optimized exposure and threshold settings individually to obtain visibly stained cells in both channels. Further, we applied the instant computational clearing algorithm with standard settings to reduce background fluorescence.

2.7.3 Symbiont gene expression upon NO exposure *in vitro*

To assess the symbiont's transcriptomic response to NO exposure *in vitro*, we generated six technical replicates per *Streptomyces* species and gas treatment (NO vs. N_2). For each *S. philanthi* replicate, an inoculum from 7-d-old liquid cultures growing at 25 °C was transferred to fresh solid medium 1 wk before NO exposure. Cells were collected from liquid media by mild centrifugation (1,000 rpm for 4 min) and omission of the supernatant. The concentrated cell suspension was transferred in 20-µL droplets to individual wells of 24-well plates. For each *S. coelicolor* replicate, an inoculum of spores was washed twice with YEME broth and transferred onto solid medium. The wells of the multi-well plate had a total volume of 3 mL, and contained 1 mL of solid medium and 2 mL headspace. We used Grace agar (see above) for *S. philanthi* and YEME agar (see above) for *S. coelicolor*. *S. philanthi* cultures were incubated for 7 d at 25 °C, *S. coelicolor* for one day, due to its much faster growth. Prior to gas exposure, plates were sealed with adhesive plastic foil (VWR, Germany) and resealed with adhesive tape (Tesa, Germany) after piercing the foil with syringes during gas application. Colonies were exposed to either 1% NO in N_2 (purity NO: 2.0; purity N_2: 5.0; 2% error tolerance; Westfalen AG, Germany) or pure N_2 (purity 5.0, Westfalen AG, Germany). The 2 mL air in the headspace of the 24-well plate wells was displaced 10 times with 1 mL fresh NO/N_2 or N_2 every 6 min. We used gas-tight glass syringes as described above.
Bacterial biomass was scraped off the agar plates using a sterilized spatula and shock-frozen in liquid nitrogen. Three of the technical replicates per species and treatment were harvested 2 h after exposure, the remaining three 6 h after exposure. RNA was extracted after all treatments were collected by lysing cells in 100 µL TE25S buffer (25 mM Tris-HCl [pH 8], 25 mM EDTA [pH 8], 0.3 M sucrose) containing 2 mg/mL lysozyme for 60 min at 37 °C. Lysates were further processed using the

innuPREP DNA/RNA Mini Kit (Analytik Jena, Germany) following the manufacturer's protocol. RNA was eluted with 30 µL RNase-free water and quantified using a NanoDrop1000 (Peqlab, Germany). 1.2 to 1.5 µg of total RNA was used for a further DNase digestion with 5U DNase (Qiagen, Germany) for 15 min at 37 °C, followed by a 10-min DNase inactivation step at 75 °C.
Library preparation and sequencing was performed at the Max Planck-Genome-Centre Cologne, Germany (https://mpgc.mpipz.mpg.de/home/). All replicates were subjected to rRNA depletion with the Ribo-zero magnetic gold kit for bacteria (Illumina), and then a directional RNAseq library was prepared with the NEBNext Ultra™ Directional RNA Library Prep Kit for Illumina (NEB) followed by sequencing as 100-bp single-end libraries at a sequencing depth of 4 million reads per sample on a HiSeq2500 device (Illumina, USA). Sequences were quality-controlled and trimmed using FastQC (96) and Trimmomatic (97). The retained reads were mapped to either the *S. coelicolor* or *S. philanthi* genome (50) using Bowtie2 (v.2.3.2) and StringTie (v.1.3.3b) implemented in KBase (98) using default settings. rRNA sequences were removed from the dataset before differential gene expression was analyzed using DESeq2 (v.1.22.2) (99) in RStudio (v1.1.453 with R v3.5.0). During the analysis, transcripts with less than 30 counts were discarded. Genes with significant difference in gene expression between NO and N_2 treatments (FDR-adjusted *P*-values following Benjamini–Hochberg of Wald test < 0.05) and at least twofold expression difference were considered differentially expressed. For subsequent visualization of the data in ggplot2, LFC shrinkage was applied using the "apeglm" shrinkage estimator. Homologous genes were identified by reciprocal mapping of relevant genes on the genomes of *S. philanthi* and *S. coelicolor* in Geneious Prime (V2021.1.1).

2.7.4 Beewolf cultivation for *in vivo* bioassays

Females of the European beewolf *P. triangulum* were collected in Berlin (Germany) for the proteome experiments and Mainz (Germany) for the *in situ* reaction of *S. philanthi* to NO released by the beewolf egg. Females were kept in observation cages (54) at 24 to 27 °C with a ~16/8 h day/night cycle, and provided ad libitum with honey and workers of the European honeybee *Apis mellifera*. The nesting compartment was covered with glass plates to allow for observation of the underground nesting behavior (Suppl. Fig. 12). A plastic foil was introduced below the glass cover to enable the sampling of the AGS from the brood cell ceiling by cutting out the respective piece of foil containing the AGS, as the AGS is visible with to naked eye as up to ten whitish spots on the plastic foil.

2.7.5 Proteome of symbiont-containing brood cell secretion (AGS)

AGS samples from fifty brood cells were collected at random time points within the approximately 10 d between females finishing a brood cell and larvae starting to spin their cocoon, and frozen at −80 °C until analysis. After thawing, samples were combined and suspended in 50 µL PBS, vortexed vigorously, and centrifuged for 10 min at 6,000 g to separate soluble proteins from cellular debris. 200 µL of rehydration buffer was added to sample supernatant (7 M urea, 2 M thiourea, 2% Chaps, 0.75% IPG buffer 3–11, bromophenol blue). Samples were immediately adsorbed onto 11 cm immobilized pH 3–11 gradient (IPG) strips (GE Healthcare Bio-Sciences) and rehydrated overnight. Isoelectric focusing was performed on an Ettan IPGphor II (Amersham plc) unit by using the following program: 500 V for 1 h, 500 to 1,000 V for 1 h, 1,000 to 6,000 V for 2 h, 6,000 V for 40 min. After focusing, the IPG strips were equilibrated for 15 min in 10 mL of equilibration buffer containing 6 M urea, 30% [v/v] glycerol, 2% [w/v] SDS, 75 mM Tris–HCl, 0.002% [w/v] bromophenol blue and 1% (w/v) DTT. Then, the strips were incubated for 15 min in the same buffer containing 2.5% (w/v) iodoacetamide instead of DTT. For the separation of proteins in the second dimension a 12.5% Criterion Tris-HCl Gel (Biorad)

was used. The proteins were separated for 3 h at 100 V and stained with Roti Blue (Carl Roth) and visualized by a densitometer (Biorad GS-800).

The protein sample was diluted in Roti-Load buffer (Carl Roth) and loaded on a 12.5% Criterion Tris-HCl precast 1D gel (Bio-Rad) according to the manufacturer's instructions. Proteins were allowed to migrate for 1 h at a constant voltage of 200 V. After migration, the gel was stained overnight with Roti Blue (Carl Roth) staining solution, rinsed twice with deionized water, destained in 25% methanol, and scanned using a densitometer (Biorad GS-800). Protein bands/spots were cut from the gel matrix and tryptic digestion was carried out as described (100). For LC-MS analysis, the extracted tryptic peptides were reconstituted in 10 µL aqueous 1 % formic acid. Depending on staining intensity, 1 to 5 µL of sample was injected into the LC-MS/MS system. The samples were acquired on a nanoAcquity UPLC system online connected to a Q-ToF Synapt HDMS mass spectrometer (Waters). The peptides were concentrated on a Symmetry C18 trap-column (20 × 0.18 mm, 5 µm particle size) using a mobile phase of 0.1% aqueous formic acid at a flow rate of 15 µL/min and separated on a nanoAcquity C18 column (200 mm × 75 µm ID, C18 BEH 130 material, 1.7 µm particle size) by in-line gradient elution at a flow rate of 0.350 µL/min using the following gradient: 1 to 15% B over 5 min, 15 to 40% B over 25 min, 40 to 55% B over 10 min, 55 to 80% B over 10 min, 80 to 95% B over 1 min, isocratic at 95% B for 1 min, and a return to 1% B (Buffers: A, 0.1 % formic acid in water; B, 100 % acetonitrile in 0.1 % formic acid). Data were acquired using data-dependent acquisition (DDA) and data-independent acquisition (DIA, referred to as enhanced MSE). The acquisition cycle for DDA analysis consisted of a survey scan covering the range of *m/z* 400-1800 Da followed by MS/MS fragmentation of the five most intense precursor ions collected at 1.5-s intervals in the range of 50 to 2,000 *m/z*. Dynamic exclusion was applied to minimize multiple fragmentations for the same precursor ions. For LC-MSE analyses, full-scan LC-MS data were collected using alternating modes of acquisition: low-energy (MS) and elevated-energy (MSE) modes at 1.5 s in the range *m/z* of 50 to 1,900. The collision energy of low-energy MS mode and high-energy mode (MSE) were set to 4 eV and 20 to 40 eV energy ramping, respectively. A reference compound, human Glu-Fibrinopeptide B [650 fmol/mL in 0.1 % formic acid/ACN (v/v, 1:1)], was infused through a reference sprayer at 30-s intervals for external calibration. The data acquisition was controlled by MassLynx v4.1 software (Waters).

DDA raw data were processed and searched against a subdatabase containing common contaminants (human keratins and trypsin) using ProteinLynx Global Server (PLGS) version 2.5.2 (Waters). The following search parameters were applied: fixed precursor ion mass tolerance of 10 ppm for survey peptide, fragment ion mass tolerance of 0.02 Da, estimated calibration error of 0.002 Da, 1 missed cleavage, fixed carbamidomethylation of cysteines, and possible oxidation of methionine. Spectra remaining unmatched by database searching were interpreted de novo to yield peptide sequences and subjected to homology-based searching using MS BLAST program (101) installed on a local server. MS BLAST searches were performed against beewolf and *Streptomyces* subdatabases both obtained from *in silico* translation of beewolf and *Streptomyces* transcriptome data and against insect and bacteria databases downloaded from https://www.ncbi.nlm.nih.gov/ on June 02, 2017. In parallel, pkl-files of MS/MS spectra were generated and searched against beewolf and *Streptomyces* subdatabases combined with NCBInr database (https://www.ncbi.nlm.nih.gov/, downloaded on January 19, 2017, containing 112,583,993 sequences) using MASCOT software version 2.6.2.

The acquired continuum LC-MSE data were processed using ProteinLynx Global Server (PLGS) version 2.5.2 (Waters). The thresholds for low-/high-energy scan ions and peptide intensity were set at 150, 30, and 750 counts, respectively. The processed data were searched against beewolf and *Streptomyces* protein subdatabases combined with Swissprot database downloaded from

http://www.uniprot.org/. The database was searched at a false discovery rate (FDR) of 4%. The minimum number of product ion matches per peptide was set to 3, and the minimum number of product ion matches was set to 7. The minimum number of peptide matches and the maximum number of missed tryptic cleavage sites were both set to 1. Searches were restricted to tryptic peptides with a fixed carbamidomethyl modification for Cys residues.

2.7.6 Symbiont gene expression upon NO exposure in beewolf brood cells

NO emission by the beewolf egg starts approximately 16 to 24 h after oviposition at our beewolf rearing temperature of 24 °C to 27 °C (39). To examine the effects of NO exposure on symbiont gene expression in the AGS, brood cells with one or two bees were manipulated within 1 h after oviposition. Each brood cell was checked for premature NO emission via the characteristic sharp, sweet NO smell. To obtain an NO-unexposed sample, the bee holding the beewolf egg was removed from the brood cell and discarded. To obtain NO-exposed samples, the bee carrying the egg was removed and then reintroduced into the respective brood cell after a 1-min removal. After 24 h, the piece of plastic foil bearing the AGS of both treatments was cut out and transferred to a 1.5-mL reaction tube. Given the 16 to 24 h that it takes until NO emission by the beewolf egg and the manipulation of the brood cell within 1 h after oviposition, sampling 24 h after manipulation corresponds to an average of 4 to 5 h after NO exposure. Samples were frozen in liquid nitrogen and stored at −80 °C.

RNA was extracted from five NO-exposed and five NO-unexposed AGS samples using the Epicentre MasterPureTM DNA and RNA kit (Epicenter Technologies, Madison, USA). Extraction was carried out according to the manufacturer's instruction with the following modifications: Pieces of plastic foil with the AGS were transferred to 100 µL TE25S buffer (25 mM Tris [pH 8], 25 mM EDTA [pH 8], 0.3 M sucrose) with 50 µL lysozyme (50 mg/mL) and incubated at 37 °C for 1 h at 750 rpm. Afterward, the plastic foil was removed from the samples, and the crude extracts were processed following the manufacturer's instructions. The extracted nucleic acids were resuspended in 20 µL TE buffer. DNA was digested using PerfeCTa DNase I (Quantabio, Beverly/MA, USA) according to the manufacturer's instructions. RNA samples were sequenced at the Max Planck-Genome-Centre Cologne, Germany (https://mpgc.mpipz.mpg.de/home/). All replicates were subjected to rRNA depletion. Due to low-input amounts of RNA, rRNA was depleted on the cDNA level using oligonucleotides complimentary to *S. philanthi* rRNA (Tecan Genomics, Switzerland). All replicates were sequenced individually as 150-bp paired-end libraries with a sequencing depth of 2 million reads per sample on a HiSeq3000 system (Illumina, USA). Sequences were processed and analyzed as described above for the *in vitro* dataset, with the exception of only omitting transcripts with less than five counts.

2.7.7 Effect of NO exposure on symbiont titer and antibiotic production on the cocoon

To examine whether NO exposure in the brood cell impacts subsequent symbiont growth and antibiotic production on the cocoon, we sampled cocoons with previously NO-exposed and NO-unexposed symbionts, respectively. After oviposition, the piece of plastic foil with AGS was removed and transferred to an artificial brood cell in a control cage. In this set-up, we removed the plastic foil to enable brood cell sanitation by NO emission, so that the larval provisions would not be overgrown by fungi over the course of the experiment. To obtain cocoons with NO-exposed symbionts, the AGS was removed and then reintroduced into the respective brood cells after ~1 min. To obtain cocoons with NO-unexposed symbionts, the AGS was transferred to an artificial brood cell and reintroduced into its original brood cell after 24 h, i.e. after the beewolf-produced NO had already diffused into the surrounding sand. The resulting cocoons were harvested 7 d after cocoon spinning, since the amount

of antibiotics reaches a peak around this time (55). We measured length and width of a random subset of the cocoons, dissected out the pupae and flash-froze the cocoons in liquid nitrogen before storage at −80 °C. We used a paired design, sampling one cocoon with NO-exposed symbionts and one cocoon with NO-unexposed symbionts per female, to account for genetic differences between individuals. We collected nine sample pairs in total, and measured the length and width for a subset of five random sample pairs. We approximated the cocoon surface by the product of length and width to calculate the amount of antibiotics in relation to the cocoon area.

The amounts of the three major compounds (streptochlorin, piericidin A1 and B1) (47) of the symbiont-produced antibiotic cocktail were quantified as previously described (55). The cocoons were thawed and then individually submerged in HPLC grade methanol (Carl-Roth, Germany) with 2 µg of internal standard (octadecan-1-ol in hexane, Sigma-Aldrich, Germany) for 60 min at 350 rpm shaking to extract antibiotic compounds. After removing the cocoons, the solvent was completely evaporated under a nitrogen stream, and residual compounds were redissolved in 50 µL methanol. Aliquots of 1 µL of each sample were injected into a Varian 450GC gas chromatograph coupled to a Varian 240MS mass spectrometer (Agilent Technologies, Böblingen, Germany) using a split/splitless injector operated at 250 °C in splitless mode. The GC was equipped with a DB5-MS capillary column (30 m × 0.25 mm diameter, film thickness: 0.25 µm, Agilent Technologies) and programmed from 150 to 300 °C at 5 °C/min with a 1-min initial and a 10-min final isothermal hold. Helium was used as carrier gas, with a constant flow rate of 1 mL/min. Mass spectra were recorded using electron ionization (EI-MS) in the external configuration of the ion trap held at 90 °C with a mass range of 45 to 500 *m/z*. Data acquisition and quantifications were achieved with the MS Workstation Version 6.9.3 Software (Agilent Technologies). A dilution series (1 to 500 ppm) of commercially available piericidin A1 was used as an external calibration standard for both piericidin derivatives, assuming similar ionization efficiencies based on the high structural similarity. For quantification of streptochlorin, we used a dilution series of a synthesized streptochlorin standard (55). The peaks were identified by comparison of their mass spectra with the standard spectra and with published reference spectra, and peak areas were manually integrated using the MS Workstation Software.

After antibiotic extraction, the same cocoons were used for DNA extraction (55), using the Epicentre MasterPureTM DNA extraction kit (Epicenter Technologies, Madison, USA). Extraction was carried out according to the manufacturer's instructions with the following additional steps: To better lyse the gram-positive symbiont cells, cocoons were crushed and transferred to 100 µL Tissue and Cell Lysis solution with 8 µL lysozyme (100 mg/mL). Samples were incubated at 37 °C for 1 h. After protein precipitation, an additional centrifugation step (14,000 rpm, 2 min) was added to optimize the transfer of the supernatant. Extracted DNA was resuspended in 50 µL low TE buffer.

Symbionts on the cocoons were quantified via quantitative PCR (qPCR). The 16S rDNA of *S. philanthi* was amplified using specific primers Strep_phil_fwd3mod (5′ TGGTTGGTGGTGGAAAGC 3′) and S16S_rev (5′ GTGTCTCAGTCCCAGTGTG 3′) (82), resulting in a 135-bp product. Amplification was performed on a Rotor Gene Q cycler (Qiagen, Hilden, Germany), using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia). Each sample of extracted DNA was diluted 1:100 prior to qPCR. One reaction comprised 6 µL H_2O, 2 µL EVA Green Mix, 0.5 µL of each primer, and 1 µL of the diluted DNA template. Samples were incubated at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 20 s at 72 °C. A melting curve was recorded by increasing the temperature from 60 °C to 95 °C over a time period of 20 min. The purified target amplicon of the 16S rDNA primers served as the template in a dilution series from 1.48 ng/µL to 1.48 pg/µL. The number of gene copies was then calculated (1 ng of the amplified product equals 6,758,851,515 copies). The resulting

symbiont titers, as well as antibiotic amounts, and antibiotic amounts standardized by cocoon area were compared between treatments by paired *t* tests using R V5.

2.7.8 Preparation of NO indicator

We mixed 1 mL of a 10% (w/v) potassium iodide (Carl-Roth, Germany) solution with 5 mL of a 2% (w/v) boiled starch (Carl-Roth, Germany) solution and 4 mL of ultrapure water (Merck, Germany). Filter papers were soaked in this mixture and dried at 60 °C overnight. Dried filter papers were stored in glass containers flooded with N_2 and stored until use. We employed this indicator to qualitatively visualize the presence of NO via detection of oxidation: NO or its spontaneous reaction products oxidize iodide to iodine. Elemental iodine then integrates into the starch double helix which results in a dark coloration (102).

2.7.9 Protective effect of the AGS for the symbionts in the brood cell

To test for a role of the AGS in protecting the embedded symbionts from NO exposure, we transferred freshly secreted AGS onto a piece of NO indicator filter paper and reintroduced it into the corresponding brood cell. We monitored changes in its coloration after NO emission by the beewolf egg, which we documented using a digital camera (Canon DS126311, Canon, Japan) and an X-Rite ColorChecker classic 24-color chart to standardize coloration between pictures. We further confirmed the presence and localization of symbiont-bearing AGS on the NO indicator, making use of its autofluorescence, with either a M165FC (Leica, Germany) or an Axio Zoom.V16 (Zeiss, Germany) and GFP filter sets. Samples were imaged without further treatment using reflective light and fluorescence illumination.

Standardized images of the NO-exposed and control indicators were evaluated in Photoshop CD5 V12.064. To compare the change in coloration between the colored area, the area bearing the AGS, and a control indicator paper, all images were first converted to grayscale. For each of these areas, we identified the mean gray value, i.e. the mean darkness of the selected pixels, using the wand tool with the default setting, and normalized it by the mean gray value of the respective imaging standard. We analyzed the results in R (V4.15) using a one-way ANOVA.

2.7.10 Protective effect of beewolf hydrocarbons from NO *in vitro*

The AGS contains high amounts of saturated and unsaturated linear hydrocarbons (53). Previous studies revealed similar hydrocarbon compositions of the AGS, the postpharyngeal gland (PPG), the hemolymph, as well as the epicuticle of female *P. triangulum* (53, 75, 76, 85). In addition, the hydrocarbon profiles of females and males were found to be comparable (103). Given the easier access to male beewolves than to AGS samples or female beewolves, we used CHC extracts of adult male beewolves to test for the protective activity of the beewolf CHCs against NO. To avoid contamination with the male sex pheromone (103), we removed the head from twelve male beewolves and extracted CHCs by immersing the two bodies per extract in hexane for 10 min, producing six replicate extracts. Wasp bodies were removed, and hexane was evaporated under a gentle flow of nitrogen. Each CHC extract was resuspended in 10 µL hexane and used to cover 40 µL of an iodide-starch indicator solution (see above) in a qPCR tube (diameter: 3 mm; Biozym, Germany). A control was prepared with a microcentrifuge tube containing 40 µL of the indicator solution and 10 µL hexane on top; six controls were generated. For treatments and controls, the hexane was allowed to evaporate. The tubes were positioned in a 24-well plate, and the well plate was placed in a sealed

exposure chamber. Half of the remaining headspace was replaced with 0.01% NO in N_2 every 6 min over the course of 1 h. We chose this concentration since this procedure resulted in clear NO detection, i.e. a complete change in coloration of the indicator solution, in a preliminary experiment. The qPCR tubes were then removed from the exposure chamber, and the indicator solution was transferred to 500-µL microcentrifuge tubes. After centrifugation at 10,000 g for 25 s, the supernatant was transferred to a 384-well plate and the absorbance at 540 nm was measured in a VarioSkan Lux (Thermo Fisher Scientific, Germany). We analyzed the results in R (V4.15) using the Wilcoxon rank sum exact test.

2.7.11 Protective effect of (Z)-9-tricosene on symbiont survival upon NO stress *in vitro*

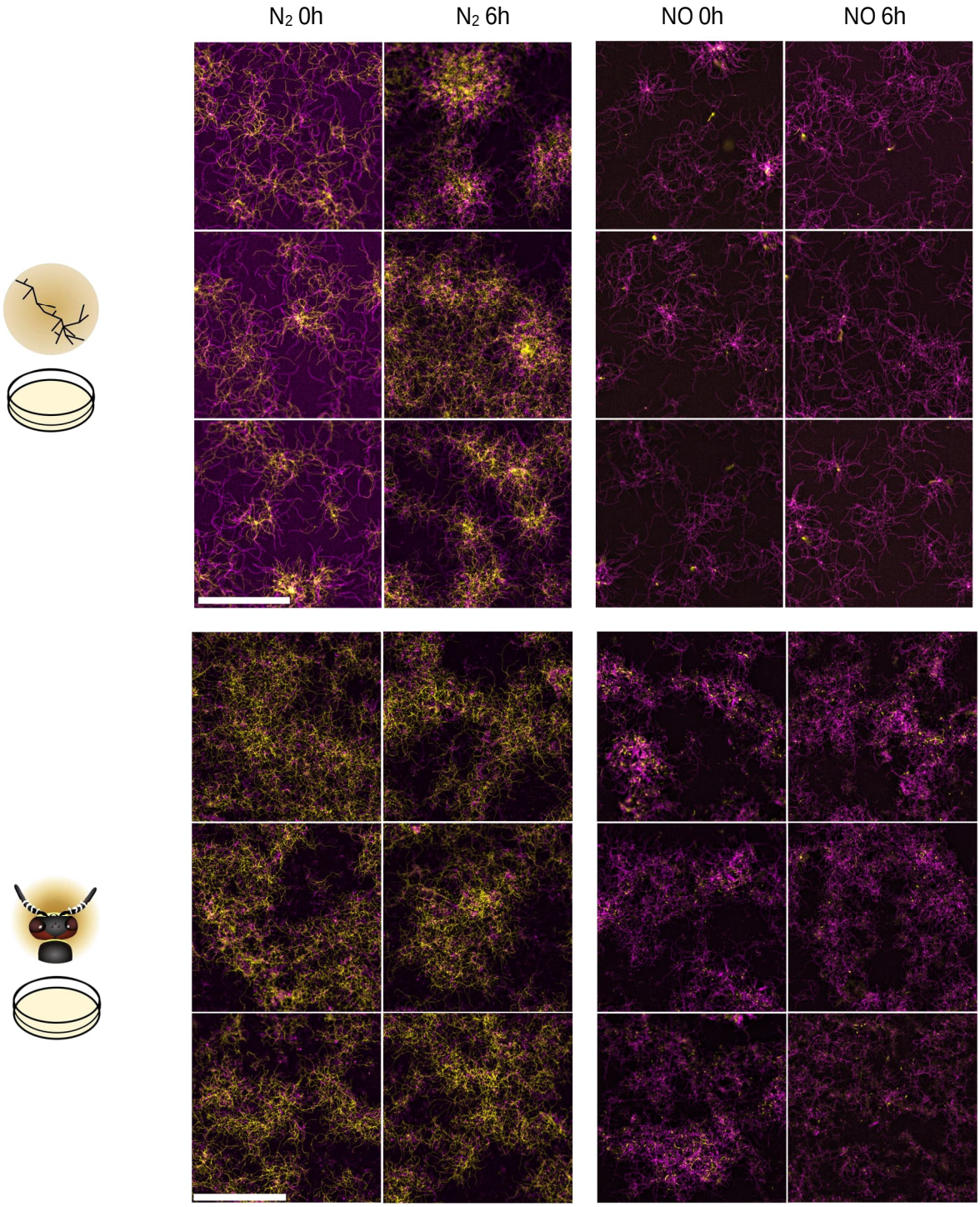
S. philanthi 23Af2 was cultured in a 1:1 mixture of Sf-900 II SFM medium (Gibco, Thermo Fisher Scientific, Germany) and Grace's insect medium (see above) at 30 °C in 24-well plates. To test whether HCs can protect symbiont cells *in vitro*, we transferred 210 µL of a growing culture of *S. philanthi* to a 1.5-mL reaction tube and gently yet thoroughly resuspended it. After division into ten aliquots of 20 µL each, the bacterial cells were pelleted by centrifugation, and the supernatant was reduced. Five control aliquots were plated on 500 µL of a mix of Grace's insect medium, Sf900 medium and 3% agar (ratio 1:1:2; pH 8) in a 48-well plate. The remaining aliquots were mixed with 20 µL (Z)-9-tricosene and then transferred to the same well plate. After 1-h incubation at RT, the plate was positioned in a sealed exposure chamber. Half of the remaining headspace was replaced with 1% NO in N_2 every 6 min over the course of 1 h. Afterward, (Z)-9-tricosene was removed with a pipette. The cultures were monitored for macroscopically visible growth for 8 d.

2.7.12 Ultrastructure of *S. philanthi in vitro* and in the AGS

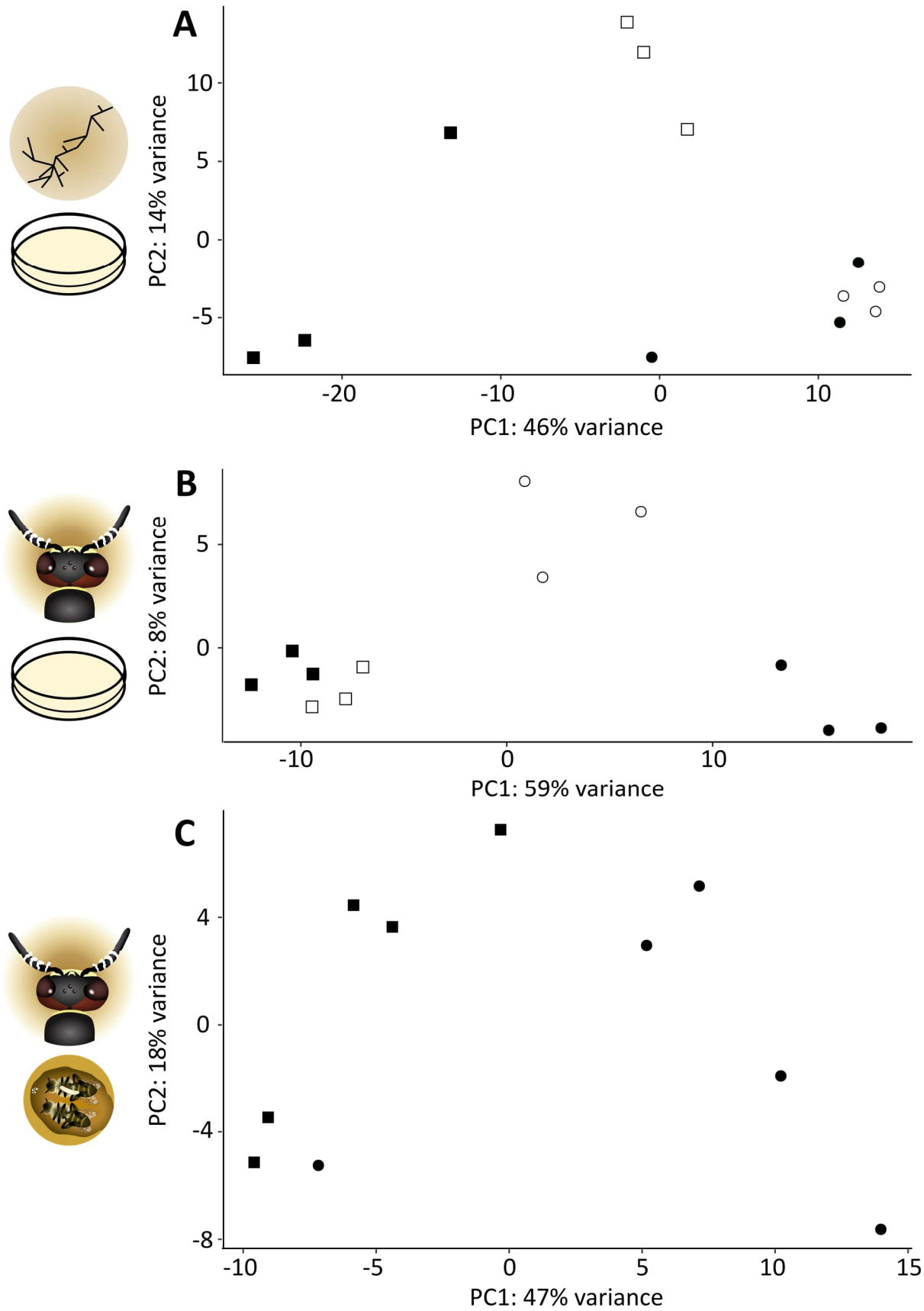
S. philanthi 23Af2 *in vitro* cultures from Grace's insect medium were subjected to SEM as described previously (95). Similarly, AGS applied by beewolf females to plastic foils that had been introduced into beewolf brood cells (see above) were harvested from three brood cells and inspected by SEM as described previously (45).

2.8 Supplement

2.8.1. Supplementary figures

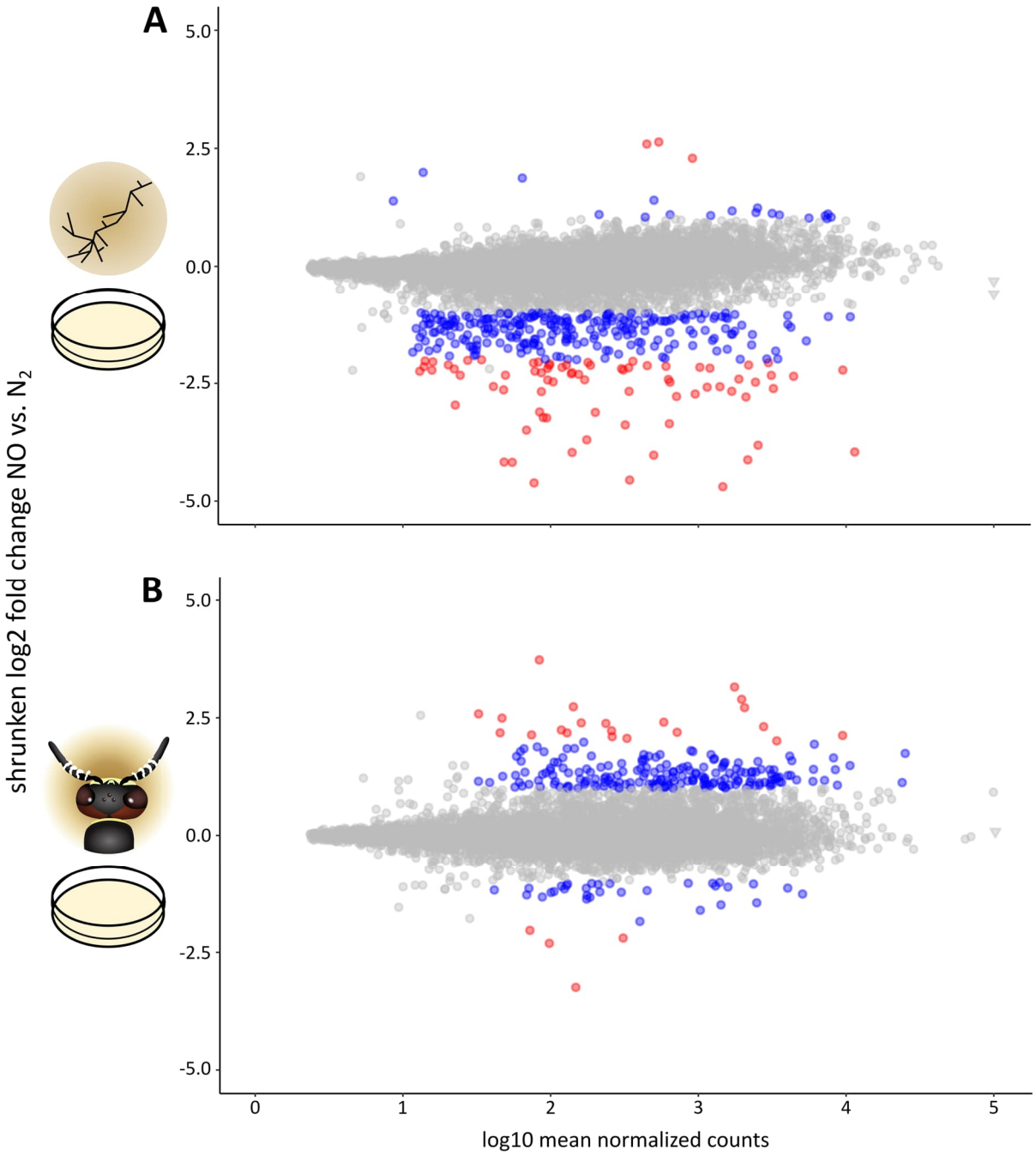


Suppl. Fig. 1: Activity-guided survival assessment of free-living *S. coelicolor* (top, indicated by the symbol of filamentous bacteria) and symbiotic *S. philanthi* (bottom, indicated by the beewolf head) upon exposure to 1% NO diluted in N_2 or pure N_2 *in vitro* (indicated by the petri dish), using a fluorescent live-dead stain on a petri dish. Columns show fluorescent images of three technical replicates of *S. coelicolor* and *S. philanthi* 0 h and 6 h after N_2 or NO treatment, respectively. Yellow: SYTO9 stain of cells with intact cell membranes, i.e. living cells. Magenta: propidium iodide stain of cells with compromised cell membranes, i.e. dead cells. Scale bars: 200 µm.



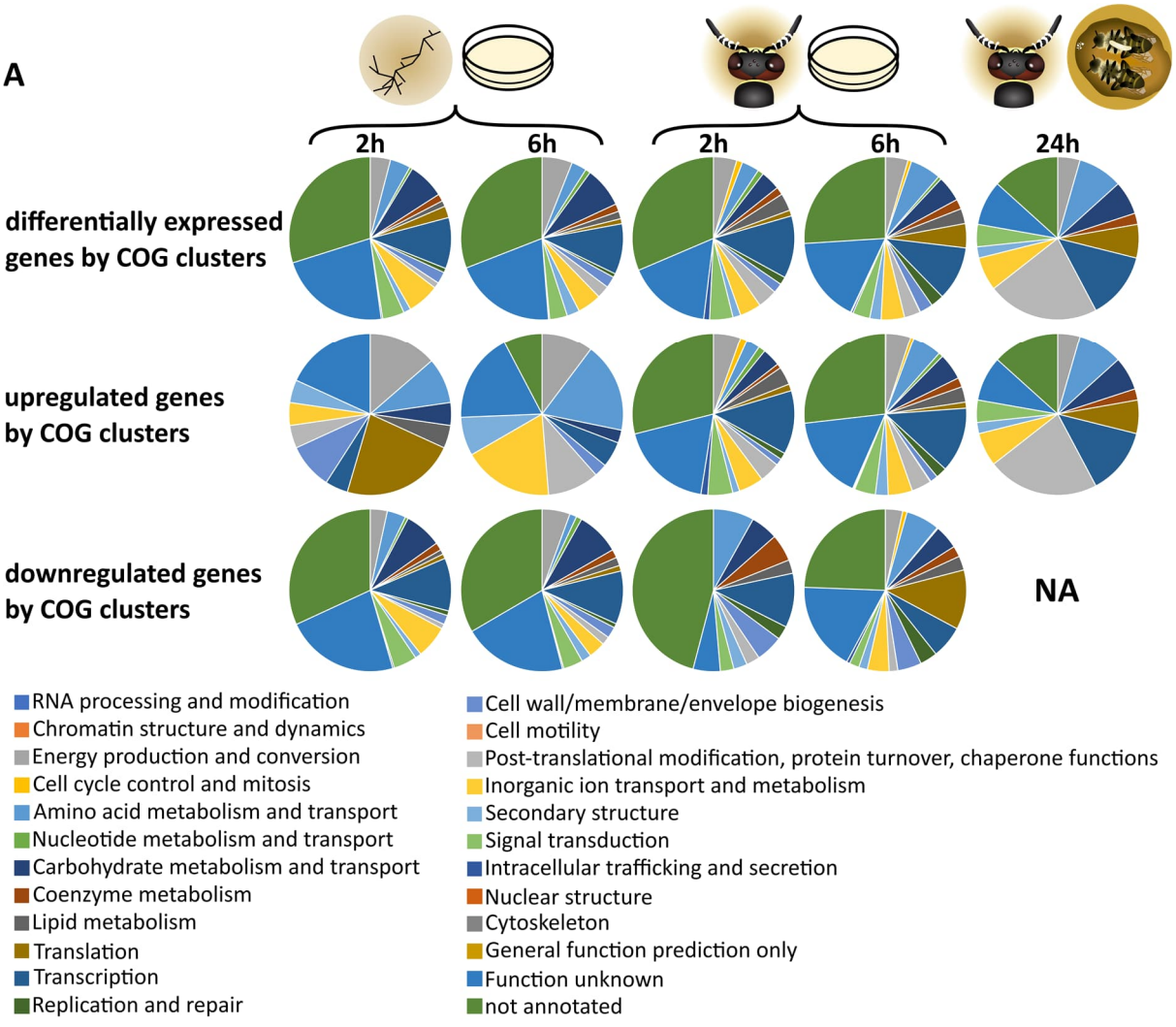
Suppl. Fig. 2: PCA plots of transcriptome experiments of (A) free-living *S. coelicolor* (indicated by the symbol of filamentous bacteria), (B) symbiotic *S. philanthi* (indicated by the beewolf head) 2 h (non-filled symbols) and 6 h (filled symbols) after *in vitro* exposure (indicated by the petri dish) to pure N_2 (squares) or 1% NO diluted in N_2 (circles), and (C) symbiotic *S. philanthi*

in antennal gland secretions incubated in beewolf brood cells (indicated by the beewolf brood cell) without (squares) or with an egg (circles). Expression values were rlog-transformed for PCA analysis.

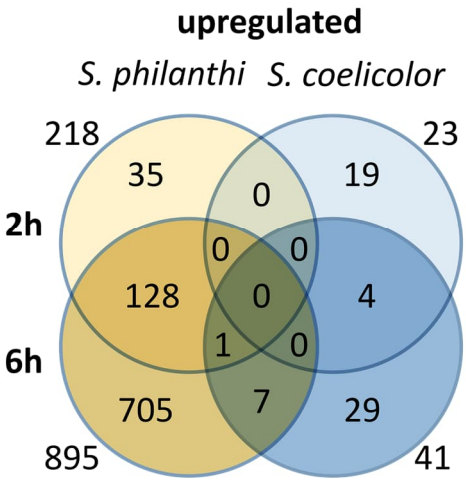


Suppl. Fig. 3: Changes in gene expression in (A) free-living *S. coelicolor* (indicated by the symbol of filamentous bacteria) and (B) symbiotic *S. philanthi* (indicated by the beewolf head) 2 h after *in vitro* exposure (indicated by the petri dish) to NO in comparison to exposure to N_2. Significant gene expression differences are highlighted in color (adjusted $p<0.05$ and 2-4 fold differential expression in blue and more than 4-fold change in red). Log-transformed values of some extremely highly expressed genes were set to 5 to improve readability and are indicated with triangles instead of circles.

A

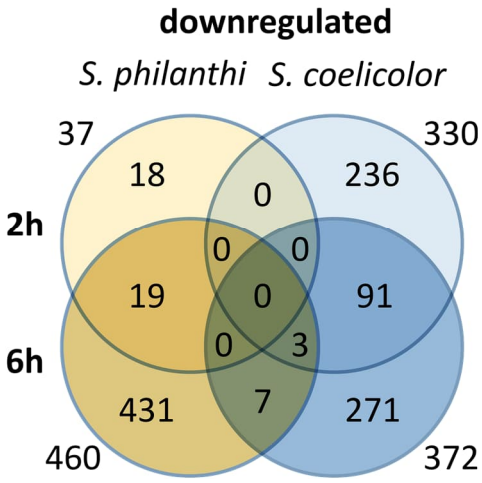


B



- 1x CSP_6103 Fe-S assembly protein SufD

- CSP_4231 thiosulfate sulfurtransferase
- CSP_5685 zinc-binding oxidoreductase split by frameshift fragment 1/3

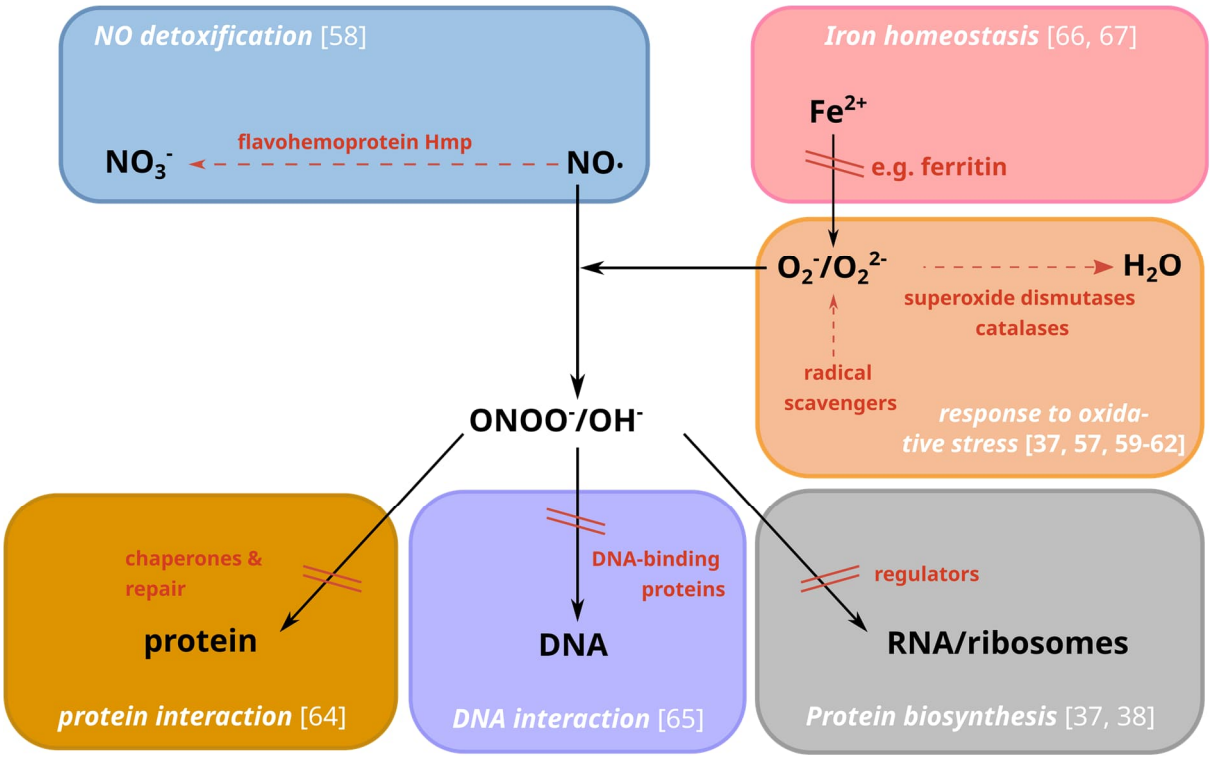


- CSP_3078 hydrolase fragment (mate CSP_3077)
- 3x CSP_4296 conserved hypothetical protein split by frameshift (mate CSP_4295)
- CSP_5183 Lrp/AsnC family transcriptional regulator, regulator for asnA, asnC and gidA

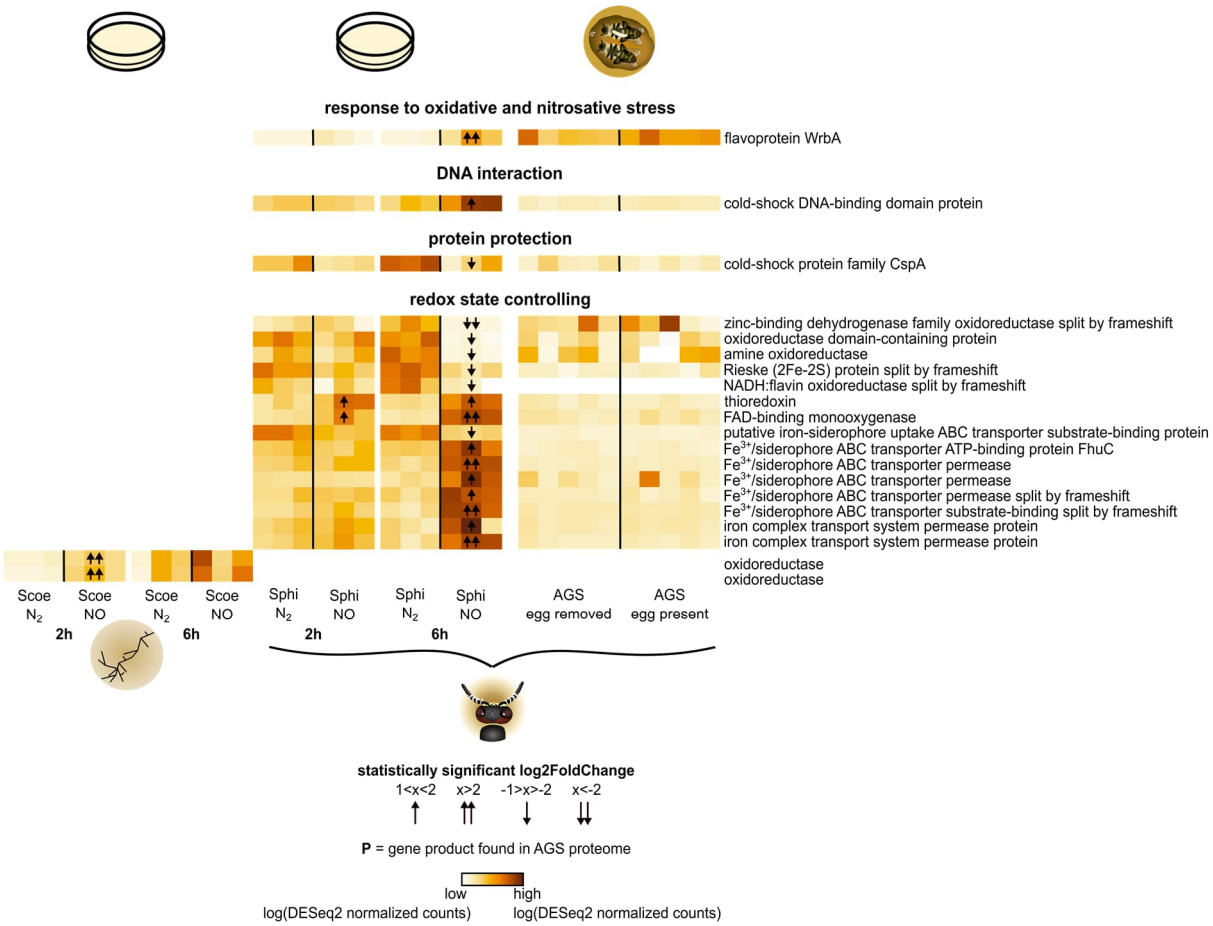
- CSP_1765 conserved hypothetical protein fragment 1/3

- CSP_6104 Rieske (2Fe-2S) iron-sulfur domain-containing protein
- 7x CSP_6105 ABC transporter ATP-binding subunit
- CSP_6106 cysteine desulfurase/selenocysteine lyase
- CSP_6107 SUF system FeS assembly protein
- CSP_6108 conserved hypothetical protein
- CSP_2230 magnesium (Mg^{2+}) and cobalt (Co^{2+}) transporter CorA
- CSP_2310 oligopeptide ABC transporter ATP binding protein OppF
- 7x CSP_2418 conserved hypothetical membrane protein
- CSP_2419 response regulator receiver protein fragment (mate CSP_2422)
- CSP_4875 ABC transporter substrate-binding protein partial (mate CSP_4876)
- CSP_5640 pseudogene the fragment of CSP_5641

Suppl. Fig. 4: (A) COG clusters of differentially expressed up-regulated and down-regulated genes of the gene expression analyses. The columns on the left and in the middle show COG clusters for free-living *S. coelicolor* (indicated by the symbol of filamentous bacteria) and symbiotic *S. philanthi* (indicated by the beewolf head) 2 h and 6 h after NO exposure (compared to N_2 exposure) *in vitro* (indicated by the petri dish). The columns on the right show COG clusters for symbiotic *S. philanthi* in antennal gland secretions incubated in beewolf brood cells for 24 h after oviposition (indicated by the beewolf brood cell). (B) Differentially expressed genes upon NO exposure versus N_2 exposure in *S. philanthi* and *S. coelicolor* 2 and 6 hours after exposure. The upper/lighter colored circles denote differentially expressed genes after 2 h, the lower/darker colored circles after 6 h, while orange colored circles denote *S. philanthi* and blue colored ones *S. coelicolor* differentially expressed genes. For the few genes that were differentially expressed in both *S. coelicolor* and *S. philanthi*, the annotations based on the *S. philanthi* genome are listed below the Venn diagrams.

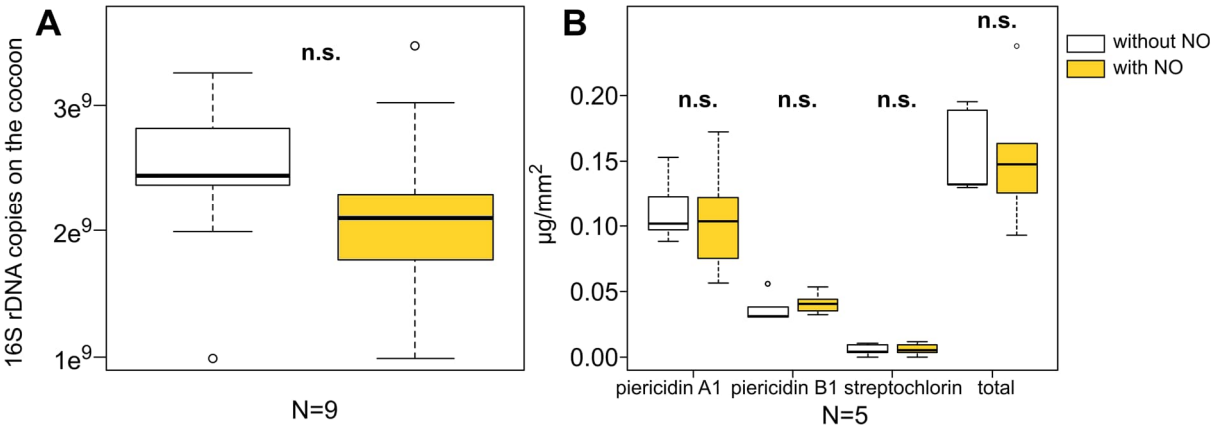


Suppl. Fig. 5: Potential damage to different macromolecules inflicted by NO and NO-derived reactive nitrogen and oxygen species (black arrows), and cellular detoxification mechanisms (red). In addition to direct detoxification by flavohemoprotein (58), the detrimental effects of NO can be indirectly mitigated: Chaperones, DNA-binding proteins and other regulators attenuate damage to proteins and nucleic acids inflicted by NO-derived reactive nitrogen and oxygen species (37, 38, 64, 65). To prevent the formation of these highly reactive species, ferrous iron and super- and peroxide can be intercepted (37, 57, 59-62, 66, 67). NO_3^- = nitrate, NO· = nitric oxide, Fe^{2+} = ferrous iron, O_2^- = superoxide, O_2^{2-} = peroxide, $ONOO^-$ = peroxynitrite, OH^- = hydroxide. References are given in square brackets.

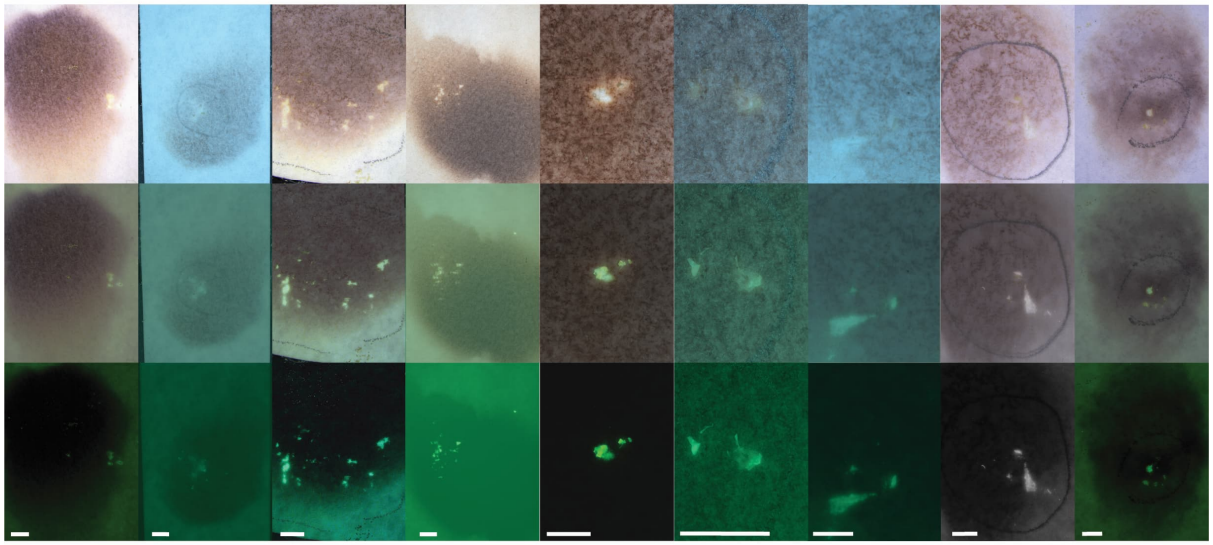


Suppl. Fig. 6: Differentially expressed genes upon nitric oxide exposure *in vitro* that are unique to one of the bacterial strains. Scoe = free-living *S. coelicolor* (also indicated by the symbol of filamentous bacteria), Sphi = symbiotic *S. philanthi* (also indicated by the beewolf head), AGS = antennal gland secretion. The petri dish indicates gene expression *in vitro*, the beewolf brood cell symbolizes gene expression within the AGS.

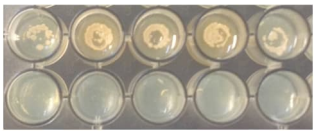
Suppl. Fig. 7: 2D gel image of the soluble proteins extracted from a pooled sample of AGS from 70 beewolf brood cells. 90 spots were excised and used for LC-MS analysis.



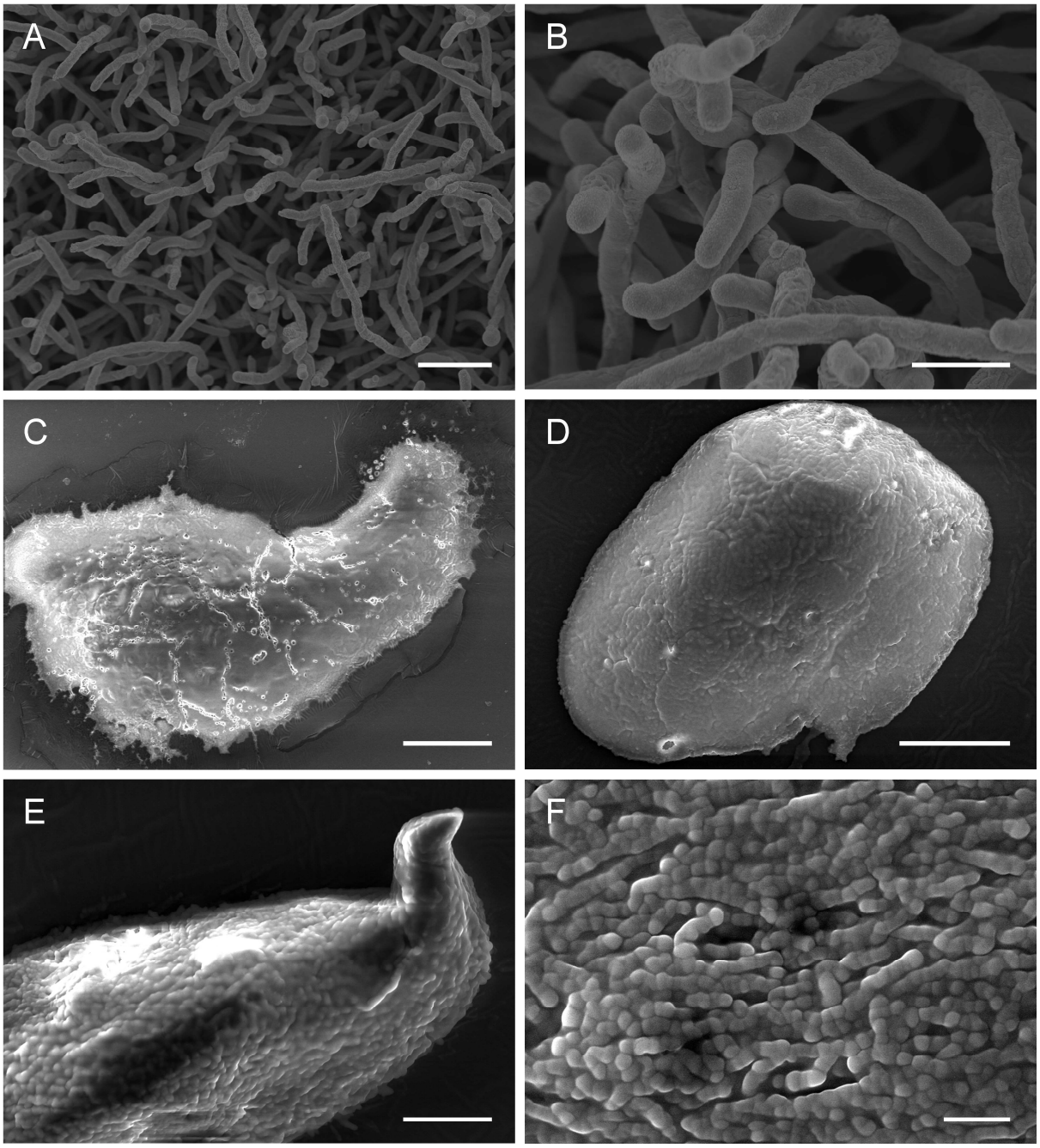
Suppl. Fig. 8: Titers and antibiotic production of symbiotic *S. philanthi* on the cocoon remain unaffected by exposure to NO in the brood cell. (A) Titers of symbionts on the cocoon after exposure to NO released by the beewolf egg within the secreted AGS from beewolf brood cells, compared to titers of symbionts transiently removed from the brood cells during NO release. Titers on the cocoon surface were quantified seven days after cocoon spinning. Paired t-test, t = -0.835, df = 8, p = 0.428, N = 9. (B) Amount of antibiotics on the cocoon surface. The same cocoons as in (A) were used. Paired t-tests, p > 0.05.



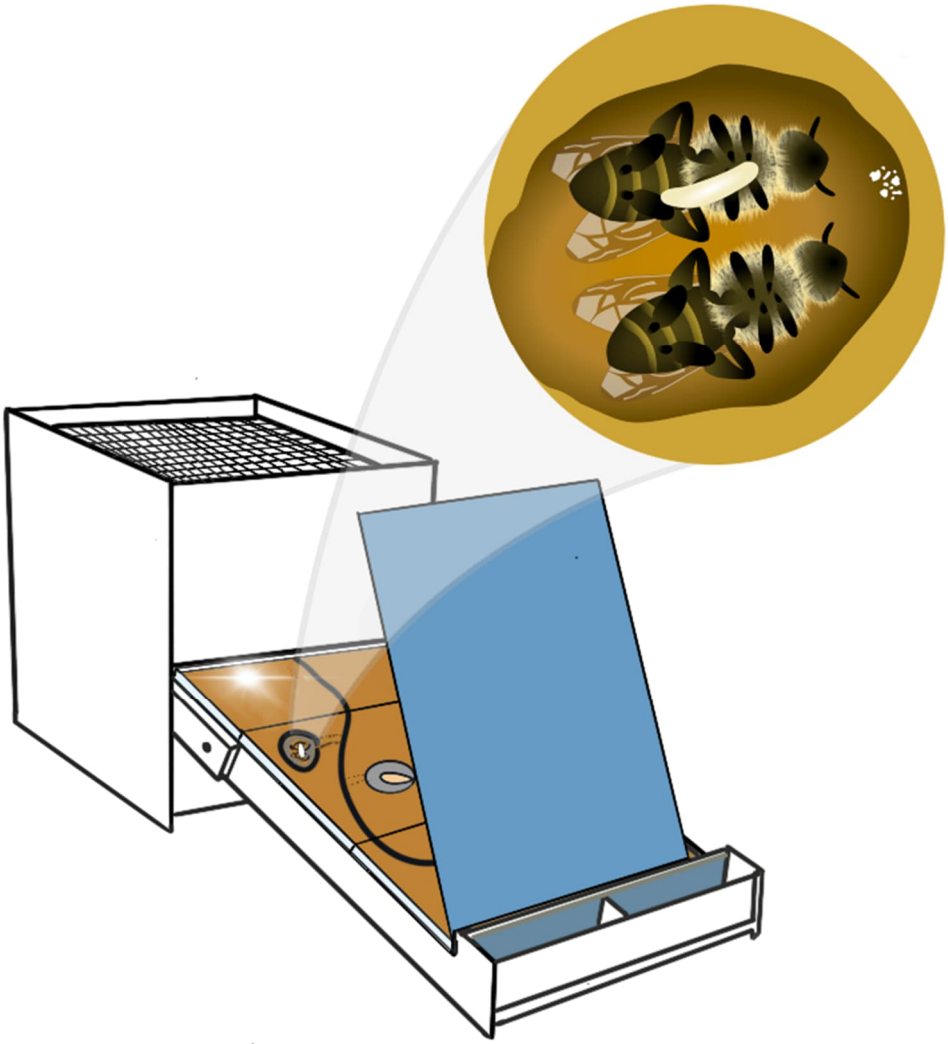
Suppl. Fig. 9: The AGS prevents diffusion of NO to a filter paper containing NO indicator solution (iodine-starch solution). The figure shows the raw data to the data summarized in Figure 4D of the main manuscript. Each scale bar indicates 2 mm. (A) Light micrographs of filter paper with AGS after exposure to NO in brood cells. (B) Autofluorescence micrographs of the same areas as in (A). (C) Superimposed images.



Suppl. Fig. 10: Synthetic hydrocarbons protect growing *S. philanthi* cultures against lethal NO exposure *in vitro*. Top row: cultures covered in (Z)-9-tricosene during exposure. Bottom row: control cultures without hydrocarbons.



Suppl. Fig. 11: Ultrastructure of *S. philanthi* cells *in vitro* and in the AGS. (A,B) Scanning electron micrographs (SEM) of *S. philanthi* growing *in vitro* in Grace's medium. (C-E) SEM micrographs of the AGS from three different beewolf brood cells. (F) Close-up of (E), showing individual *S. philanthi* cells covered by the AGS matrix that is rich in hydrocarbons. Scale bars: (A) 5 µm, (B) 2 µm, (C) 200 µm, (D) 50 µm, (E) 20 µm, (F) 5 µm.



Suppl. Fig. 12: Setup of a beewolf observation cage, with an exemplary brood cell (enlarged), showing a beewolf egg on the provisioned honeybees as well as the AGS on the ceiling of the brood cell.

2.8.2 Supplementary tables

Suppl. Tab. 1: Number of expressed protein-coding genes in the beewolf symbiont *S. philanthi* (7930 protein-coding sequences in total) and the free-living *S. coelicolor* (7825 protein-coding sequences in total) for each treatment-time point combination of the *in vitro* gene expression analysis (three replicates per species, treatment and time point).

Replicate	*S. philanthi* (#)	*S. coelicolor* (#)
N_2 2h (1)	7898	7626
N_2 2h (2)	7914	7653
N_2 2h (3)	7907	7636
N_2 6h (1)	7900	7621
N_2 6h (2)	7911	7656
N2 6h (3)	7913	7624
NO 2h (1)	7902	7613
NO 2h (2)	7852	7613
NO 2h (3)	7852	7616
NO 6h (1)	7870	7586
NO 6h (2)	7827	7606
NO 6h (3)	7880	7648

Suppl. Tab. 2: Number of protein-coding genes expressed by symbiotic *S. philanthi* (7930 protein-coding genes in total) for each treatment of the *in vivo* gene expression analysis.

Treatment	Replicate	# expressed genes
NO-unexposed	B	7421
	G	7313
	J	7622
	K	7515
	N	6944
NO-exposed	A	7503
	C	7617
	E	7660
	L	7526
	M	7647

Suppl. Tab. 3: Impact of NO exposure in the brood cell on symbiont titers and antibiotic production on the cocoon. Shown are the statistical analyses of DNA copies on the cocoon and amount of the major antibiotics per cocoon area.

Dataset	N	Shapiro-Wilk normality test of differences	Paired t-test
DNA copies	9	W=0.840, p=0.058	t=-0.835, df=8, p=0.428
piericidin A1 [µg/mm^2]	5	W=0.921, p=0.536	t=-0.691, df=4, p=0.528
piericidin B1 [µg/mm^2]	5	W=0.944, p=0.693	t=-0.520, df=4, p=0.631
streptochlorin [µg/mm^2]	5	W=0.861, p=0.232	t=-0.081, df=4, p=0.939
total amount of antibiotics [µg/mm^2]	5	W=0.966, p=0.847	t=-0.179, df=4, p=0.867

Suppl. Tab. 4: Impact of NO exposure in the brood cell on symbiont titers on the cocoon, given as DNA copies on the respective cocoons.

Individual	Brood cell	Treatment	DNA copies
1	6_1	NO	$2.096e^{9}$
1	24_1	w/o NO	$2.363e^{9}$
4	28_1	NO	$3.022e^{9}$
4	19_1	w/o NO	$2.527e^{9}$
5	19_2	NO	$1.766e^{9}$
5	10_1	w/o NO	$9.850e^{8}$
6	17_1	NO	$9.822e^{8}$
6	17_2	w/o NO	$2.813e^{9}$
8	27_4	NO	$1.281e^{9}$
8	14_3	w/o NO	$3.258e^{9}$
9	27_2	NO	$3.476e^{9}$
9	27_4	w/o NO	$2.933e^{9}$
10	15_3	NO	$2.297e^{9}$
10	15_4	w/o NO	$2.444e^{9}$
11	18_8	NO	$2.267e^{9}$
11	18_3	w/o NO	$2.441e^{9}$
11	5_7	NO	$2.086e^{9}$
11	5_9	w/o NO	$1.992e^{9}$

Suppl. Tab. 5: Cocoon measurements, cocoon area, and antibiotics per cocoon area of brood cells in which the symbionts were or were not exposed to the NO produced by the beewolf egg. PA1 = piericidin A1, PB1 = piericidin B1, S = streptochlorin, total = total amount of antibiotics.

Individual	Brood cell	Treatment	Length [mm]	Width [mm]	Area [mm^2]	PA1 [µg]	PB1 [µg]	S [µg]	total [µg]
11	18_8	NO	15.2	4.6	69.92	8.57	2.48	0.38	11.42
11	18_3	w/o NO	14.1	4.1	57.81	7.11	3.24	0.57	10.92
11	5_7	NO	15.0	5	75.00	4.23	2.46	0.27	6.96
11	5_9	w/o NO	13.4	3.7	49.58	4.38	1.90	0.19	6.46
4	28_1	NO	16.1	4.8	77.28	5.84	3.16	0.73	9.73
4	19_1	w/o NO	20.5	6.3	129.15	13.16	3.99	0.00	17.13
6	17_1	NO	13.75	3.6	49.50	5.12	2.20	0.00	7.31
6	17_2	w/o NO	19.3	5.2	100.36	9.76	3.07	0.46	13.30
8	27_4	NO	17.0	4.5	76.50	13.16	4.09	0.90	18.15
8	14_3	w/o NO	17.35	4.85	84.15	12.88	2.65	0.92	16.44

Suppl. Tab. 6: Protective activity of the AGS against NO diffusion in the beewolf brood cell. Given are the mean gray values of the NO-indicator filter paper bioassay.

Brood cell	Treatment	Mean gray value
1	AGS	1.755
	Filter paper NO	3.349
	Control	1.092
2	AGS	1.630
	Filter paper NO	1.990
	Control	1.021
3	AGS	1.333
	Filter paper NO	1.839
	Control	1.043
4	AGS	1.347
	Filter paper NO	1.690
	Control	1.107
5	AGS	1.226
	Filter paper NO	1.689
	Control	0.914
6	AGS	1.324
	Filter paper NO	1.926
	Control	1.032
7	AGS	1.757
	Filter paper NO	2.649
	Control	0.845
8	AGS	3.576
	Filter paper NO	3.920
	Control	0.934
10	AGS	1.151
	Filter paper NO	1.412
	Control	1.026

Suppl. Tab. 7: Diffusion barrier effect of beewolf hydrocarbons on an iodine starch indicator solution exposed to NO. The numbers represent spectrophotometrically measured absorbance values at 540 nm of differently treated NO indicator solutions after 1h of NO exposure.

Sample	OD_{540}					
Beewolf CHC extract	0.090	0.102	0.058	0.063	0.053	0.053
Indicator solution + hexane	1.764	1.672	1.716	1.840	1.706	1.820
Untreated indicator solution	0.053	0.057	0.046	0.048	0.053	0.053

2.9 Data, materials, and software availability

Transcriptome sequencing data have been deposited in the NCBI database under accession numbers BioProject PRJNA975590 (104), BioSamples SAMN35335670–SAMN35335672 (105-107), SRAs SRX20486080–SRX20486113 (108-141). Proteomics data are available from the Open Research Data Repository Edmond of the Max Planck Society (https://doi.org/10.17617/3.5r) (71).

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2.11 Competing interests

The authors declare no competing interests.

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CHAPTER 3: Protection of a defensive symbiont does not constrain the composition of the multifunctional hydrocarbon profile in digger wasps

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C.S.I.: conceptualization, investigation, methodology, visualization, writing—original draft; [redacted]: conceptualization, investigation, methodology, writing—review and editing; [redacted]: conceptualization, funding acquisition, investigation, methodology, project administration, supervision, writing—review and editing. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

3.1 Abstract

Hydrocarbons (HCs) fulfill indispensable functions in insects, protecting against desiccation and serving for chemical communication. However, the link between composition and function, and the selection pressures shaping HC profiles remain poorly understood. Beewolf digger wasps (Hymenoptera: Crabronidae) use an antennal gland secretion rich in linear unsaturated HCs to form a hydrophobic barrier around their defensive bacterial symbiont, protecting it from brood cell fumigation by toxic egg-produced nitric oxide (NO). Virtually identical HC compositions mediate desiccation protection and prey preservation from molding in underground beewolf brood cells. It is unknown whether this composition presents an optimized adaptation to all functions, or a compromise due to conflicting selection pressures. Here, we reconstitute the NO barrier with single and binary combinations of synthetic linear saturated and unsaturated HCs, corresponding to HCs found in beewolves. The results show that pure alkanes as well as 3 : 1 mixtures of alkanes and alkenes resembling the composition of beewolf HCs form efficient protective barriers against NO, indicating that protection can be achieved by different mixtures of HCs. Since *in vitro* assays with symbiont cultures from different beewolf hosts indicate widespread NO sensitivity, HC-mediated protection from NO is likely important across Philanthini wasps. We conclude that HC-mediated protection of the

symbiont from NO does not exert a conflicting selection pressure on the multifunctional HC profile of beewolves.

3.2 Introduction

Cuticular hydrocarbons (CHCs) protect insects from desiccation, and have often evolved secondary functions, e.g. intra- and interspecific communication in social and solitary insects (1-3). Constituents of insect CHC profiles include n-alkanes, methyl-branched alkanes, alkenes, and alkadienes of different chain lengths (2), forming complex compositions with up to more than 100 different components (1). The chemical composition influences the function of the profile (3). One profile frequently fulfills multiple functions, e.g. cuticle lubrication (4), prey protection from pathogens (5, 6) and enhancing tarsal adhesion (7), posing potentially conflicting requirements on the CHC composition (3). However, due to its complexity, it remains poorly understood how composition influences function, and how natural selection shapes composition considering the functional constraints (3).

An intriguing multifunctional hydrocarbon (HC) profile has recently been reported for a group of solitary digger wasps (6, 8). These beewolves (Hymenoptera: Crabronidae, *Philanthus triangulum*) construct brood cells in sandy soil and mass-provision their offspring with paralyzed honeybee workers (*Apis mellifera*)(9-11). Females embalm the bees in an alkene-rich HC secretion from their postpharyngeal gland (PPG), which prevents water condensation and thereby reduces fungal infestation (5, 6, 12). Two further adaptations to protect the offspring have evolved in beewolves: First, females secrete defensive "*Streptomyces philanthi*" symbionts from specialized antennal gland reservoirs into the brood cell (13, 14). After integration into the larval cocoon (13), symbiont-produced antibiotics provide efficient long-term protection against opportunistic microbes (13, 15). Second, the beewolf egg sanitizes the brood cell with toxic nitric oxide (NO) (16), preventing pathogen growth without harming the symbionts present in the brood cell during NO fumigation (8). Recently, the HCs in the antennal gland secretion (AGS) (17) were found to form a hydrophobic barrier against NO around '*S. philanthi*' (8).

The nearly identical HC profiles of the cuticle, the PPG and the AGS of *P. triangulum* are characterized by an approximately 3:1 alkene-alkane ratio, with compounds ranging from C21 to C31 in chain length (17-20). Tricosane (C23) represents the most abundant alkane (17-20), and either pentacosene (C25:1) or heptacosene (C27:1) constitute the most abundant alkene (17, 19, 20). Together with tricosane, C25:1 or C27:1 account for 51-92% of HCs (17-20). Thus, desiccation protection, prey preservation, and symbiont protection are realized by virtually the same profile (Suppl. Tab. 1, Suppl. Fig. 1) (17-20). However, it remains unclear whether the alkene-rich HC profile presents an adaptation to all three functions, or a compromise arising from conflicting selection pressures. Here we tested single and binary mixtures of synthetic linear saturated and unsaturated HCs, corresponding to those found in beewolves, for their effectiveness in blocking NO. We demonstrate that a range of individual HCs, and mixtures of alkenes and alkanes resembling beewolf HC extracts, are effective NO barriers *in vitro*. Additionally, we show that symbiont strains from multiple different host species are susceptible to NO *in vitro* in the absence of HCs, and that CHC profiles across different beewolves species have similar compositions, indicating a widespread HC-based protection of defensive symbionts across Philanthini. While our findings support the important function of HCs in the AGS, we argue that AGS-mediated symbiont protection does not exert a conflicting selection pressure on the multifunctional HC profile and thus does not constrain its composition.

3.3 Results and discussion

We assessed NO sensitivity of five '*S. philanthi*' biovariations from all host genera (21) and six free-living *Streptomyces* species (Suppl. Tab. 2) to five NO concentrations. Survival was assessed as a trinary response (no growth, growing slower than control (without NO), growing as quickly as control). Most of the symbiont strains were already affected by low NO concentrations, with growth completely ceasing at 1% NO. By contrast, most free-living strains were unaffected at concentrations below 1% and still grew at 1% NO. In the statistical analysis, our final model retained 'bacterial category/strain' and 'NO concentration' as independent variables (Supplemental methods). Symbionts were significantly more sensitive to NO than free-living *Streptomyces* (Anova factor 'bacterial category', $\chi 2 = 103.5$, d.f. = 2, $p < 2.2 \times 10^{-16}$, Fig. 1), and strains varied in their NO sensitivity (Anova factor 'bacterial category/strain', $\chi 2 = 155.4$, d.f. = 40, $p = 1.618 \times 10^{-15}$). Expectedly, the impact of NO on bacterial growth increased with concentration (Anova factor 'NO concentration', $\chi 2 = 200.3$, d.f. = 2, $p < 2.2 \times 10^{-16}$). Given the symbionts' sensitivity towards NO, we hypothesize that other beewolf hosts may use their HCs in the AGS to protect their symbionts from NO fumigation.

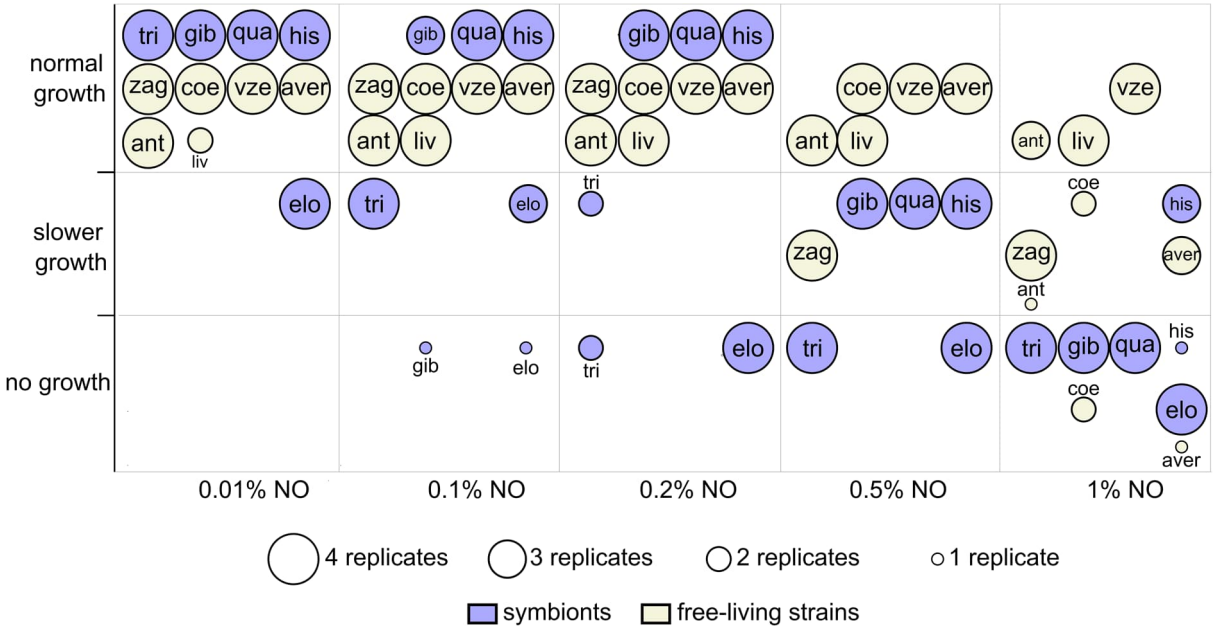


Fig. 1: Nitric oxide (NO) sensitivity of '*S. philanthi*' biovariations and free-living *Streptomyces* at different NO concentrations. Symbionts (purple) were more sensitive to NO than free-living *Streptomyces* (off-white) (ANOVA, $\chi^2 = 103.5$, d.f. = 2, $p < 2.2 \times 10^{-16}$), and growth inhibition increased with NO concentration (ANOVA, $\chi^2 = 200.3$, d.f. = 2, $p < 2.2 \times 10^{-16}$). In addition, the strains varied in their NO sensitivity (ANOVA, $\chi^2 = 155.4$, d.f. = 40, $p = 1.618 \times 10^{-15}$). The sizes of the circles indicate the number of replicates in the different growth categories. See Suppl. Tab. 2 for strain designations.

To examine the link between HC composition and NO barrier function, we tested synthetic binary alkene-alkane (3:1) mixtures mimicking the AGS composition of *P. triangulum*, and their individual constituents for their ability to protect an NO indicator solution against oxidation. CHC extracts of *P. triangulum* (56-349 µg CHCs, $\bar{x}$ = 161.0 µg) served as positive controls. Their alkene-alkane ratio diverged slightly from previous reports (Suppl. Tab. 1, Suppl. Fig. 1). Expectedly, beewolf extracts protected the indicator solution from oxidation. The degree of protection was not correlated with CHC amounts (Spearman's rank correlation, N = 19, rho = -0.335, S = 1522, p = 0.161; Suppl. Fig. 2), as they probably fell into the maximal range of protection. The combination of (Z)-9-pentacosene (C25:1) and heptacosane (C27) was as effective as the extracts (Tukey's HSD, N = 8, $p < 0.05$, Fig. 2), while C27,

C23, and C25:1+C23 (Tukey's HSD, N = 8, $p < 0.05$, Fig. 2) exhibited attenuated protection. The effect was still observed after a 10-fold reduction of the applied amount of HCs (Suppl. Fig. 3). C25:1 did not prevent NO from reacting with the indicator solution (Tukey's HSD, N = 8, $p < 0.05$, Fig.2).
Our experiments revealed that the hydrophobic NO barrier can be reconstituted by single and binary HC combinations and is not specific to certain HCs, implying a general effect. As previously observed in phase behaviour (22), binary combinations did not behave as predicted from individual HCs: While ineffective individually, C25:1 enhanced the effect of C27, but not of C23. C25:1 did not provide a barrier to NO, but C23:1-covered '*S. philanthi*' survived an otherwise lethal NO exposure (8). This may be explained by the difference in the HC amount applied in both experiments resulting from the need to apply the HC without a harmful solvent in the previous study. Interestingly, C25:1+C27 protected better from NO than C25:1+C23, although the latter more closely resemble *P. triangulum* CHC extracts (17).

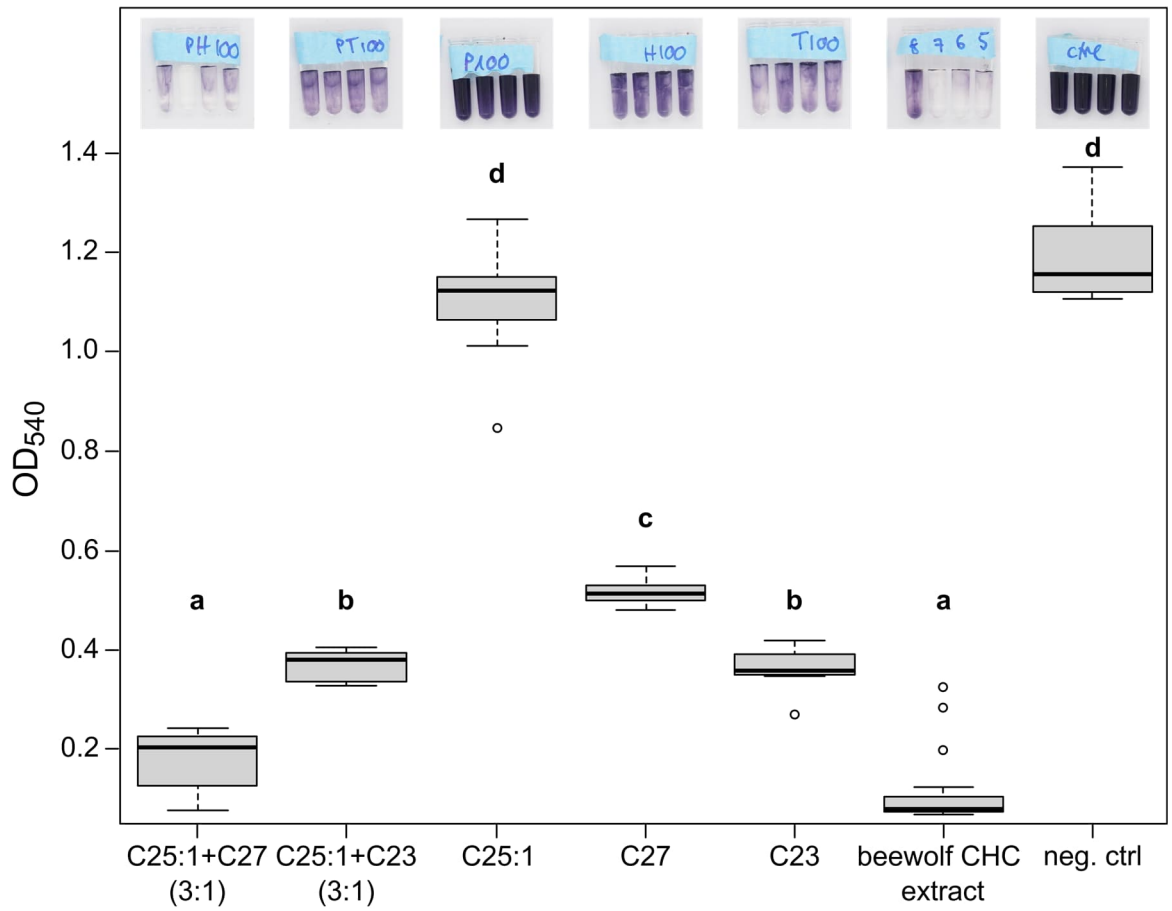


Fig. 2: Reconstitution of the nitric oxide (NO) barrier effect with single and binary combinations of synthetic hydrocarbons (HCs). A total of 100 µg of HCs was applied for all treatments. Effectivity of HCs in blocking NO was measured as the change in coloration in an NO indicator solution, with higher OD_{540} values indicating stronger oxidation and thus less protection against NO by the HC layer (see representative images on the top; for all images see Suppl. Fig. 4). Beewolf CHC extracts served as positive, and hexane as negative control. All HCs and the alkene–alkane ratio of binary combinations are found in *P. triangulum* AGS and HC extracts (Suppl. Tab. 1, Suppl. Fig. 1). Letters indicate significant differences (Tukey's HSD, N = 8, $p < 0.05$).

Assuming a common site of production and/or a shared pathway for HC biosynthesis for the AGS, PPG, and cuticle, the HC composition is likely shaped by selection acting simultaneously on symbiont

protection (AGS), prey preservation (PPG), and desiccation resistance (cuticle). The more complex profile of the AGS (as well as the cuticle and the PPG) – as opposed to a simpler mixture of C25:1 and C27 - might be explained by the wider melting range of a more complex composition ensuring adequate viscosity or establishing a biphasic secretion under varying environmental conditions (23). Furthermore, less abundant HCs may serve a role in AGS localization by the larva or as nutrients for '*S. philanthi*' (17, 24-26). Alternatively, unspecific enzymes in the HC biosynthesis may produce a homologous HC series as a byproduct, without the minor HCs being selectively favoured (3).

In addition to *P. triangulum*, NO defense has been observed for the North American *P. gibbosus* and *P. basilaris* (M. Kaltenpoth & T. Engl, personal observation), suggesting a widespread distribution across Philanthini. Therefore, we assessed new and previously published HC profiles from available host species to speculate on their potential for symbiont protection from NO. CHC extracts of *P. histrio* were quantified using gas chromatography-mass spectrometry (GC-MS) and consisted of 76% alkenes/alkadienes, and 24% alkanes. Tritriacontene (C33:1), hentriacontene (C31:1), and hentriacontadiene (C31:2) accounted for approximately 67% of CHCs. Heptacosane (C27) and pentacosane (C25) (approx. 9% each) were the most abundant alkanes (Suppl. Tab. 3, Suppl. Fig. 5). Chain lengths of the dominant HCs varied across species (Suppl. Tab. 3, Suppl. Fig. 5). The alkene/alkadiene-alkane ratio of *P. histrio* resembled previously published profiles of *Trachypus elongatus* (64%/36%; Suppl. Tab. 3, Suppl. Fig. 5) (27). Assuming that alkenes and alkadienes have similar physicochemical properties, it is also comparable to previously published alkene/alkane ratios of *P. triangulum* (C25 chemotype: 76%/23%; C27 chemotype: 72%/26%) (17) and *P. gibbosus* (87%/13%) (28). Their equally alkene- and alkene/alkadiene-rich HC profiles may qualify other beewolves to provide a hydrophobic NO barrier to protect their symbionts if they fumigate their brood cells. The reported NO fumigation in *P. triangulum*, *P. gibbosus* and *P. basilaris*, and the ecology shared among beewolves renders a widespread NO fumigation across Philanthini wasps plausible.

Apart from blocking NO, similar HC profiles mediate prey preservation (5, 6, 12) and protect *P. triangulum* from desiccation. Previous studies indicate a selection for alkene-rich profiles in Philanthinae digger wasps, including beewolves (Philanthini) and part of the Cercerini, to efficiently preserve decay-prone Hymenopteran prey (e.g. Apidae and Halictidae) (29). By contrast, basal Cercerini providing unembalmed Coleoptera possess diversifying HC profiles, with often lower amounts of alkenes (29). Furthermore, Aphilanthopsini are unlikely to embalm their ant prey, which is presumably less susceptible to microbial threats (28). Thus, prey preservation likely evolved independently in the derived Hymenoptera-hunting Cercerini and in the ancestor of the Philanthini, the latter probably coinciding with the acquisition of defensive symbionts about 68 mya (30). Although additional evidence is needed, we speculate that the evolutionary origin of NO fumigation and the HC-mediated protection of the symbionts may have coincided with the origins of symbiosis and prey embalming (Suppl. Fig. 6).

Prey preservation likely selects for high proportions of alkenes (29), and our experiments indicate their suitability for NO protection. For desiccation protection, the large qualitative variety of CHC in insects (3) suggests that this function can be realized by very different compositions, provided they form a biphasic layer (23). Thus, HC-mediated protection of the symbionts does not appear to impose a conflicting selection pressure on the composition of the beewolf AGS that could otherwise compromise the efficiency of the same HC profile for desiccation resistance and prey preservation.

3.4 Methods

3.4.1 Bacterial cultivation

Bacterial strains were cultured in a 1:1 mixture of Sf-900 II SFM medium (Gibco, Thermo Fisher Scientific, Germany) and Grace's insect medium (Supplemental methods; Sigma-Aldrich, Germany) at 30°C in 24-well plates.

3.4.2 Comparative cultivation assay

We assessed the NO sensitivity of five symbiont strains representing all three host genera and different geographic origins (21), and six free-living *Streptomyces* strains obtained from DSMZ (Braunschweig, Germany). After NO exposure (Supplemental methods), we analyzed the trinary growth observation (no growth, growth observed later than in the control, growth observed at the same time as in the control; N = 2-4) using a multinominal regression model. Growth was examined as a function of NO concentration and bacterial category (symbiont versus free-living *Streptomyces*), with 'strain' as a nested factor within bacterial category. Starting from the full factorial model, we used a step-wise reduction of model complexity to select the best-fitting model. Statistical analyses were performed in R i386 4.1.2 using the 'nnet' (31) and 'car' (32) packages.

3.4.3 Extraction and quantification of beewolf CHCs

P. triangulum females collected in Berlin, Germany, were reared in observation cages (33). We assessed CHC extracts from 19 females regarding their efficacy as an NO barrier. The females' antennae were removed. One female per extract was submerged in 1 ml hexane. After a 10 min extraction under stirring at RT, the female was removed, and hexane was evaporated under argon flow. CHCs were re-dissolved in 100 µl hexane. A 95 µl aliquot of each extract was evaporated under argon flow and stored at -20°C. The remaining 5 µl were used for GC-MS (Supplemental methods). We characterized the CHC composition of *P. histrio* using a single female from a collection near Knysna, Western Cape Province, South Africa, in 2005. After removing the head, thorax and abdomen were extracted for 30 minutes in hexane. The extract was subjected to GC-MS (Supplemental methods).

3.4.4 Cuticular hydrocarbon experiments

We purchased HCs from Sigma Aldrich, Germany, and Cayman Chemical, Michigan, USA. C25:1 was combined with C23 or C27 in a 3:1 ratio to mimic the alkene-alkane ratio found in beewolves. We transferred 10 µl of hexane containing 100 µg, 50 µg, or 10 µg of each treatment (N = 7-8 each), on top of 40 µl NO indicator solution (Supplemental methods) in tubes (diameter = 3 mm; Biozym, Germany), respectively. As a positive control, we transferred each beewolf CHC extract in the same way. Indicator solutions treated with 10 µl hexane served as negative controls (N = 8). The applied HCs form a distinct layer on top of the indicator solution due to their hydrophobicity. After NO exposure (Supplemental methods), the content of the tubes was centrifuged in 0.5 ml tubes at maximum speed for 30 s. We measured the OD_{540} of 20 µl of the supernatant in a 384 well plate (VarioSkan Lux, Thermo Scientific, Germany). We performed OD_{540} comparisons across all 100 µg treatments and both controls, and within treatments, using a one-way ANOVA. The correlation between the amount of beewolf CHCs and the OD_{540} was analyzed using Spearman's rank correlation. Statistical analyses were conducted in R (V4.15).

3.5 Supplement

3.5.1 Supplemental methods

3.5.1.1 Model fitting for comparative cultivation assay

m0 <- multinom(growth observation~bacterial category/strain*NO concentration)
m1 <- multinom(growth observation~bacterial category/strain+NO concentration)

anova(m0, m1)

Model	Resid. df	Resid. Dev	Test	Df	LR stat.	Pr(Chi)
m1	412	70.27460		NA	NA	NA
m0	392	51.77915	1 vs 2	20	18.49545	0.5548057

m2 <- multinom(growth observation~bacterial category/strain + 1)
anova(m1, m2)

Model	Resid. df	Resid. Dev	Test	Df	LR stat.	Pr(Chi)
m2	414	270.5957		NA	NA	NA
m1	412	70.2746	1 vs 2	2	200.3211	0

m3 <- multinom(growth observation~1+NO concentration)
anova(m1,m3)

Model	Resid. df	Resid. Dev	Test	Df	LR stat.	Pr(Chi)
m3	432	329.1685		NA	NA	NA
m1	412	70.2746	1 vs 2	20	258.8939	0

Anova(m1)
Analysis of Deviance Table (Type II tests)
Response: growth observation

	LR Chisq	Df	Pr(>Chisq)
bacterial category	103.50	2	< 2.2e-16 ***
NO concentration	200.32	2	< 2.2e-16 ***
bacterial category/strain	155.39	40	1.618e-15 ***

3.5.1.2 Grace's insect medium preparation

We dissolved 45.4 g Grace medium base in 1 l distilled water and supplemented it with 50 µL phenol red, as well as tryptose-phosphate broth to a final concentration of 0.2g/L tryptose, 0.02g/L dextrose, 0.05g/L NaCl, 0.025g/L disodium phosphate (all from Carl-Roth, Germany). The pH was adjusted to pH 6.5 with 2M NaOH (21).

3.5.1.3 NO exposure

For the comparative cultivation assay, we transferred 10 µl of each bacterial starting culture to 500 µl of a mix of Grace's insect medium, Sf900 medium and 3% agar (ratio 1:1:2; pH 8) in a 48 well plate. For each NO concentration, two 48 well plates were prepared. The well plates were incubated for 1h,

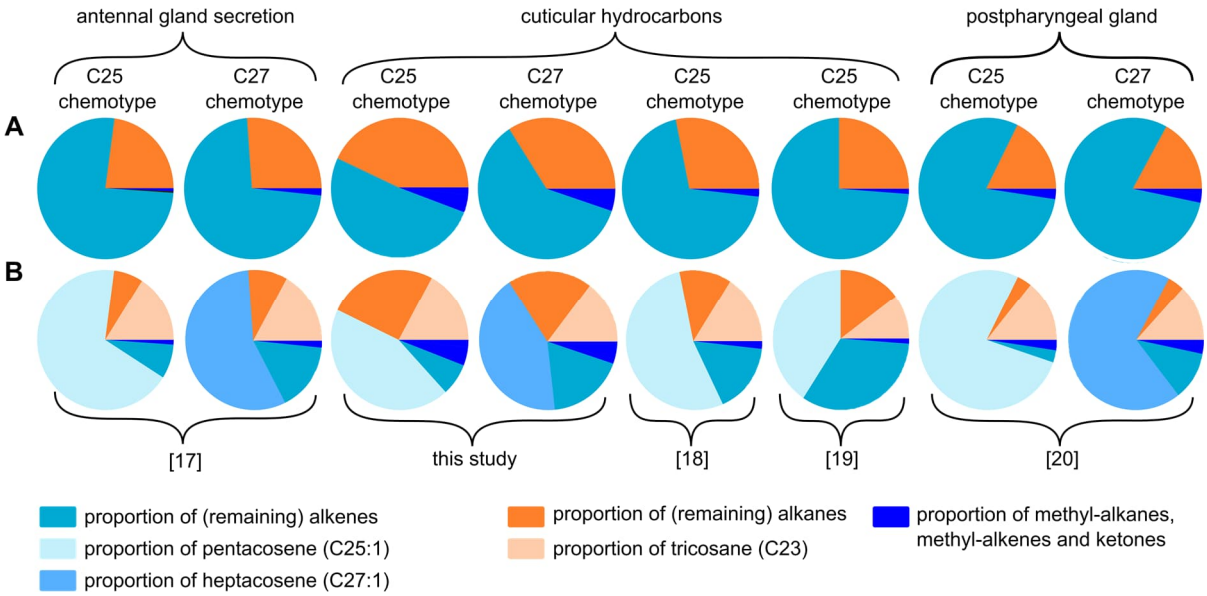
then exposed to 1%, 0.5%, 0.2%, 0.1%, 0.01%, or 0% NO in N_2 (purity ≥ 99.999 mol %, Air Liquide Deutschland GmbH, Germany) in a sealed exposure chamber. NO was released into the headspace of the chamber from a gas cylinder. Half of the headspace of the exposure chamber was replaced with NO 10x every 6 min. Afterwards, well plates were incubated at 30°C and monitored daily to record macroscopically visible growth.
For the CHC experiments, tubes were exposed to 0.01% NO in N_2 (purity NO: 2.0; purity N_2: 5.0; 2% error tolerance; Westfalen AG, Germany) after hexane had evaporated. NO exposure took place as described above. Untreated indicator solutions changed coloration upon NO exposure in the chamber, proving that the water-soluble NO diffuses from the headspace into the aqueous indicator solution, where NO and/or its spontaneous reaction products oxidize iodide to elemental iodine. The latter integrates into the starch double helix, resulting in a dark blue coloration of the previously transparent indicator solution.

3.5.1.4 Preparation of NO indicator
An indicator solution for NO detection via oxidation was prepared by mixing 10% (w/v) potassium iodide, 2% (w/v) starch (both Carl-Roth, Germany) and ultrapure water (Merck, Germany) in a 1:5:4 ratio. This indicator is based on NO and its spontaneous reaction products oxidizing iodide to elemental iodine. The latter integrates into the starch double helix, resulting in a dark blue coloration (34).

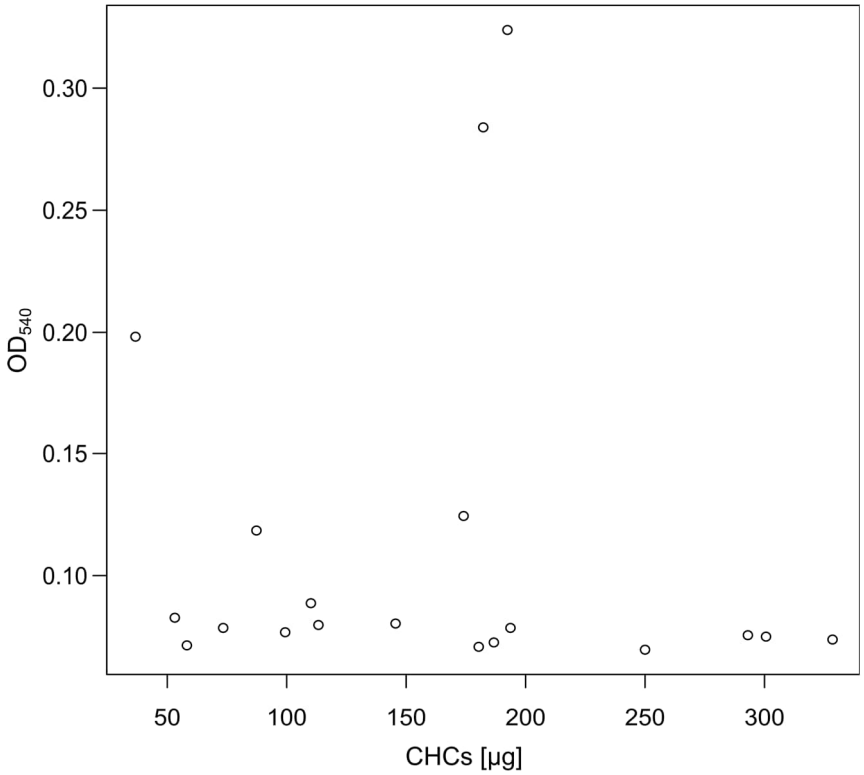
3.5.1.5 Gas chromatography-mass spectrometry (GC-MS) of beewolf CHC extracts
A 5 µl aliquot of each CHC extract from *P. triangulum* females was transferred to a micro-insert containing 1 µg of octadecane (Sigma-Aldrich, Germany) as an internal standard. A 1 µl aliquot of each extract was injected into an Agilent mass selective detector equipped with an inert extractor ion source (MSD5977B Inert Plus EI) coupled to a Agilent 8890 gas chromatograph. The GC was equipped with a HP-5 ms column (30 m × 0.25 mm ID; 0.25 µm df; Agilent Technologies, Santa Clara, CA, USA). The temperature of the split/splitless GC injector was set to 250°C, and the injector was operated in the splitless mode. The GC oven temperature was programmed as follows: 150°C for 1 min, heated with 5°C/min to 300°C and a final isothermal hold of 10 min. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. Mass spectra were recorded with electron impact ionization with a mass range uf m/z=40-550 after a 5min solvent delay until the end of the GC run time. The transfer line was operated at a temperature of 320°C, the ion source of 230°C and an ionization voltage of 70 eV and the quadrupol at 150°C. Compounds were identified in the MassHunter Quantitative Analysis software (V10.0, Agilent Technologies) based on retention time, comparison with an external series of linear alkanes ranging from decane to tetracontane and manual interpretation of mass spectra following (5). Quantification was performed with the Masshunter Quantitative Analysis software (V10.2, Agilent Technologies, Santa Clara, CA, USA). Absolute amounts were calculated based on the internal octadecane standard.
For the *P. histrio* extract, GC-MS was performed with an Agilent 6890N Series gas chromatograph (Agilent Technologies, www.agilent.com) coupled to an Agilent 5973 mass selective detector. The GC was equipped with a DB-5 ms column (30 m × 0.25 mm ID; 0.25 µm df; Agilent Technologies; temperature program: from 60°C to 300°C at 5°C/min, held constant for 1 min at 60°C and for 10 min at 300°C). Helium was used as the carrier gas with a constant flow of 1 ml/min. A split/splitless injector was installed at 250°C in the splitless mode for 60 sec. The electron impact mass spectra were recorded with an ionisation voltage of 70 eV, a source temperature of 230°C and an interface

temperature of 315°C. The software MSD ChemStation for Windows (Agilent Technologies) was used for data acquisition.

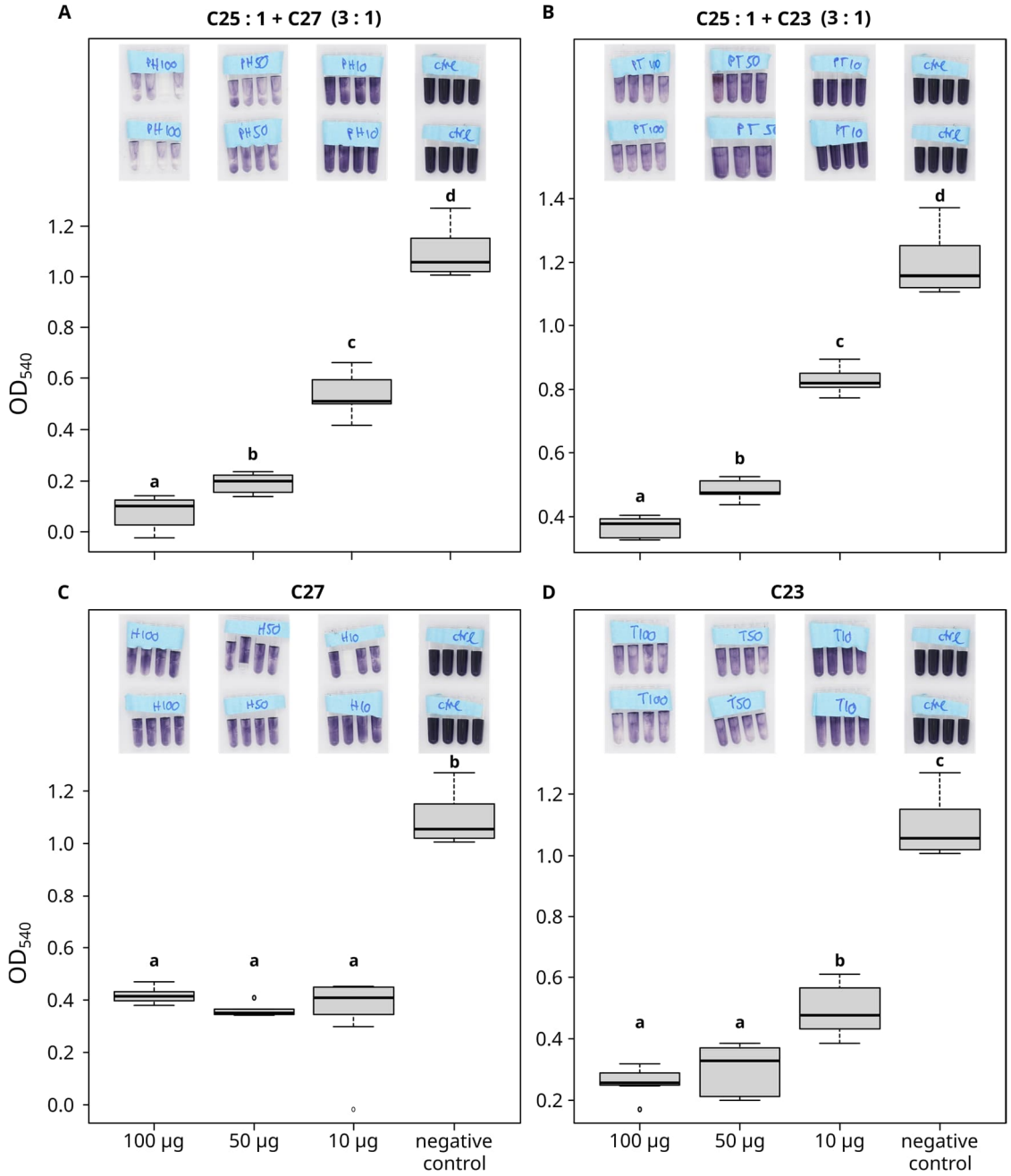
3.5.2 Supplementary figures



Suppl. Fig. 1: Hydrocarbon composition of the antennal gland secretion (17), on the beewolf cuticle (18, 19) and in the postpharyngeal gland (20) of *Philanthus triangulum* females. The pie charts in (A) and (B) show identical datasets with emphasis on (A) alkene-alkane ratio of the entire mixture and (B) the most abundant alkene and alkane. Note that all figures represent a visualization of the data presented in Suppl. Tab. 1. Mean relative amounts are given in percent. The proportions of methyl-alkanes, methyl-alkenes and ketones have been subsumed in this figure for better visibility. Two distinct chemotypes with either a C25 or C27 backbone of the major component occur for *P. triangulum* in nature (17, 20).

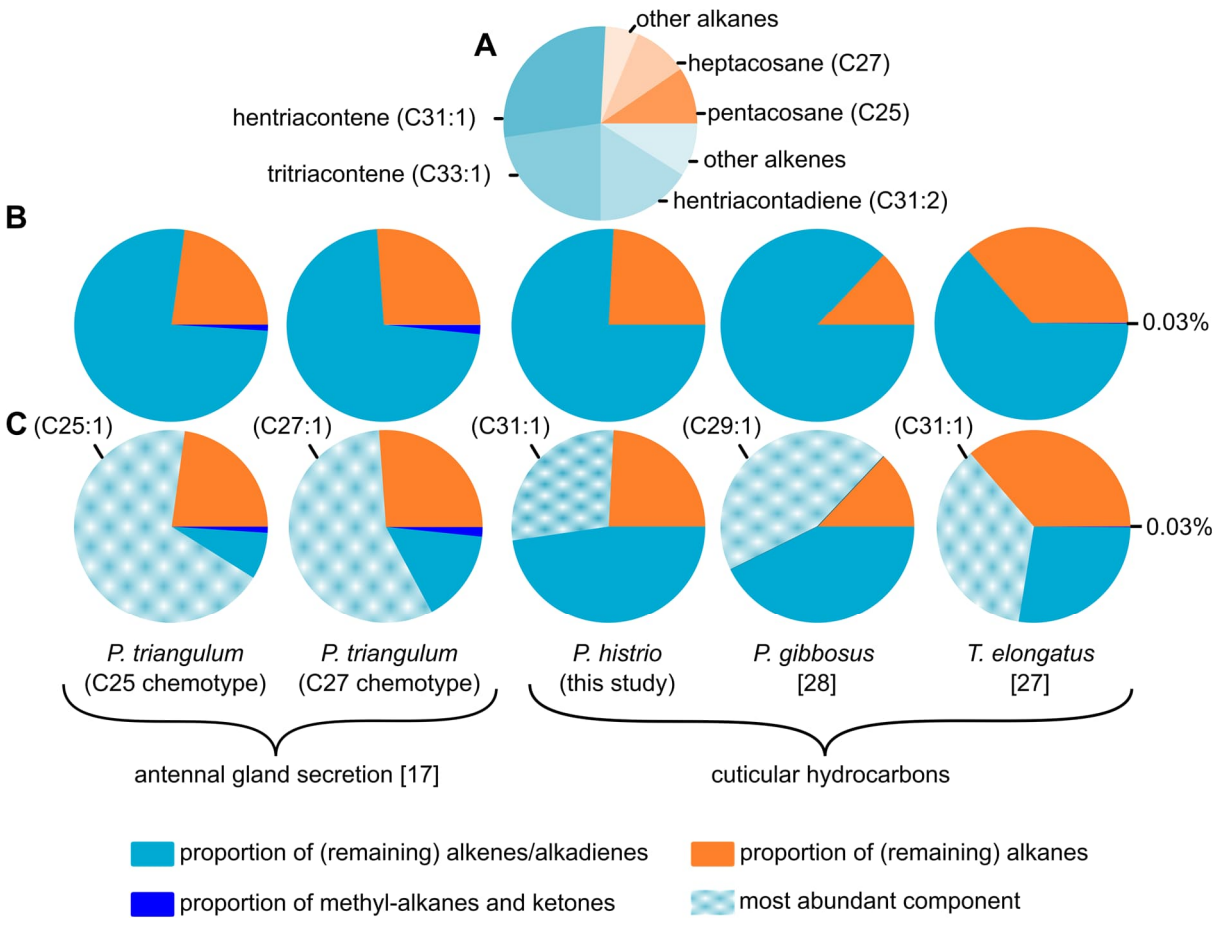


Suppl. Fig. 2: Correlation between the amount of CHCs in beewolf extracts and OD_{540} of the NO indicator solutions treated with CHC extracts of female beewolves after NO exposure (Spearman's rank correlation, N = 19, rho = -0.335, S = 1522, p = 0.161). Please note that the highest ODs observed for indicator solutions treated with beewolf extracts (around 0.32) still reflect a considerable diffusion barrier towards NO, as compared to the OD of the negative controls, which exceed 1.0 (see Fig. 2, Suppl. Fig. 3, 4).



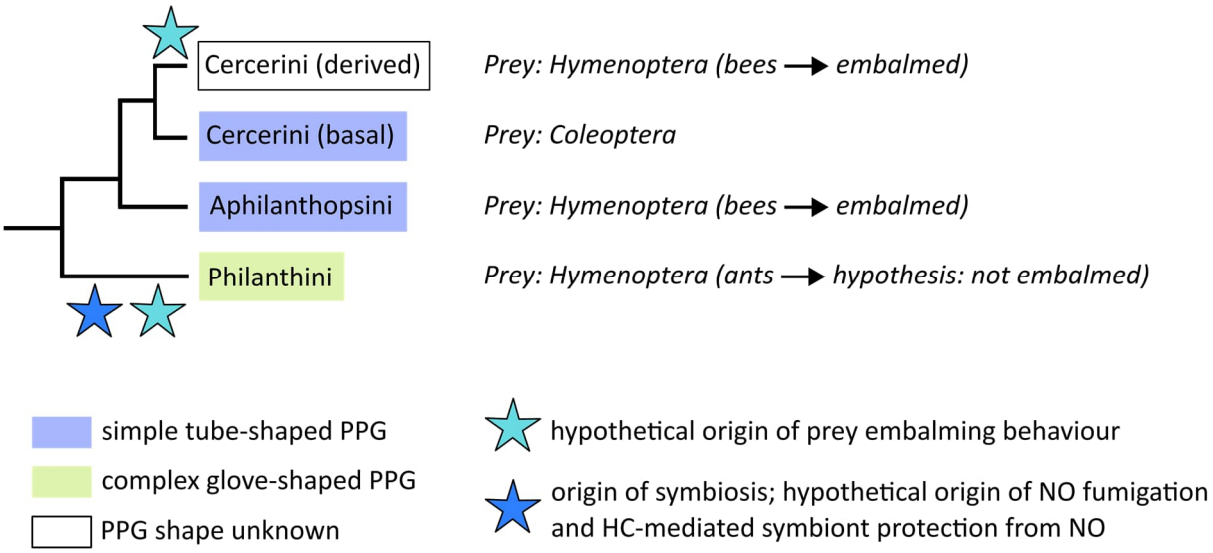
Suppl. Fig. 3: Concentration-dependence of the NO diffusion barrier effect reconstituted with single and binary combinations of synthetic HCs (N = 7-8). (A) C25:1-C27 (3:1), (B) C25:1-C23 (3:1), (C) C27, (D) C23). NO diffusion was measured as change in coloration in an NO indicator solution (top). Hexane extracts served as negative control. All HCs and the alkene-alkane ratio of binary combinations are found in *P. triangulum* (Suppl. Tab. 1). Letters indicate significant differences (Tukey's HSD, N = 7-8, $p < 0.05$).

Suppl. Fig. 4: Indicator solutions treated with 100 µg, 50 µg and 10 µg of different single hydrocarbons and binary hydrocarbon mixtures after 1h exposure to 0.01% NO. ctrl = hexane (N=8), P = C25:1 (N=8), H = C27 (N=8), T = C23 (N=8), PH = 75% C25:1 + 25% C27 (N=8), PT = 75% C25:1 + 25% C23 (N=7), blank = untreated indicator solution (N=4). 1-17 + 19, 20 = female beewolf cuticular hydrocarbon extracts.



Suppl. Fig. 5: Composition of hydrocarbon profiles in *Philanthus triangulum* (17), *P. histrio* (this study), *P. gibbosus* (28) and *Trachypus elongatus* (27). The pie charts show identical datasets with emphasis on (A) the detailed cuticular hydrocarbon composition of *P. histrio* (result of this study; blue hues represent alkenes/alkadienes, orange hues represent alkanes), (B) the alkene-alkane ratio and (C) the chain length of the most abundant component of the hydrocarbon profiles of different host species. Note that all figures represent a visualization of the data presented in Suppl. Tab. 3. Mean relative amounts are given in percent. The proportions of methyl-alkanes and ketones have been subsumed in this figure for better visibility. Note

that alkadienes were only found in *P. histrio* and *T. elongatus*; alkenes and alkadienes were subsumed due to their similar physicochemical properties. Two distinct chemotypes with either a C25 or C27 backbone of the major component occur for *P. triangulum* in nature (17, 20). C25:1 = pentacosene, C27:1 = heptacosene, C29:1 = nonacosene, C31:1 = hentriacontene.



Suppl. Fig. 6: Hypothetical evolutionary scenario for the origin of prey embalming, the beewolf-*Streptomyces* symbiosis (30), NO fumigation and HC-mediated protection of beewolf symbionts from NO. Schematic phylogeny derived from (28, 30, 35); length of the branches do not represent phylogenetic distances. Other phylogenies with Philanthini and Aphilanthopsini forming one clade are less well supported (29). Members of the Cercerini hunt both beetles (Coleoptera) and bees (Hymenoptera) (36), but only bees are embalmed in alkene-rich secretions (29). The same is true for bee-hunting Philanthini (5). Aphilanthopsini have been suggested to not embalm their ant prey (28). Aphilanthopsini and beetle-hunting Cercerini possess a simple tube-shaped postpharyngeal gland (PPG), while Philanthini possess a complex glove-shaped PPG (28). The latter is presumably associated with prey embalming (28). There is no information on the gland shape of bee-hunting Cercerini (28, 29). Prey embalming may have evolved independently in Cercerini and in the ancestor of all Philanthini, the latter coinciding with the acquisition of defensive symbionts about 68 mya (30). Although additional evidence is needed, we speculate that the evolutionary origin of NO fumigation and the HC-mediated protection of the symbionts may have coincided with the origins of symbiosis and prey embalming.

3.5.3 Supplementary tables

Suppl. Tab. 1: Hydrocarbon composition of the antennal gland secretion (AGS) (17), on the beewolf cuticle (CHCs) (18, 19) and in the postpharyngeal gland (PPG) (20) of *P. triangulum* females from different studies. Note that two distinct chemotypes with either a C25 or C27 backbone of the major component occur for *P. triangulum* in nature (17, 20).

	AGS (17)		CHCs (this study)		CHCs (18)	CHCs (19)	PPG (20)	
Component	C25-type	C27-type	C25-type	C27-type	C25-type	C25-type	C25-type	C27-type
heneicosane	0.18	0.37	0.53	0.37	0.36		0.2	0.18
docosane	0.33	0.35	0.36	0.20	0.23		0.16	0.13
x-tricosene	0.74	0.20	1.10	0.16			0.36	0.08
(Z)-9-tricosene					0.55			
(Z)-7-tricosene					0.11			
tricosane	15.90	16.91	17.04	14.67	16.00	10.20	14.06	12.93
x-methyltricosane	0.34	0.23					0.1	0.05
9-methyltricosane			0.44	0.22				
3-methyltricosane			3.07	0.36		0.39		
11-,9-,7-methyltricosane					0.17			
5-methyltricosane					0.04			
x-tetracosene	2.65	0.37			1.63		1.19	0.14
tetracosane	0.31	0.40	0.66	0.43	0.34		0.17	0.14
x-pentacosene	68.35	8.84	44.06	9.17			77.48	8.29
(Z)-9- and (Z)-7-pentacosene					54.00			
(Z)-9-pentacosene						40.80		
pentacosane	4.34	5.39	10.62	9.48	7.16	5.80	2.05	2.82
16-pentacosen-8-one	0.65	0.15			0.95	0.31	1.13	0.25
x-methylpentacosane							0.06	0.08
11-,13-methylpentacosane			0.33	0.25	0.14			
7-methylpentacosane			0.08	0.10	0.04			
5-methylpentacosane			0.04	0.11	0.02			
x-hexacosene	0.43	3.07	0.64	4.12	0.67	0.44		
(Z)-9-hexacosene							0.13	1.62
hexacosane			0.31	0.19	0.13		0.05	0.05
x-methylhexacosane			1.60	0.31				
x-heptacosene	3.10	56.74	5.24	42.79				
(Z)-9- and (Z)-7-heptacosene					12.10			
(Z)-9-heptacosene						31.20	1.00	69.8
heptacosane	0.60	1.36	5.20	3.42	1.94	2.51	0.38	0.31
18-heptacosen-10-one	0.03	1.18			0.27	0.53	1.02	1.73
x-methylheptacosane			0.17	0.14	0.04			
x-methyl-x-heptacosene			0.02	0.12				
x-octacosene			0.01	0.24	0.05			
octacosane	0.09	0.12	0.24	0.15	0.34		0.03	0.03
x-methyl-x-octacosene			0.03	3.50				

x-nonacosene	0.87	3.08	0.15	4.06	0.98			
(Z)-9-nonacosene	0.82	1.07				1.38	0.04	0.95
nonacosane			5.97	4.13	1.27	4.01	0.3	0.32
x-methylnonacosane					0.07			
triacontane					0.17			
x-hentriacontene			0.12	0.35				
(Z)-9-hentriacontene							0.01	0.04
hentriacontane	0.24	0.18	1.95	0.99	0.30	2.47	0.07	0.06
proportion of alkanes	22.83	26.15	42.89	34.02	28.23	24.99	17.47	16.97
proportion of alkenes/alkadienes	76.15	72.30	51.32	60.88	70.08	73.82	80.21	80.92
proportion of methyl-alkanes	0.34	0.23	4.13	1.17	0.51	0.39	0.16	0.13
proportion of ketones	0.69	1.33	0.00	0.00	1.22	0.83	2.15	1.98
proportion of methyl-alkenes	0.00	0.00	1.66	3.92	0.00	0.00	0.00	1.00

Suppl. Tab. 2: Symbiotic and free-living *Streptomyces* species used in this study.

Code	Strain	Host species	Host's geographic origin
elo	elo_217	*Trachypus elongatus*	South America
qua	qua_16-1	*Philanthinus quattuordecimpunctatus*	Eurasia/Africa
gib	gib_464-1	*Philanthus gibbosus*	North America
his	his_161	*Philanthus histrio*	Eurasia/Africa
tri	tri_23Af2	*Philanthus triangulum*	Eurasia/Africa
Code	Strain	Bacterial species	Isolated from
coe	M145	*Streptomyces coelicolor* A3(2)	soil
aver	DSM 46492	*Streptomyces avermitilis*	soil
ant	IMET 43440	*Streptomyces antibioticus*	soil
cat	DSM 46488	*Streptomyces cattleya*	soil
liv	PM66	*Streptomyces lividans*	
vze	DSM 40230	*Streptomyces venezuelae*	tropical soil
zag	DSM 42018	*Streptomyces zagrosensis*	rhizospheric soil

Suppl. Tab. 3: Composition of hydrocarbon profiles in *Philanthus triangulum*, *P. histrio*, *P. gibbosus* and *Trachypus elongatus*. Mean relative amounts are given in percent. Data for *P. triangulum, P. gibbosus,* and *T. elongatus* were obtained from the literature (17, 27, 28), those for *P. histrio* were the result of this study. Note that two distinct chemotypes with either a C25 or C27 backbone of the major component occur for *P. triangulum* in nature (17, 20).

	P. triangulum (AGS)		Cuticular hydrocarbons		
Substance	C25-type	C27-type	*P. histrio*	*P. gibbosus*	*T. elongatus*
heneicosane	0.18	0.37			0.99
docosane	0.33	0.35			0.60
tricosene	0.74	0.20			
tricosane	15.90	16.91	0.78	2.60	2.26
methyltricosane	0.34	0.23			
tetracosene	2.65	0.37			
tetracosane	0.31	0.40		0.14	0.52
pentacosene	68.35	8.84	0.20	5.70	0.37
pentacosane	4.34	5.39	9.49	8.54	22.64

methylpentacosane					0.03
hexacosene	0.43	3.07		0.53	
hexacosane			1.08		0.06
Δ-16-pentacosen-8-one	0.65	0.15			
heptacosene	3.10	56.74	0.86	31.25	0.31
heptacosane	0.60	1.36	9.16	0.89	4.65
octacosene				1.00	
octacosane	0.09	0.12	0.23		0.03
Δ-18-heptacosen-10-one	0.03	1.18			
nonacosene	0.87	3.08	6.75	44.24	2.75
nonacosane	0.82	1.07	3.46	0.79	2.53
triacontene			1.10		0.11
triacontane					0.03
nonacosen-6-one				1.10	
hentriacontadiene			16.03		3.13
hentriacontene			28.07	3.02	36.26
hentriacontane	0.24	0.18			1.98
tritriacontadiene					6.90
tritriacontene			22.80		13.85
proportion of alkanes	22.83	26.15	24.19	12.97	36.30
proportion of alkenes/alkadienes	76.15	72.30	75.81	86.84	63.67
proportion of methyl-alkanes	0.34	0.23	0.00	0.00	0.03
proportion of ketones	0.69	1.33	0.00	0.00	0.00

3.6 Ethics

This work did not require ethical approval from a human subject or animal welfare committee.

3.7 Data accessibility

The raw data underlying this publication are available from the Edmond repository of the Max Planck Society: https://doi.org/10.17617/3.HPDVQM (37).

3.8 Declaration of AI use

We have not used AI-assisted technologies in creating this article.

3.9 Conflict of interest declaration

We declare we have no competing interests.

3.10 Funding

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CHAPTER 4: Transmission of mutualistic bacteria in social and gregarious insects

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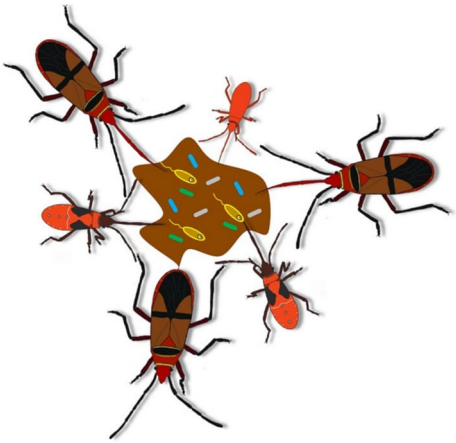
4.1 Highlights

- Mutualistic bacteria confer functional traits to many social and gregarious insects.
- Group living and social behavior shape host microbial communities.
- Social transmission of symbionts entails costs and benefits.
- Social symbiont transfer may reinforce the evolution and maintenance of sociality.

4.2 Abstract

Symbiotic microbes can confer a range of benefits to social, sub-social, and gregarious insects that include contributions to nutrition, digestion, and defense. Transmission of beneficial symbionts to the next generation in these insects sometimes occurs transovarially as in many solitary insects, but primarily through social contact such as coprophagy in gregarious taxa, and trophallaxis in eusocial insects. While these behaviors benefit reliable transmission of multi-microbial assemblages, they may also come at the cost of inviting the spread of parasites and pathogens. Nonetheless, the overall benefit of social symbiont transmission may be one of several important factors that reinforce the evolution of social behaviors and insect eusociality.

4.3 Graphical abstract



4.4 Symbiont-conferred functional benefits in social and gregarious insects

Many insects engage in mutualistic interactions with bacteria that confer novel traits to their hosts, enabling them to utilize a wide range of previously inaccessible resources or colonize new habitats (1). In many instances, these partnerships have become so intimate that the partners cannot survive without each other (2). The functional importance of mutualistic bacteria in social and gregarious insects has gained much attention over the past few decades, as they frequently play important roles in host nutritional ecology. In this review, we briefly summarize known functions of mutualistic bacteria in social insects and then focus on their transmission routes from one generation to the next, discussing the possible costs and benefits of social symbiont transfer and its implications for the evolution of social behaviors. As the social transmission of microorganisms between conspecifics occurs from gregarious all the way to eusocial taxa, we will broadly cover bacterial mutualisms within insects across all levels of sociality.

In social and gregarious insects, symbionts have been described or implied to be involved in the biosynthesis of nutrients in bees and bugs (3-6), pectin and lignocellulose degradation in bees, termites and wood roaches (1, 6, 7), and carbohydrate metabolism in ants and bees (7, 8). In addition, several bacterial symbionts have putative roles in host nitrogen metabolism (1, 8-10). In termites, the hindgut community is essential for nitrogen fixation, recycling and upgrading, mitigating the low nitrogen content of their cellulose-based diet (10, 11). In ants, putative roles include recycling nitrogenous waste to essential amino acids in *Camponotus* and *Cephalotes* (1, 12), nitrogen fixation in certain *Tetraponera* species (9), and providing a tyrosine precursor for cuticle formation in *Cardiocondyla obscurior* (13). In fact, symbiotic microbes may be one factor explaining why herbivorous ants can successfully exploit nitrogen-poor arboreal habitats (8, 14).

Apart from influencing host nutrition, symbionts present an important component of the defensive arsenal in social and gregarious insects, providing protection to host individuals, their food sources and/or nesting environment against pathogens, parasites, and parasitoids (15). In the cockroach *Cryptocercus punctulatus*, the application of feces to the nest plays a putative role in fungal defense. Antifungal compounds in the feces — potentially of microbial origin — may sanitize the nest, preventing growth of antagonistic fungi (16). Similarly, in the termite *Zootermopsis angusticollis*, there is evidence that the hindgut microbiota synthesizes multiple functionally active β-1,3-glucanases with a putative role in fungal pathogen defense (17). Recent studies in bees (*Apis mellifera* and *Bombus terrestris*) revealed that individuals with a native, undisturbed gut microbial community were less susceptible to *Lotmaria passim* and *Crithidia bombi* trypanosomatid parasites, respectively, likely due

to competitive exclusion of the parasites by bacterial gut symbionts (18, 19). Other studies observed an effect of the whole gut microbiota and individual bacterial symbionts on the host immune system (20, 21). Kwong *et al.* (21) found that the native, non-pathogenic microbiota of the honeybee *A. mellifera* induces host immune responses, particularly an upregulation of genes coding for the antimicrobial peptides (AMPs) apidaecin and hymenoptaecin in gut tissue and, subsequently, an elevated apidaecin concentration in the gut lumen and hemolymph. Thus, immune priming by the native symbionts may play a role in regulating the microbiota and/or protecting against pathogens. In fungus-farming ants, antimicrobial compounds produced by actinobacterial symbionts protect the fungal cultivars from specialized *Escovopsis* fungal pathogens (22). Interestingly, recent studies on the burying beetle *Nicrophorus vespilloides* (Coleoptera: Silphidae), which provide parental care for their offspring, suggested a potential food-preserving role of the symbionts in this taxon as well (23, 24).
Lastly, growing evidence suggests that symbiotic bacteria can influence host social interactions (25). In the German cockroach, volatiles emanating from feces-associated bacterial symbionts promote gregarious host behavior (26). Similarly, in the desert locust *Schistocerca gregaria*, some of the bacterial gut symbionts play a role in host aggregation by producing components of the locust's cohesion pheromone (27). In addition, bacterial symbionts have putative roles in nestmate recognition in eusocial insects (25). For example, in the termite *Reticulitermes speratus* and the harvester ant *Pogonomyrmex barbatus*, bacterial associates have an effect on the hosts' chemical profiles and therefore on recognition (28, 29).

4.5 Symbiont transmission routes

Insects that depend on symbionts to perform key functions are faced with the challenge of ensuring acquisition or successful transmission of specific symbionts in every generation. Many solitary insects have evolved complex mechanisms that ensure high fidelity of symbiont passage to the next generation, including symbiont invasion of the oocytes prior to embryogenesis, specialized secretions or structures that harbor extracellular symbionts until egg hatching, or organs that allow for the selective colonization of specific environmental bacteria (30, 31). While social and gregarious insects can exploit similar mechanisms for symbiont transmission, their frequent contact with conspecifics opens up the possibility for direct symbiont transfer and thereby adds another dimension to the transmission of beneficial microbes. Behaviors such as coprophagy, trophallaxis and shared resources that are predominant in social and gregarious insects facilitate transmission or acquisition of appropriate microbes in addition to the transfer of nutrients or digestive enzymes.

4.5.1 Coprophagy

Coprophagy refers to the consumption of conspecifics' feces after excretion (Fig. 1A) and is considered a major force shaping gut microbial communities of gregarious insects such as bugs, beetles, cockroaches and, to some extent, of eusocial bees and termites. In bumblebees and honeybees, for instance, naïve individuals reared in the presence of fresh feces collected from nurse bees acquire bacterial communities similar to those of nurses or wild bees (18, 32). Gregarious Pyrrhocoridae and Reduviidae bugs (Hemiptera) exhibit a high degree of coprophagy, likely as a behavioral adaptation for symbiont acquisition (33, 34). For instance, when kissing bugs, *Rhodnius prolixus* (Reduviidae), the vectors of Chagas disease, hatch under sterile laboratory conditions and are raised separately from conspecifics, the nymphs lack the important nutritional symbiont *Rhodococcus rhodnii*, resulting in stunted growth (33). However, molting and development can be easily restored when the nymphs are

exposed to freshly collected feces or conspecifics harboring the *R. rhodnii* symbiont (33). In *Pyrrhocoris apterus* and *Dysdercus fasciatus* firebugs (both Pyrrhocoridae), initial acquisition of essential microbes is mediated by vertical transmission to offspring via egg surface smearing with feces (35, 36). Additionally, the gregarious nature of these insects facilitates horizontal transmission of microbes when individuals probe conspecifics' feces. Bugs lacking the typical gut microbiota display high symbiont infection frequencies when reared with symbiotic adult conspecifics, contaminated egg shells or feces (35, 36). Coprophagy can also mediate transition between these two modes of transmission that is from horizontal symbiont transfer (uptake of feces from the environment) to vertical transfer, when feces are deposited along with the eggs or offspring (31).

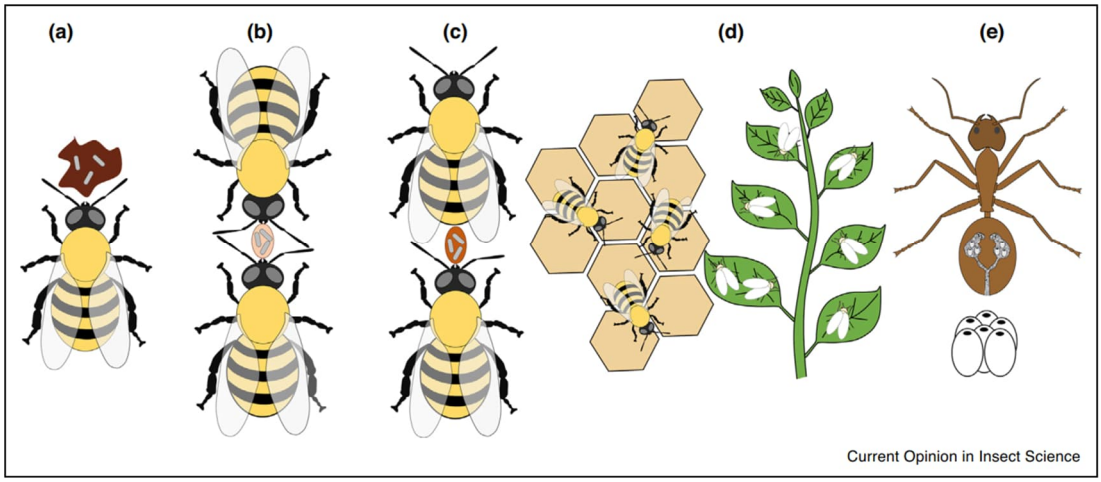


Fig. 1: Symbiont transmission routes in social and gregarious insects. (A) Coprophagy, (B) mouth-to-mouth feeding (stomodeal trophallaxis), (C) anus-to-mouth feeding (proctodeal trophallaxis), (D) transmission through shared environment, and (E) transovarial transmission.

Carrion beetles (Coleoptera, Silphidae), which exhibit biparental care, utilize small vertebrate carcasses as an ephemeral nutritional resource for their larvae. When preparing the carcass, they apply anal secretions containing a distinct bacterial community (mostly Xanthomonadaceae, Enterococcaceae, and Enterobacteriaceae) and *Yarrowia* yeasts (23, 24, 37). The treated carcass then acts as a medium for vertical symbiont transmission when hatched larvae feed on the secretions, which is reflected in a high similarity of symbiont composition in adult and larval guts as well as prepared carcass surfaces (23, 24, 38). Similarly, transmission of symbionts in *Euoniticellus* dung beetles (Coleoptera, Scarabaeniae) is mediated by maternal fecal secretions deposited in the dung balls together with the eggs. Characterization of the bacterial community composition of larvae, adult females and males, brood balls and maternal secretions revealed that larval gut communities are more similar to female and brood ball communities than to those of males, suggesting that larvae acquire the symbionts vertically upon consuming provisioned maternal secretions (39). In Dictyoptera, coprophagy was likely ancestral (40) and remains an essential behavior in the transmission of bacterial symbionts in cockroaches and termites. In *Blattella germanica*, whose gregarious nature has been partially attributed to volatile carboxylic acids associated with fecal bacteria (26), survivorship and growth of early instar nymphs largely depends on access to nutrients present in conspecifics' feces (41). Moreover, high similarity of bacterial composition in the feces and gut reveals that this behavior is responsible for inoculation, re-colonization and succession of gut microbiota in the cockroach (42,

43). In termites, coprophagy also mediates the exchange of nutritional fluids, symbiotic protists and bacteria, complementing trophallactic transfer between colony members (see below) (11, 44).

4.5.2 Trophallaxis

Trophallaxis, the direct transfer of oral fluids via mouth-to-mouth feeding (stomodeal trophallaxis) (Fig. 1B) or hindgut content via anus-to-mouth feeding (proctodeal trophallaxis) (Fig. 1C), likely evolved from coprophagy (40). Apart from the transfer of nutrients within a colony (44, 45), many social insect taxa exploit trophallaxis for initial inoculation or re-acquisition of symbionts. In social corbiculate bees (honeybees, bumblebees, and stingless bees), bioassays demonstrated that both stomodeal and proctodeal trophallaxis are essential for the acquisition and maintenance of the distinctive bee gut microbiota after symbiont-free emergence (32, 46). When newly emerged bees are exposed to nurse workers or their hindgut homogenates, they mainly acquire core gut symbionts (*Snodgrassella*, *Gilliamella*, *Bifidobacterium*, and *Lactobacillus*), whereas those limited to oral contact with nurse workers are largely colonized by *Lactobacillus* and low amounts of the other taxa (32, 46). In bumblebees, social contact within the hive has been shown to facilitate symbiont transfer between nestmates, including daughter queens. The latter subsequently transmit the symbionts vertically to their offspring when founding new colonies after hibernation (18, 47).

Similarly, the cellulolytic hindgut communities of endosymbiotic flagellates and bacteria in the eusocial lower termites and in subsocial wood roaches, *C. punctulatus* (Cryptocercidae), are stably maintained by sociality. Group living in termites assures members reliable and continuous access to nutritionally essential gut microbes via trophallaxis (10, 48). Additionally, re-acquisition of microbes lost shortly before ecdysis is achieved by proctodeal trophallaxis between newly molted juveniles and adult conspecifics (11, 44). In *C. punctulatus*, social contact as well as biparental brood care are key in shaping intestinal flagellate and bacterial communities (49). Essential microbes, which take a long time to stably establish in juveniles, and nutrients are repeatedly acquired from adult conspecifics via proctodeal trophallaxis for a period of at least one year until the juveniles gain nutritional and microbial independence (44, 48-50). This behavior also seems to occur in some of their solitary relatives such as *Shelfordella lateralis* (Blattidae), where individuals inoculated with non-native bacteria were shown to re-acquire the native gut microbiota via social contact with conspecific adults (51).

Intimate biparental care of carrion beetles (Silphidae) is key to progeny survival, as it grants larvae access to nutrients and symbionts essential for development. In addition to the transmission of microbes via the carcass surface, acquisition of symbionts by larvae is assured when they engage in stomodeal trophallaxis with adults, whose oral secretions are known to contain symbionts (23). Likewise, eusocial *Polistes* paper wasp males (Vespidae) add oral secretions containing digestive enzymes to pre-chewed pieces of solid food before offering it to their young in regurgitated form (48). This process may also include the addition and transmission of relevant gut symbionts.

4.5.3 Environmental transmission

In some insects, direct contact or other forms of exchange between conspecifics or parents and offspring are not necessary for successful symbiont transmission. Rather, symbiotic partners are acquired horizontally from a shared environment or food resource contaminated with symbionts (Fig. 1D), as reported in whiteflies, thrips, bees and leaf cutter ants. Caspi-Fluger *et al.* (52) and Li *et al.* (53) demonstrated that in whiteflies (*Bemisia tabaci*, Hemiptera: Aleyrodidae), which overcome plant defenses when feeding gregariously, plant-mediated horizontal transmission of the secondary

endosymbiont *Rickettsia* can occur when infected and non-infected whiteflies of the same or different species share a host plant. These studies observed the transfer of the *Rickettsia* endosymbiont to phloem cells of host plant leaves, as well as its retention and distribution throughout the phloem network before its subsequent re-acquisition by non-infected individuals at high rates (52, 53). Similarly, in *Frankliniella occidentalis* (Thysanoptera; Thripidae), which tend to feed gregariously, transmission of symbionts via shared host plants has been demonstrated. Female thrips prefer depositing eggs on grazed leaves, which are subsequently fed on by hatched larvae that acquire symbionts present in regurgitations or feces of previous feeders (54, 55).
In bumblebees and honeybees, the bacterial gut symbionts *Snodgrassella*, *Bifidobacterium*, and *Lactobacillus* are taken up during or after natural emergence, when eclosed bees come in direct contact with symbiont-contaminated hive material such as wax, hive frames, pollen, brood cell, honey comb and bee-bread (32, 46, 47). Furthermore, intra-colony and inter-colony symbiont transfer frequently happens when bees from the same or different colonies pick up symbionts deposited on shared flowers by previous foragers (47), which can then be passed on to nestmates upon return to the hive. Recent examinations of leaf-cutter ant symbioses revealed how nest environments mediate transmission of mutualistic bacteria. In *Acromyrmex* ants, physical contact of newly eclosed symbiont-free worker ants to adult workers and fungus gardens ensures a stable colonization with mutualistic defensive ectosymbiotic *Streptomyces* sp. and *Pseudonocardia* bacteria a few days post-eclosion (56, 57).

4.5.4 Transovarial transmission

In social insects, as in solitary insects, long-term host–symbiont co-evolution can result in strict dependence of symbionts on hosts for survival and transmission. In these circumstances, transovarial transmission is an efficient method of ensuring successful transfer of obligate intracellular symbionts. Transovarial transmission occurs when endosymbiont-filled bacteriocytes or endosymbionts released from bacteriocytes, nurse cells or other organs invade and establish in developing oocytes, eggs or embryos in the mother's ovaries (Fig. 1E). In addition to being widespread among solitary insects, this type of transmission has been demonstrated for some social and gregarious taxa such as *Blattabacterium* in *B. germanica* (58), *Blochmannia* in *Camponotus* ants (59), and *Westeberhardia* in *Cardiocondyla* ants (13). In addition, the presence of endosymbionts in reproductive organs, such as those occasionally found in *Camponotus floridanus* male testis follicles (59), suggests that copulation and/or paternal transmission may play a role for symbiont transmission in social or gregarious insects (60).

4.6 Benefits and costs of social transmission routes

4.6.1 Benefits of social symbiont transmission

Social contact provides more opportunities for effective transmission and maintenance of beneficial microbes than in solitary insects (Fig. 2A, Tab. 1). For example, social bees are known to harbor a more consistent gut microbiota than solitary bees (61), and some sub-social stink bugs exhibit egg-tending behavior, followed by post-hatch symbiont secretions by mothers that ensures offspring uptake of symbionts that may not be able to survive long enough outside the host (62). Some microbes may even influence the social behavior of their hosts, like in locusts and cockroaches where their gut

bacteria are involved in producing aggregation pheromones, likely enhancing the further transmission of these bacteria when hosts congregate (26, 27).

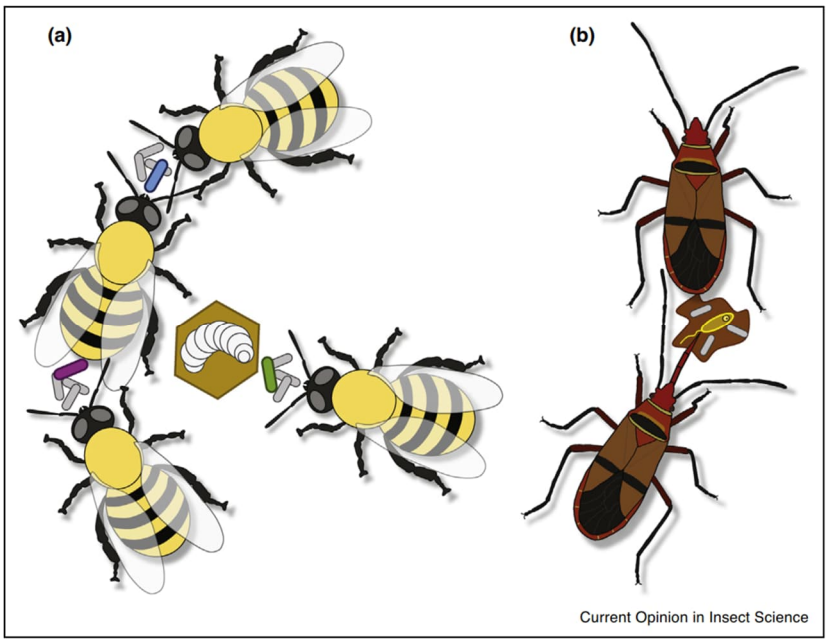


Fig. 2: Important benefits and costs of social transmission routes. (A) More opportunities for transmission of multiple beneficial microbes and at different life stages. (B) Parasites, such as trypanosomes, may co-opt the transmission routes of mutualists for their own advantage. For a more detailed account of costs and benefits associated with different transmission routes, please refer to Tab. 1.

Social behaviors can also promote the establishment of multi-partite symbioses, where multiple microbes together contribute to the overall health of the host (Fig. 2A). In a recent study, for example, honeybees were found to gain or lose metabolic potential based on the experimental addition or removal of individual microbes (63), suggesting strong selective pressures for behaviors that ensure their maintenance. Other insects may suffer serious fitness defects when transmission routes of one or more of their symbionts are accidentally disrupted, so gregarious behavior as observed in many Heteroptera can be adaptive by increasing the chance of symbiont transmission.
In some cases, mass-provisioning insects transmit their symbiotic microbes in ways that help create an environment that is more suitable for the development of their offspring by inhibiting microbial competitors and pathogens and/or upgrading nutritional content. Carrion beetles, which exhibit cooperative brood care, inoculate animal carcasses with specific bacteria and yeasts that are then transmitted to the larvae and help to preserve the carcass that would otherwise be overgrown with opportunistic microbes (23, 24). Beewolves, which provide paralyzed bees for their larvae, have also evolved specialized symbiont deposition behaviors whereby antibiotic-producing *Streptomyces* bacteria left by mothers ensure protection and symbiont transmission to their larvae (64, 65). And dung beetles, before enclosing their offspring inside dung balls, deposit symbiont-containing ‘gifts’ that are important for the early nutrition of their offspring inside the dung ball (39).

4.6.2 Cost of co-transmitting parasites and pathogens

In addition to facilitating the transmission of beneficial microbes, social symbiont transmission can also entail significant costs, as social contact may open the door to pathogens that can take advantage

of these transmission routes for their own means (Fig. 2B) (66, 67). In social bees such as *A. mellifera* and *B. terrestris*, *Crithidia* trypanosomes were found to infect workers during brood-care of infected larvae (68), although other socially acquired bacteria can protect against these infections (18, 69). Similarly, *Blastocrithidia* and *Leptomonas* trypanosomes in firebugs such as *P. apterus* and *D. fasciatus* are known to co-opt their gregarious hosts' coprophagy and egg-smearing symbiont transmission routes for their own transmission (Fig. 2B) (36, 70). The foraging behavior of *Acromyrmex* leaf-cutter ant workers appears to be the culprit for acquisition and spread of the fungal garden parasite, *Escovopsis*, to ant nests. It was originally thought that *Escovopsis* is vertically transmitted in fungal pellets carried by ant foundress queens on their nuptial flights, but recent studies instead found that the parasitic fungus sporulates in external ant refuse dumps where spores are spread intraspecifically and interspecifically by sticking to the cuticle of foraging workers from other colonies (71-73).

Parasitic entomopathogenic fungi, bacteria, and nematodes, too, may spread quickly through social insect societies, as they can be rapidly transmitted, potentially decimating colonies. However, social insects can alleviate these costs and reduce pathogen loads through social allogrooming that removes pathogens and primes the host's immune system (44, 74, 75). For example, in ants and termites, contact with fungi by either exposure to non-infective doses of spores or trophallaxis with immunized individuals increases their resistance to future pathogen exposures (76-78). Social immunity is not always effective against all pathogens, however, as proctodeal trophallaxis from *Serratia marcescens*-infected termites was found to increase susceptibility of recipient termites to later encounters with the same pathogen (79).

4.7 Ecological and evolutionary implications

Among insects associated with mutualistic symbionts, selection favors those that successfully endow offspring with their beneficial partners. While the transovarial mode of transmission for intracellular symbionts is shared between some solitary and social insects, they differ in the predominant transmission route for extracellular symbionts (Tab. 1). Solitary insects mostly rely on depositing fecal droplets, glandular secretions, symbiont capsules or caplets in close contact to the eggs or in the nesting environment (31, 80). As these insects usually do not have direct contact with their offspring, depositing the symbionts in locations where they are likely to be encountered by the hatchlings increases the likelihood of successful transmission (31). By contrast, social insects predominantly exchange symbionts via direct contact, that is stomodeal or proctodeal trophallaxis (Tab. 1). Presumably, this mechanism of symbiont transmission has several advantages over the transmission routes that are accessible to solitary insects: Direct transfer reduces the risk of failure to transmit beneficial symbionts and allows for simultaneous provisioning of digestive enzymes that aid digestion in immature individuals (31, 44, 45); the immediate acquisition of microbial partners from conspecifics minimizes the time that the microbes spend outside of their host's body, ensuring the survival of multiple host-adapted symbionts, and although social interactions are known to increase the risk of pathogens spreading across large groups of closely related conspecifics, the direct transfer between individuals in social insect colonies likely reduces the risk of acquiring opportunistic microbes (81), at least in comparison to an unspecific uptake of feces from the environment or an extended exposure of maternal symbiont-containing secretions to external contamination (Tab. 1).

Tab. 1: Demonstrated or hypothesized requirements, benefits and costs associated with different symbiont transmission routes in social and gregarious insects.

	Extracellular transmission route				
	Trophallaxis	Coprophagy	Egg surface smearing	Specialized structures	Environmental
A. Requirements for symbiont transmission					
Host social behavior	Eusociality	Eusociality/ gregariousness	Mostly solitary	Mostly solitary	Gregariousness
Host adaptations	Behavioral	Behavioral	Behavioral	Metabolic/ structural	Behavioral
Symbiont adaptations	Gut passage	Gut passage and survival in feces	Gut passage and survival on egg surface	Survival in specialized structures	Establishment and survival in environment
B. Benefits of transmission route					
Reliability of symbiont transmission	High	Low	Medium	High	Low
Additional nutritive uptake	Yes	Occasional	Occasional	Unknown	Yes
Host protection	Social immunity	Social immunity	Egg protection	Unknown	Unknown
Maintenance of diverse symbiont community	Yes	Yes	Sometimes	No	Unknown
C. Costs of transmission route					
Host metabolic input	Low	Low	Low	High	Unknown
Risk of parasite co-transmission	High	High	High	Low	Unknown
Acquisition of opportunistic microbes	Low	High	High	Variable	High

Considering the importance of inter-individual transmission of mutualistic bacteria in social insects, it is tempting to speculate on the role of symbiont transmission in the evolution of sociality. For termites, Cleveland *et al.* (82) and Nalepa (44) argued that proctodeal trophallaxis was intimately connected to the evolution of sociality, by integrating the social, nutritional, and microbial environment. Arguably, as lower termites need to reacquire their gut protists after each molt, recurrent contact with conspecifics is crucial for maintaining the cellulolytic microbial gut community (44, 48). Even though, as outlined above, many other social insects are associated with microbial symbionts and likewise benefit from their social transfer, most of them only require a single acquisition event for successful and lasting symbiont infection (46, 57), rendering frequent contact with conspecifics less important for symbiont transmission. For many eusocial insects, however, beneficial symbionts are as yet uncharacterized (e.g. most eusocial wasps and many ants), so more knowledge is needed to assess whether enhanced symbiont transmission is an important driving force for the evolution of sociality. In addition, quantitative empirical work on costs and benefits associated with different transmission routes (Tab. 1) remains largely lacking, prohibiting conclusions on the adaptive value of evolutionary transitions between transmission routes. Nevertheless, the opportunity for direct symbiont transmission may have reinforced sociality (48, 83) and certainly adds another dimension to the costs and benefits of living in groups of conspecifics.

4.8 Conflict of interest

Nothing declared.

4.9 Acknowledgements

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CHAPTER 5: General discussion

5.1 Symbiont protection from host chemical defence

All living organisms encounter a variety of biotic and abiotic challenges, and a plethora of adaptations have evolved to cope with them (1, 2). Exposure to predators, pathogens and parasitoids represents an example of a biotic stressor organisms are confronted with (1, 3). One possible way to cope with this stressor is employing chemical compounds for self-defence (3-5). In fact, chemical defences are widespread across all kingdoms of life (3-5). Organisms can use chemical compounds as protective signals, deterrents against predators, and growth inhibitors targeting microbial pathogens (6). Harnessing the defensive properties of such compounds entails mechanisms counteracting self-toxication (6). Since virtually all eukaryotic organisms engage in intimate associations with one or more microbial symbionts (7), avoiding toxication is not only a necessity for the host, but also for its microbial symbionts (6). However, little is known about how symbionts are maintained upon confrontation with host chemical defences.

In symbiotic associations where the symbiont is vertically transmitted to the next generation, and exposed to host chemical defences, either the symbiont or the host should evolve an adaptation to protect the symbiont. Examples for a symbiont adaptation have been described in bugs and bees. For instance, gut bacterial symbionts of the cotton stainer bug *Dysdercus fasciatus* (Hemiptera: Pyrrhocoridae) are insensitive to the host antimicrobial peptide (AMP) pyrrhocoricin (8), and gut bacterial symbionts of the honeybee *Apis mellifera* (Hymenoptera: Apidae) are resistant to various honeybee AMPs (9).

In this thesis, I describe a host adaptation protecting the symbiont from the host's chemical defence: The European beewolf *P. triangulum* protects it defensive symbiont *S. philanthi* from toxic NO released by the beewolf egg in the brood cell (10) by embedding the symbiont in the HC-rich AGS during transmission. The HC-rich AGS presents a physicochemical barrier against NO (10, 11) and is very likely required for successful symbiont transmission, since *S. philanthi* is not able to withstand brood cell-level NO concentrations by its own devices *in vitro* (10).

Apart from challenges originating from host chemical defences, symbionts may also be threatened by other biotic and abiotic stressors during transmission. In this context, the stinkbug *Megacopta punctatissima* (Hemiptera: Plataspidae) presents an example of a host adaptation stabilizing its symbiont during external transmission due to impaired symbiont integrity caused by an eroded genome, specifically the lack of a number of genes involved in bacterial cell wall synthesis (12-16). Additionally, the host adaptation may protect the symbiont from abiotic stressors during external transmission, namely exposure to intense sunlight for 7-10 days (16). Protection is achieved by embedding the symbiont '*Candidatus* Ishikawaella capsulata' (henceforth *I. capsulata*) in a host-derived capsule mainly composed of PMDP, an intestinal secretion protein (12).

Protecting the symbiont from host chemical defences and other threats should entail a cost, but whether the cost is inflicted on the symbiont or the host should depend on which of the two partners evolves the protective adaptation. Overall, experimental quantifications of the cost of protective adaptations are scarce. The protective adaptation of *Meg. punctatissima* constitutes a notable exception, since it has been shown that PMDP production reduces the female's lifespan (12). In beewolf hosts, the implications of the host adaptation for host fitness are not clear. Providing the HC-rich AGS should entail the cost of HC biosynthesis and their assumed transport from the oenocytes to the antennal gland reservoirs (11, 17). Nonetheless, the cost of producing the HCs provided in the AGS

should be quite low compared cost of producing the HCs for the PPG and the cuticle. This is due to different amounts of HCs being provided in the three locations: While the AGS contains 2.37 ± 1.72 µg HCs (18), females possess 188.4 ± 35.3 µg CHCs (19), and the PPG secretion consists of 78.6 ± 43.7 µg HCs (20). Producing, storing and applying the HCs in the PPG secretion actually requires a high amount of the female's resources, and decreases its capacity to invest in future progeny (21).
In addition to the protective adaptation of *P. triangulum*, *S. philanthi* produces several proteins with protective functions in the AGS during transmission in addition to the protective adaptation of *P. triangulum*, among them the chaperone GroEL and other stabilizing proteins (10). Likewise, *I. capsulata*-derived GroEL was found in abundance in the capsule provided by *Meg. punctatissima* (12). While there is evidence that host-derived HCs in *P. triangulum* and PMDP in *Meg. punctatissima* are the decisive factors ensuring the survival of the respective symbiont during transmission, the symbionts may still contribute to a successful transmission (10, 12). It is therefore conceivable that the protection of the symbiont from host chemical defence, and other biotic and abiotic stressors, is often realized along a continuum of joint efforts of host and symbiont, rather than achieved by isolated efforts of one partner.

5.2 Aspects affecting the evolutionary trajectory to host and/or symbiont adaptations

Whether a host and/or a symbiont evolves an adaptation to protect the symbiont from host chemical defence and other biotic and abiotic pressures experienced within the association is driven by multiple interdependent factors. Similarly, multiple interdependent factors affect the type of adaptation evolving in response to the threat to the symbiont.
Evolving protection from the stressor requires suitable genomic inventory and metabolic capabilities of host and symbiont. For example, an HC layer protecting symbionts from NO requires the ability to synthesize HCs that are quantitatively and qualitatively suitable for protection from NO. The genomic inventory and metabolic capabilities of host and symbiont particularly depend on the evolutionary history of the symbiosis, i.e. the order in which evolutionary events occur, which is especially relevant for the symbiont. In the first evolutionary scenario, the symbiosis evolves after the threat, or the evolution of symbiosis and threat coincide. If symbiont genes suitable for protection from the stressor are present in the symbiont genome, they should be maintained over evolutionary timescales, since they are important for the symbiont's fitness. Such genes might especially be present in the symbiont genome in case the symbiont already faced the same or a similar challenge before the symbiosis was established and is therefore pre-adapted.
In the second evolutionary scenario, the establishment of the symbiosis precedes the evolution of a threat. In this case, the symbiont's genomic inventory and metabolic capabilities, and therefore its ability to respond to new stressors, may already have been reduced by genome erosion following host-symbiont co-evolution. This phenomenon is driven by relaxed selection pressure on symbiont genes that become obsolete in a host-associated lifestyle, and the non-adaptive process of genetic drift during population bottlenecks, resulting in the loss of genetic material (22). The heat sensitivity of the obligate *Blochmannia* symbiont of carpenter ants presents an example of reduced flexibility of a symbiont with an eroded genome (23). Since symbionts with eroded genomes may lack genetic factors to mitigate specific risks, and may possess a lower general cellular stability (24, 25), it is tempting to infer that host adaptations might regularly occur to protect these symbionts. Indeed, *S. philanthi* and *I. capsulata* – which are both protected by a host adaptation during external transmission (see Chapter 2 and 5.1) (10, 12) – show signatures of incipient genome erosion and advanced genome erosion,

respectively (13, 26): Even though *S. philanthi* retains a fairly large genome of ~7 Mb, it exhibits signatures of incipient genome erosion (26). *I. capsulata* possesses a strongly eroded genome (~0.7 Mb)(13).

Host-symbiont co-evolution should also entail other factors rendering the symbiont less flexible in its adaptations to new challenges arising from the symbiosis. One of these factors should be the evolution of compartmentalization, i.e. the restriction of symbionts to specialized organs. This isolates the symbiont from environmental bacterial reservoirs, which renders the acquisition of new genes via horizontal gene transfer and therefore the acquisition of new symbiont capabilities impossible (27). At the same time, restricting the symbionts to specialized organs can be considered a protective host adaptation, since it can isolate the symbiont from the challenge. For instance, the bacteriocytes of the cereal weevil *Sitophilus* (Coleoptera: Curculionidae), which harbor the nutritional symbiont *Sodalis pierantonius*, show a reduced expression of immune effectors (28). This limited immune response presumably enables symbiont survival in the bacteriocytes by reducing the threat from the host immune effectors (28).

It should be noted that in the second evolutionary scenario, where a risk to the symbiont emerges after the symbiosis has been established, the emergence of a stressor should always be concomitant with an adaptation protecting the symbiont. Otherwise, the symbiosis should no longer be stable. In this context, it is important to consider that structures or mechanisms protecting the symbiont may not have originally evolved for this function, but may secondarily serve this purpose. In beewolves, for example, HCs in the AGS may have primarily served as a nutrient source for *S. philanthi* before they secondarily served to protect the symbiont from rising NO concentrations released from the beewolf egg for self-defence (17, 29).

External transmission is inherently associated with abiotic and biotic environmental challenges to the symbiont (e.g. (30)). The evolution of internal/intracellular transmission may appear to be a solution to this threat; however, it is constrained by the symbiont's ability to enter the host cell cytoplasm. In addition, transmission is also constrained by the benefit provided by the symbiont. In cases where the symbiont function is executed externally, e.g. in beewolves (host offspring protection on the cocoon) (31) and *Lagria* beetles (host egg protection)(32), extracellular external transmission is obviously the only option for vertical transgenerational transfer. Similarly, in Donaciinae beetles (Coleoptera: Chrysomelidae), pectinolytic symbionts are likely bound to extracellular transmission because of their extracellular localization in Malpighian tubules, which is important for the efficient transfer of pectinases to the host gut (33).

5.3 Potential mechanism of HC-mediated NO protection

My experiments strongly suggest that the HCs in the AGS form an efficient barrier against NO, therefore shielding *S. philanthi* from the toxic effects of NO in the brood cell (10, 11). Mechanistically, a chemical inactivation of NO by the predominantly linear unsaturated and saturated HCs in the AGS appears unlikely (10). On the one hand, these HCs should exhibit low reactivity due to the absence of functional groups (17). On the other hand, NO-exposed AGS resembles the non-exposed PPG secretion, cuticle and hemolymph of *P. triangulum* in terms of HC composition (17, 19, 34, 35). Thus, the AGS may rather form a physicochemical barrier against toxic NO concentrations. However, the exact underlying physicochemical mechanism by which a hydrophobic HC layer achieves this is puzzling, in particular because NO has traditionally been assumed to freely diffuse across chemically similar cell membranes, i.e. without involving membrane pores or transmembrane protein channels (36). The diffusion of NO through the cell membrane, but not through an HC layer may be explained

by a considerably tighter molecular packing in the HC layer, at such a rate that the permeation of a small molecule is spatially impossible. However, such tight molecular packing is very unlikely to exist in the HC layer surrounding *S. philanthi* in the AGS, since it consists of approximately 75% unsaturated hydrocarbons (17).

Several observations could not be aligned with the concept of free NO diffusion across cell membranes. First, the actual amount of NO permeating the cell membranes under physiological conditions is reduced by the short half-life of NO (~4 s)(37, 38) and its spontaneous reaction with O_2 to form NO_2 (36). In fact, this reaction has been estimated to be 2-2.5x faster in cell membranes than in the aqueous phase (39, 40). Second, the high expression of aquaporins – membrane proteins realizing water transport functions in mammals (41) – in cells producing NO (42-44) raised questions about the role of aquaporins in the transfer of NO across cell membranes (45). Indeed, experiments with pancreatic endothelial and hamster ovary (CHO-K1) cell lines confirmed such a role, which would enable the putatively crucial control of NO concentration in the cell, as well as directional NO release (45). Further, the cell membrane itself was concluded to present a significant NO diffusion barrier (45). Additionally, research on the mammalian cardiovascular system, in which NO represents a crucial signalling molecule in vasodilation, resulted in observations challenging the concept of free NO diffusion across cell membranes. In particular, inconsistencies between the time to an NO-induced vasodilation response (~2-5 s)(46), and the time until the NO concentration in the endothelial cell membrane reaches a peak (~13 s)(47) hinted at a mechanism of intercellular NO transfer differing from free diffusion across cell membranes. Indeed, a study combining experiments on rat aortic walls, rat neocortical astrocytes and human HeLa cells revealed that NO did not readily permeate cell membranes, but rather activated and passed through connexin(Cx)-based channels (48). In the absence of Cx-based channels, the cell membrane did not allow the diffusion of physiologically relevant amounts of NO (48).

Connexins (Cx) are protein subunits, of which six form a hemichannel (49). Two hemichannels of neighboring cells constitute an intercellular gap junction channel (49). Channels formed by Cx represent aqueous pores known to enable the intercellular passage of small molecules and ions with a diameter below 1.4 nm, including Ca^{2+} and ROS (e.g. H_2O_2 and O_2^-)(49-51). The outlined results indicate that their aqueous milieu is also conducive to NO diffusion from cell to cell (48), which is in line with NO diffusion being 2-10 times faster in water than in lipid membranes (36, 52). Taken together, these findings suggest that contrary to the long-standing notion, the cell membrane is actually impenetrable to NO – at least in physiologically relevant concentrations – and transmembrane proteins forming an aqueous passage are required for its fast and efficient diffusion (48). In other words, NO is able to diffuse into the cell membrane, but unable to reach the cytoplasm in relevant concentrations due to its slow diffusion through lipids in conjunction with its quick reaction with O_2 and its oxidation of membrane-bound lipids and proteins. It is important to note that the oxidation of membrane constituents, which should be further exacerbated by the production of NO_2 (39), potentially impairs cell membrane integrity and functionality and therefore inflicts a cost on the cell (53, 54). However, this may not be very pronounced in the cardiovascular system, since NO concentrations used in a signalling context are relatively low compared to the concentrations used in a defensive context (55-58).

Although realized by different types of transmembrane proteins, intercellular gap junctions and other types of aqueous pores are found in organisms across various taxonomic groups (59-61). While connexins have to date only been found in vertebrates, non-homologous innexins form gap junction channels in invertebrates (62). In Gram-negative and some Gram-positive bacteria, transmembrane

proteins called porins allow non-specific diffusion of small hydrophilic molecules across the cell membrane (61, 63). Therefore, efficient NO diffusion through cell membranes via aqueous pores may not be restricted to the mammalian cardiovascular system, but may rather constitute a more universal phenomenon.

Assuming the presence of aqueous channels as a prerequisite for efficient NO diffusion across a lipid layer, the effective blocking of NO by HCs in the AGS, but not by the cell membrane, could generally be explained by the absence of such aqueous pathways in the HC layer covering *S. philanthi*. Host- and symbiont-derived protective proteins likely enhance this physicochemical blocking by biochemical scavenging of NO (10), similar to NO 'scavenging' by membrane-bound proteins and lipids in the cell membrane (53). In contrast to the cell membrane, however, the integrity of the HCs is unlikely to be endangered by this biochemical scavenging (10).

5.4 Potential for indirect long-term effects of NO on *S. philanthi*

Whereas physiologically relevant NO concentrations did not cross the cell membrane in the study described above (see 5.3) (48), my data indicates a physiological effect of NO on the symbionts, namely the upregulation of several molecular chaperones in response to NO fumigation *in vivo* (10). In addition, the AGS did not completely inhibit NO detection on an indicator filter paper in the brood cell, implying that NO can still diffuse into the AGS to a certain degree (10). How can this difference be explained, assuming aqueous pores as a prerequisite for efficient NO diffusion?

On the one hand, the NO concentrations used for external defense in the beewolf should be much higher than the NO concentrations used in a signalling context (29, 57, 58), which includes NO-induced vasodilation (58). Even after the HC layer slows down diffusion (due to a lack of aqueous pores) and the protective enzymes scavenge NO, there might still be a higher number of NO molecules overcoming these barriers and reaching the symbionts merely by virtue of a much higher NO concentration to begin with. Moreover, NO should also be able to react with O_2 in the HC layer to form NO_2 (39), but in contrast to vasodilation, where NO_2 does not have a signalling effect, NO_2 can also damage proteins, lipids and nucleic acids (36). In other words, the 'scavenging' of NO by O_2 would reduce the amount of signalling molecules in the context of vasodilation, but not the amount of harmful molecules that need to be fended off by the HC layer and the protective proteins in the beewolf system. Last but not least, the thickness of the HC layer around *S. philanthi* may also influence the effectivity of the NO blocking, since a thicker layer should slow down the NO molecules even more. Given that the filamentous morphology of *S. philanthi* translates to a relatively large surface area, the ratio of HC to symbiont surface area may be relatively small in the AGS. This may result in a relatively thin layer of HCs allowing physiologically relevant amounts of NO to cross the barrier and reach the symbionts.

The non-lethal NO concentrations *S. philanthi* putatively experiences in the AGS may indirectly affect the symbionts by imposing a mutagenic burden on their genome, as NO is known to inflict oxidative damage on pyrimidine bases (64, 65). In bacterial cells, the base excision repair (BER) machinery recognizes and subsequently repairs this damage (66-68). Briefly, DNA glycosylases cleave the glycosidic bond of the damaged nucleotide, and enzymes with apurinic/apyrimidinic endonuclease and exonuclease function excise the concerned nucleotide from the DNA (68). After that, DNA polymerases replace the removed nucleotide, and DNA ligases seal the gap (68). In the event that the BER fails to correctly repair the damage, error-prone DNA polymerases are able to replicate the DNA in spite of the damage (69). However, this frequently introduces point or frameshift mutations (69), a

mutagenic burden that should be exacerbated if the BER pathway is impaired. Indeed, five out of 14 genes of the BER pathway have been pseudogenized in the genome of *S. philanthi* (26).
Even though it is unknown whether the NO concentrations faced by *S. philanthi* in the brood cell are sufficient to cause a mutagenic burden on the symbiont, it is tempting to speculate that recurrent NO exposure in conjunction with the impaired BER pathway (26) may contribute to the incipient genome erosion in *S. philanthi*. Determining the exact NO concentration experienced by *S. philanthi* in the AGS *in vivo*, complemented by experimental evolution experiments *in vitro*, will be critical to elucidate whether NO (and/or the impaired BER genes) influences *S. philanthi* genome degradation. The first part could be achieved with the help of NO-sensing reporter strains or NO-detecting ultramicroelectrodes (70-74). Concerning the evolution experiments, the *S. philanthi* symbiont of the North American beewolf *P. gibbosus* could be examined alongside the symbiont of *P. triangulum*, since the former does not show signatures of incipient genome erosion, and possesses an intact BER repair pathway (Mario Sandoval-Calderón, unpublished data). To explore the isolated and synergistic effects of NO and impaired BER genes on genome evolution without the background of the differently eroded genomes in the *S. philanthi* symbionts, evolution experiments with *E. coli* knockdown mutants (75) may complement experimental evolution with the beewolf symbiont.

5.5 Implications of HC-mediated NO protection for the self-defense of the beewolf egg from NO

How the beewolf egg itself avoids self-toxication by NO has remained enigmatic (29, 76). Strohm et al. (29) proposed an NO transport via carrier proteins, similar to nitrophorins of blood-sucking hemipterans (77), from the site of NO production in the embryonic tissue to the site of emission into the brood cell (i.e. the egg shell). This hypothesized adaptation would prevent oxidative damage within embryonic tissue from free NO.
Concerning the diffusion of high NO concentrations back into the brood cell, Engl (76) proposed that the outer cuticle and the epithelial layer of the egg may mediate the protection of the developing embryo. Further, he put forward the idea of the high observed NO concentrations in the cuticle acting as scavengers towards NO_2, a toxic product of the autoxidation of NO (36) in the brood cell. Finally, he hypothesized that the embryo may increase DNA repair and macromolecule recycling efforts to counteract the detrimental effects of high NO concentrations in the brood cell.
Since host-derived HCs protect *S. philanthi* from NO in the AGS (10, 11), it seems possible that the beewolf egg might be protected from NO via a similar mechanism. Indeed, HC- or HC-containing eggshell layers have been described in insect eggs, namely the maternal-derived wax layer, which is part of the chorionic layers, and the extraembryonic wax-containing serosal epicuticle (78). While the serosal epicuticle is made of protein, it is generally assumed to additionally contain waxes and lipids. In the kissing bug *Rhodnius prolixus* (Hemiptera: Reduviidae), the serosal epicuticle is reportedly impregnated with a wax-like substance (79). In the house cricket *Acheta domesticus* (Orthoptera: Gryllidae), the serosal epicuticle is thought of as a lipoid layer (80, 81). A wax layer has also been suggested to be part of the serosal cuticle of the mosquito *Aedes aegypti* (Diptera: Culicidae) (82). These two eggshell layers may prevent NO from diffusing from the brood cell back into the egg, thereby protecting the embryo.
If this is the case, how would a unidirectional NO release from the egg into the brood cell still be possible, given that the chorionic wax layer and the serosal epicuticle completely surround the embryo? As previously described, connexin-based aqueous pathways enable the efficient NO diffusion

through vertebrate cell membranes (see 5.3) (48). Could a unidirectional NO diffusion from a hypothetically HC-covered egg into the brood cell be realized in an analogous manner, i.e. via non-lipid pathways? Aeropyles might constitute candidate egg structures for such a non-lipid NO release pathway. Aeropyles are microscopic apertures on the chorionic surface of the egg, which are connected to the interior of the egg via chorion-traversing channels (78). They provide passage ways for gas exchange for the embryo, and differ in number, distribution and arrangement on the chorionic surface across insect species (78). NO might be produced in the embryo and loaded onto nitrophorin-like carrier proteins, as previously suggested (29, 77). NO might then be released from the carrier proteins into the chorion-traversing channels, from where they might diffuse into the lumen of the brood cell via the aeropyles. To prevent the re-entry of NO into the egg, the external surface of the chorion-traversing channels might also be covered in a wax layer. A similar functional principle has been described in the *Chrysomela* larvae (Coleoptera: Chrysomelidae), where a cuticular lining protects the glandular reservoirs from the detrimental effects of the stored salicylaldehyde, the major compound of its defensive secretion (83). NO transport via carrier proteins, and subsequent release via aeropyles, might therefore allow for a unidirectional NO release. To examine whether HCs may be involved in protecting the beewolf egg from NO, hexane extracts from beewolf eggs could be tested for the presence of HCs. In addition to quantifying the amount of egg-associated HCs in the hexane extracts, one should also qualitatively analyse their composition to find out whether their profile corresponds to the HC profile in the AGS, in the PPG and on the cuticle, and is therefore suitable for protection from NO.

Although there is currently no data available, it appears very likely that HCs are also part of beewolf eggshells, given that HCs are found on the cuticle, in the PPG and in the AGS of beewolves. Nevertheless, it is important to note that the eggs of some insects lack a chorionic wax layer, and a serosal cuticle has not yet been described in eggs of insects in the order Hymenoptera (78). The original waterproofing function of these two missing structures should then be exclusively executed by other components of the eggshell layer, namely by the vitelline membrane and the innermost chorionic layer (78). In the unlikely case that both the chorionic wax layer and the HC-containing serosal epicuticle are lacking in beewolf eggshells, could there be an alternative mechanism underlying the egg's resistance against NO? In the study on the mammal cardiovascular system mentioned above (see 5.3), NO was capable of opening and permeating certain Cx-based channels, whereas it closed another type of these channels (48). The latter could only be permeated by NO if it was previously opened by a divalent cation-free extracellular solution (48). These results indicate that NO diffusion through cell membranes via gap junction channels can be regulated (48, 84). As described above, gap junction channels in invertebrate tissue are formed by innexins, which are non-homologous to vertebrate connexins (59, 60). They can be directly opened by changes in membrane potential, hypoxic conditions, mechanical stressors, and an increase in cytoplasmic concentration of Ca^{2+} ions (85). Additionally, they can be indirectly activated when ligands bind to membrane receptors (85, 86). Innexin inactivation can occur through acidification and low pH of the cytoplasm (85, 86).

Since innexins are present in the epithelial cells of invertebrate embryos (87), one could speculate that the embryo may be protected from the diffusion of NO back into the egg via the inactivation of innexin-based gap junctions in the embryonal epithelial layer. This inactivation may either directly be induced by NO, or via an NO-induced signalling pathway. Once innexin-based channels are inactivated, NO would not be able to diffuse through them, and the lipid layer of the epithelial membranes would not be conducive to efficient NO diffusion in the first place (48). As a consequence, NO would not be able to diffuse from the brood cell back into the embryo – in other words, the embryo would be

protected from NO released into the brood cell. In support of this scenario, NO can indeed cause intracellular acidification, which can inactivate innexins (88). However, this again raises the question how a unidirectional NO release would be possible in the face of a mechanism based on innexin-inactivation. On the one hand, NO could be released via carrier proteins and aeropyles as described above. Alternatively, nitrophorin-bound NO might be transferred through the eggshell layers without the involvement of aeropyles: While innexin-based channels enable the transfer of ions and other small molecules through the cell membrane (59), they are likely too small to allow the passage of macromolecules such as nitrophorin-like proteins. Therefore, the transfer of nitrophorin-like proteins through the embryonal tissue might rely on a different membrane protein. The nitrophorin-mediated NO transport to the site of release would thus not be affected by the hypothetical innexin inactivation. Even though a potential closure of innexin-based gap junction channels may hinder the diffusion of NO through the epithelial tissue of the embryo, NO likely still oxidizes membrane lipids and transmembrane proteins, compromising membrane integrity (53, 54). It is therefore conceivable that preventing efficient NO diffusion alone is not sufficient to ensure the survival of the embryo in this scenario. Instead, the protection of the embryo could be realized in conjunction with an increased expression of genes involved in macromolecule recycling, as proposed by Engl (76). For instance, eggshell peroxidase, an enzymatic component in the innermost chorion layer and endochorion of some insect eggs, may aid in egg self-defense via enzymatic scavenging of NO and its noxious reaction products (78).
Despite these considerations, the exact mechanism by which eggs avoid self-toxication remains mysterious.

5.6 Potential for HC-mediated desiccation protection of *S. philanthi* in the AGS

The HC profile of beewolves is not only suitable for blocking NO, but also prevents desiccation on the cuticle of adult wasps (11). This begs the question whether the HCs in the AGS may not only protect the symbiont from NO, but also from desiccation. Indeed, the symbiont-containing AGS remains on the ceiling of the brood cell for approximately 9 days between secretion and uptake by the larva (89, 90). This translates to a considerable amount of time outside of the antennal gland reservoirs, an external environment most likely entailing a considerable desiccation stress (as opposed to the antennal gland reservoirs, in which the host should maintain water homeostasis). Therefore, it is conceivable that HCs in the AGS additionally serve to protect *S. philanthi* from desiccation in the brood cell.

5.7 Defense of symbionts from ROS and RNS in different contexts

Apart from the to date unique case of *S. philanthi* being threatened by NO during external transmission (29), other symbionts also experience RNS and ROS as a host immune effector within the host, and as a host-derived effector and signalling molecule during host colonization (56, 91, 92). In these systems, host and symbionts have evolved different strategies to cope with the resulting oxidative stress, mirroring the context in which ROS in encountered, as well as the particularities of the respective systems.
ROS are commonly produced by insect gut epithelial cells to maintain microbial gut community homeostasis (92). Pathogen infection generally causes an oxidative burst, effectively eliminating antagonists, while low levels of ROS are produced in response to the native microbiota (92-94). This differential regulation of ROS release is crucial to prevent damage to the gut epithelial tissue from

otherwise constant oxidative stress (92, 95-97). The manner in which this differential regulation is achieved putatively differs between systems. For instance, observations in *Wolbachia*-infected *Ae. aegypti* mosquitoes (Diptera: Culicidae) gave rise to the suggestion that the host evolved tolerance towards *Wolbachia* infection (92). Indeed, the ROS levels in the gut of naturally *Wolbachia*-infected and aposymbiotic mosquitos are comparably low (98). However, the ROS level is higher when aposymbiotic mosquitoes are artificially infected with *Wolbachia* (98). Since these higher levels of ROS are linked to a higher expression of the immune genes *Duox* and *Nox* (99), they likely represent an immune response to *Wolbachia* infection. Co-evolution may have led to a reduction in host-produced NO to avoid continuous oxidative damage to the epithelial tissue, hence resulting in host tolerance towards *Wolbachia* (92).

In the honeybee *Apis mellifera* (Hymenoptera: Apidae), *Gilliamella* embodies a core gut microbial symbiont, which persists in the gut by not stimulating ROS generation: In contrast to the native *Gilliamella* symbiont, a non-native strain triggered immune regulatory pathways, which resulted in high levels ROS in the gut, and ultimately the inhibition of the non-native strain (100). The presence of the non-native *Gilliamella* was associated with higher levels of prostaglandin in the gut; however, the underlying molecular mechanism of differential host immune regulation remains enigmatic (100). Additional experiments confirmed that the native *Gilliamella* was not resistant to increased ROS levels (100). The fact that the native *Gilliamella* symbiont did not cause elevated levels of prostaglandin may be a result of host-symbiont co-evolution (101).

Similarly, gut symbionts in *Drosophila* persist in the host environment by not triggering a host immune response (102, 103). Here, pathogenic gut bacteria import extracellular uridine from the insect gut lumen, where it is converted to ribose and uracil by a nucleoside hydrolase (103). While ribose is putatively involved in quorum sensing regulation and subsequent pathogenesis, uracil is released from the bacterial cell (103). In the gut lumen, uracil is detected by host dual oxidase, which induces the generation of high concentration of ROS, effectively eliminating the pathogens (103). In contrast, resident gut symbionts lack the ability to import and hydrolyse uridine, so they do not release uracil and hence do not evoke ROS generation (103). This may embody an adaptation of the resident gut microbes to the gut environment (102, 104).

In the case of the *Gilliamella* symbiont of honeybees, it is known that the symbiont itself is not resistant to high ROS concentrations (100). Therefore, it likely does not survive the ROS burst upon infection with non-native microbes. The collateral elimination of the symbiont by host defences should entail that the symbiont can be reliably replaced in the concerned system (6). Indeed, several routes of *Gilliamella* re-acquisition are possible in honeybees, which are mostly rooted in honeybee eusociality: Symbionts can be re-acquired via trophallaxis, coprophagy of conspecific feces, ingestion of honey and via contact with hive material (105, 106). Assuming a susceptibility of *Drosophila* gut residents to elevated ROS concentrations in the gut upon pathogen infection, it is conceivable that they are replenished by the ingestion of food, which embodies a suitable substrate for the growth of microbes (94, 107).

Mosquitoes maintain a hematophagous lifestyle, which entails oxidative challenges in the gut upon a blood meal due to the high amounts of contained heme (108). The gut microbiota experiences a pronounced compositional shift upon blood feeding: First, the bacterial abundance increases (108), even though bacterial proliferation is ultimately constrained by the peritrophic matrix (PM)(108), an extracellular structure of organic macromolecules produced by midgut epithelial cells (108-110). Second, the general bacterial diversity is reduced, and Enterobacteriaceae species become enriched (108, 111). While it is not clear what causes this compositional change, it has been suggested that it

either mirrors the change in the majorly available nutrient in the gut – from predominantly carbohydrates upon nectar feeding to predominantly proteins upon blood feeding (108) – or genomic inventories and metabolic capabilities more suitable to deal with oxidative stress resulting from the blood meal (111), which may be linked to the suggested active role in hemolysis of some of these enriched microbes (112-116). Additionally, the enriched bacterial taxa may be more adept at avoiding excretion with the digested blood meal (111).
Arising from the need to protect the gut tissue from damage, there are several host-derived mechanisms in place to alleviate the oxidative challenges posed by blood digestion (108). For instance, the iron storage and transport protein ferritin and catalase appear to play a role in the mosquito's response to blood feeding (108, 117-124). Further, heme perception by the mosquito results in the upregulation of several key metabolic genes, which appears to ensure the sufficient production of molecules with reducing properties (108). Overall, these mechanisms are putatively connected with the net reduction of ROS in the midgut post blood ingestion (108, 125). The PM also separates the blood meal and the epithelial cell layer from each other and can effectively sequester heme (108, 126, 127). While these host defensive strategies are primarily implemented to protect gut epithelial cells, they might secondarily aid in the protection of the gut microbiota from blood feeding-related oxidative stress. However, a possible spatial isolation of host protective measures from the gut microbes via the PM would be at odds with this notion. Moreover, the loss of diversity in the gut composition does not support this idea, or would at least suggest that host protection is restricted to a subset of hypothetically beneficial gut microbes which might aid the host in blood meal digestion. Eventually, the gut microbes lost during blood feeding can be recovered (128), supposedly via reacquisition by feeding on nectar and consuming water, via contact with soil, the plant rhizosphere, or conspecific pupae (108).
These four examples illustrate that the threat of ROS to the gut microbiota can be mitigated in various ways, within the constraints and factors influencing their evolutionary trajectory as outlined in 5.2.

Apart from the gut environment, symbionts are known to encounter ROS during the colonization of specialized host organs (56, 92). For example, *Aliivibrio fischeri*, the symbiont of the Hawaiian bobtail squid *Euprymna scolopes*, is confronted with a burst of host-derived NO during host colonization (129). To withstand this challenge, *Al. fischeri* overexpresses flavohemoprotein (*hmp*) (130), which converts NO to nitrate (131).
Similarly, nitrogen-fixing symbionts of legumes (Fabaceae) encounter an oxidative burst during the colonization period (92), which mainly consists of H_2O_2 and superoxide (132). Following a complex cross-talk between host and symbiont, *Sinorhizobium* (*Ensifer*) *meliloti* excretes the exopolysaccharide succinoglycan (EPS-I)(91, 133). This macromolecule is composed of repeating octasaccharides, each carrying three non-sugar modifications, and occurs in a high molecular weight (HMW) and in a low molecular weight (LMW) form (134). In contrast to the HMW form, which consists of several hundred subunits, the LMW form occurs as monomers, dimers and trimers (135). In addition, some strains are also known to produce a second exopolysaccharide, namely galactoglucan (EPS-II) (135). EPS-I, specifically the LMW form, is required for a successful colonization and nodulation in their plant hosts in the genus *Medicago* (136, 137). EPS-II improves host colonization in conjunction with EPS-I (138), and can even mediate host colonization in the absence of EPS-I (139). However, it is not sufficient for nodulation in hosts other than *Medicago* (139).
EPS-I and EPS-II were shown to facilitate *Sin. meliloti* survival when challenged with H_2O_2 *in vitro* (140), suggesting that a layer of exopolysaccharides (EPS) enables host colonization by protecting *Sin.*

meliloti from H_2O_2-induced oxidative stress. The same protective effect of EPS has been described in *Azorhizobium caulinodans* during the colonization of *Sesbania rostrate* (141). Concerning EPS-II, the fact that its presence alone is not sufficient for symbiosis establishment in some host plants led to the speculation that EPS may fulfil additional functions (140). The size and biochemical nature of the LMW form of EPS-I have been implied to play a role in its protective effect against H_2O_2. One the one hand, it has been brought forward that the LMW form may be more mobile or diffusible compared to the HMW form, resulting in an improved radical scavenging ability (140). Alternatively, it has been hypothesized that it is mediated by a higher number of reducing sites compared to the total number of sugars (140). Similarly, an increased number of succinyl groups in the LMW form may be an explanation, since these are putative targets of H_2O_2 (140, 142).

5.7.1 Protection from ROS and RNS via matrices of organic macromolecules

The symbiont being coated in organic macromolecules for physicochemical protection against oxidative stress is a defensive strategy employed in the beewolf-*Streptomyces* symbiosis (10, 11), the Plataspid-*Ishikawaella* system (12) and in the legume-rhizobium association (140, 141). Nevertheless, the type of macromolecules realizing the coating, and the underlying mechanism of protection may differ between the systems: In beewolves, majorly linear saturated and unsaturated HCs coating *S. philanthi* may form an effective primary physiochemical barrier towards toxic NO encountered during external transmission (10, 11), but are unlikely to biochemically scavenge NO due to the lack of functional groups in the HCs (see 5.3; (10)). Instead, additional protective enzymes of host- and symbiont origin embedded in the HC matrix may take over this role (10), resulting in a putative two-fold protective mechanism. In the legume-rhizobium association, the protective effect of EPS excreted by and coating *Sin. meliloti* upon oxidative stress during host colonization may be based primarily on biochemical radical scavenging via reducing ends of the EPS (140). During external transmission in Plataspid bugs, *I. capsulata* is engulfed in a capsule mainly composed of the host-derived PMDP peptide, stabilizing the symbiont and putatively shielding it from oxidative stress due to prolonged exposure to intense sunlight (12). It is currently unknown how PMDP mediates symbiont survival under these conditions. However, it has been speculated that PMDP stabilizes *I. capsulata* by binding to its surface (12). Concerning protection from putative oxidative stress, free radicals might also be scavenged by receptive ends of the peptide.

The origin of the critical protective macromolecules presents another difference between the systems: HCs and PMDP are provided by the respective host (12, 17), while *Sin. meliloti* itself provides the EPS for its coating (134). This is likely related to the different modes of symbiont transmission in these systems (closed system with vertical transmission vs. open system with environmental symbiont acquisition in every generation). Since the beewolf-*Streptomyces* and Plataspid-*Ishikawaella* symbiosis present rather co-evolved systems in which the symbiont cannot be readily replaced (14, 143-145), it should be in the best interest of the host to implement measures for a successful vertical symbiont transmission (6), especially given their eroded genomes (13, 26). In contrast, *Sin. meliloti* is environmentally acquired, and the oxidative burst by the host plant may constitute a partner choice mechanism ensuring symbiont specificity (27), requiring the prospective symbiont to protect itself for successful host colonization.

In contrast, a common aspect of symbiont coating in organic macromolecules may be the presence of additional symbiont-derived protective proteins in the primary coating matrix, although this has only been demonstrated for the HC layer around *S. philanthi* and the capsule around *I. capsulata* (10, 12).

Nevertheless, the genome of *Sin. meliloti* encodes three catalases and two superoxide dismutases (146), and one or more copies of these genes were also found in *Mesorhizobium loti*, another nitrogen-fixing bacterium (147). Mutations in one or more copies of these genes resulted in an aberrant symbiotic phenotype (148, 149). Additionally, both *Sin. meliloti* and *Mes. loti* encode several glutathione-*S*-transferases, which play a role in cellular antioxidant activity (147, 150). Another nitrogen-fixing bacterium, *Rhizobium tropici*, possesses the genetic information of a glutathione synthetase, and a mutation in this gene led to outcompetition by the wild type during host colonization (151). These gene products therefore play an important role in the protection of nitrogen-fixing symbionts during the colonization process, although they may not be excreted into the extracellular milieu.

Another aspect that symbiont coating in the beewolf and the legume system might have in common is that their components may fulfil more than one function. In addition to protection from NO, HCs in the AGS may serve as nutrients for *S. philanthi*, protect the symbiont from desiccation, and provide olfactory cues for the localization of the AGS by the larva (17). Concerning EPS, the existence of one or more additional functions of EPS-II has been hypothesized for EPS-II (140). On a broader level, EPS are part of surface polysaccharides (SPS) of bacterial envelopes, which may sequester antibiotics, repel AMPs via electrostatic repulsion, and may imitate host cellular compounds via structural plasticity (141). It may therefore be conceivable that EPS in nitrogen-fixing bacteria have more than one function in the symbiosis with legumes. EPS are generally excreted by a wide range of bacteria and exhibit a broad diversity in chemical structures and physical characteristics, a prolonged lifespan and a high molecular weight (10-1,000 kDa)(152). EPS, among other organic polymers such as proteins and lipids, are crucial constituents of biofilms (152). Within the biofilm, EPS enable intercellular communication between bacteria, trap metabolites and nutrients, confer stability, prevent desiccation and deflect environmental stress (152). Apart from diverting H_2O_2, EPS also inhibit the activity of a wide array of other radicals, and also prevent the entry of liquids, gases, and antimicrobials (153). Therefore, biofilms play a key role in many different contexts, including bacterial pathogenicity and symbiosis (141, 153-159). Independent of oxidative stress, biofilms are likely essential for the establishment of various symbiosis, e.g. for the respective symbiont(s) of the bobtail squid *Eup. scolopes* (158, 159), of the bean bug *Riptortus pedestris* (154), of the honeybee *Apis mellifera* (155), and of the tsetse fly *Glossina morsitans morsitans* (156).

Our study, together with the study on Plataspid bugs, demonstrates that coating in organic matrices is not only a strategy employed by bacteria to defend themselves against a wide array of stressors, but can also be used by eukaryotic hosts to protect their microbial symbionts during stressful external transmission. Research in other systems may find that this functional principle is far more widespread among insect hosts than we are currently aware (see 5.9). It is important to note that this functional principle is also harnessed outside the context of symbiosis, as illustrated by the PM in the mosquito gut (see 5.7) (108-110, 126, 127), and therefore has universal application in the face of a plethora of stressors.

5.8 Potential threat of insect chemical defence via volatile compounds to symbionts in other systems, and potential for host protective adaptation

Using volatile compounds for external chemical defence is not restricted to beewolf NO defence, but has been observed – or at least inferred – in other insects in the orders Dermaptera, Hemiptera, Coleoptera, Hymenoptera and Blattodea (83, 160-167). The potential threat to a symbiont during

transmission in these systems – and therefore the necessity to protect it – should depend on the mode of transmission (influenced by intra- and extracellularity of symbionts), on the temporal overlap of external defense and symbiont transmission, the degree of toxicity of the volatile(s) to the symbiont, and the degree and length of symbiont exposure to the volatile(s). The latter should be influenced by the level of sociality of the host insect (solitary, gregarious, sub-social, social) and the level of confinement of their habitat (168).

Nest fumigation via volatiles has been described in the solitary emerald cockroach wasp *Ampulex compressa* (Hymneoptera: Ampulicidae) (160, 168). The larvae parasitize *Periplaneta americana* cockroaches (Blattodea: Blattidae), which they sterilize by applying a secretion containing micromolide and (*R*)-(-)-mellein (168). The latter vaporizes from the cockroach, fumigating the subterranean nest (160). The antimicrobial effect of (*R*)-(-)-mellein was confirmed by incubating bacterial and fungal isolates in the headspace of parasitized cockroaches, and of a filter paper impregnated with synthetic mullein (160).
To the best of the author's knowledge, there a no reports on an association between *Am. compressa* and one or more microbial symbionts. Due to the similar ecology of *P. triangulum* and *Am. compressa*, one may be tempted to infer a defensive symbiosis with an antibiotic-producing microbe in *Am. compressa* for long-term cocoon protection. Indeed, *Am. compressa* larvae incorporate antimicrobials into their cocoon (160). These antimicrobials, however, correspond to the antimicrobials found in the secretion applied to the cockroach, including micromolide and (*R*)-(-)-mellein (160). Therefore, a symbiosis similar to that of the beewolf-*Streptomyces* association is unlikely in *Am. compressa*. Currently available data suggests that the defensive symbiosis with *S. philanthi* is confined to the tribe Philanthini in the family Crabronidae (169).

In addition, there is evidence for microenvironment sanitation in chrysomelid beetles (Coleoptera). For instance, larvae of the willow leaf beetle *Phratora vitellinae* constitutively emit volatile, salicylaldehyde-rich secretions from glandular reservoirs, and salicylaldehyde was detected in the larval headspace (161). Synthetic salicylaldehyde hampered the growth of the bacterial entomopathogen *Bacillus thuringiensis in vitro*, and removal of salicylaldehyde from the glandular reservoirs increased the larval susceptibility to the fungal entomopathogens *Metarhizium anisopliae* and *Beauveria bassiana* (161). Similarly, larva of *Chrysomela vigintipunctata* and *Chrysomela lapponica* release salicylaldehyde-rich secretions from nine pairs of dorsal glands (83). Corroborating the insights from *Phr. vitellinae*, larval secretions, as well as pure salicylaldehyde, inhibited fungal growth *in vitro*, and exposure to the gaseous phase led to a decrease in fungal germination and growth (83). Additionally, the unspecific cytotoxicity of salicylaldehyde against prokaryotic and eukaryotic cells, including insect cells, was demonstrated (83). Since the secretion is emitted in response to various triggers, for instance upon attack, upon disturbance by conspecifics, and mechanical stimuli, the larvae of *Chr. vigintipunctata* and *Chr. lapponica* are almost constantly surrounded by a protective cloud (83).
However, similarly *to Am. compressa*, knowledge on a symbiotic association in these beetle species (or related species in the tribe Chrysomelini) is presently unavailable (170).

Sanitation of the microenvironment with volatile compounds may also be a defensive strategy in stinkbugs. Laboratory colonies of the stinkbugs *Euschistus heros*, *Nezara viridula* and *Chinavia ubica* (Hemiptera: Pentatomidae) were observed to not succumb to fungal diseases, in spite of rearing

conditions favorable to their introduction into the colonies (163). Concordantly, stinkbugs were less susceptible than fungal infection-prone *Dichelops melacanthus* stinkbugs when challenged with the entomopathogenic fungus *Beau. bassiana*. This was attributed to various volatile components harboured in the metathoracic glands of adults and abdominal glands of nymphs (163). Bioassays confirmed a fungistatic and fungicidal effect of these volatiles in the gaseous phase (163).
Accounts of midgut-associated symbiont(s) can be found for *Eus. heros* and *Ne. viridula*, which are likely vertically transferred via egg smearing (171-176). Since the egg masses are deposited on the bottom of a leaf in nature (177), and no further care is provided by the adults, the symbionts should not be exposed to the defensive volatile compounds, at least not in relevant concentrations, and therefore not require specific protection.

Nasutitermes costalis and *Nasutitermes nigriceps* soldier termites (Blattodea: Termitidae) may employ volatile compounds pervading the nest material to inhibit fungal growth in their microenvironment. When exposed to fungal spores *in vivo*, subcolonies of these termites were less susceptible compared to another termite species whose soldiers rely on mechanical defense (164). Additionally, α-pinene and limonene, two volatile components of the frontal gland secretion of soldiers, decreased spore germination of a fungal isolate *in vitro* (164). The same was observed for a mixture of both compounds (164). Similarly, workers and soldiers of *Reticulitermes speratus* (Blattodea: Rhinotermitidae) appear to excrete mellein and apply it to the walls of their nest, from where it is suggested to volatilize for microenvironment sanitation (178). Mellein showed a wide-ranging antimicrobial activity and was effective against *Met. anisopliae* and *Beau. bassiana in vitro* (178).
Termites are generally known to harbour intracellular *Wolbachia* symbionts in reproductive, but also in somatic tissue (179, 180). Indeed, infections with *Wolbachia* were found in several *Nasutitermes* species, among them *Na. nigriceps* (179). According to long-standing beliefs, these symbionts should be exclusively transovarially transmitted and therefore not directly exposed to the defensive volatiles during transmission (however, it should be noted that there are various observations suggesting additional horizontal transfer (181-184)). Moreover, termites possess a complex gut microbial community aiding in the breakdown of their diet consisting of complex material, such as wood (185). This community is majorly transmitted via social acquisition (105, 186, 187), which should not pose a considerable risk of exposure to the defensive volatiles to the microbiota, since the transfer is anticipated to take a minimal amount of time.

Microenvironment sanitation via volatiles may also play a role in the burrowing bug *Scaptocoris talpa* (Hemiptera: Cydnidae). It was initially observed that banana plants withstanding banana wilt caused by *Fusarium oxysporum* exhibited *Sc. talpa*-infested roots (165). Concordantly, exposure of microbial isolates in the headspace of the burrowing bug *Sc. talpa* (Hemiptera: Cydnidae) showed that unidentified volatiles from adult and nymphal scent gland excretions acted as fungistatics and fungicidals against *Fu. oxysporum* and 14 other ecologically relevant fungal isolates from soil (165). In the congeneric *Sc. castanea*, 11 constituents were found in the metathoracic gland secretion, among which (*E*)-2-decenal, 2-octenal, 2-hexenal, and 1-octen-3-yl acetate were the most abundant (188).
Studies on the congeneric *Sc. castanea* found putative extracellular symbionts in the lumen of the fourth ventricle of the host gut (189). Females additionally harbour bacteriocytes filled with intracellular bacteria in the fifth gut ventricle (189). While intracellular bacteria are usually transovarially transmitted, the putative extracellular symbionts in the fourth ventricle might be vertically transmitted via egg smearing, capsule transmission or coprophagy, which are widespread

transmission mechanisms of gut crypt-associated symbionts in the infraorder Pentatomomorpha (105). Members of the subfamily Cephalocteinae, such as *Scaptocoris* burrowing bugs, generally deposit single eggs underground in the vicinity of the host plant and provide no further maternal care (190). The depth of oviposition is influenced by environmental conditions, and the offspring hatches after 1-4 weeks (190). An assumed vertical transmission of the extracellular gut bacteria in the fourth ventricle is not expected to pose a major threat to the putative symbionts, since it does not appear likely that the eggs are constantly surrounded by adults.

Harmonia axyridis ladybird beetles (Coleoptera: Coccinellidae) have been hypothesized to be enveloped in a cloud of antimicrobials, putatively protecting beetle aggregations from pathogen infestation during overwintering periods (166). Three components of the headspace of *H. axyridis*, namely phenol, (+)-α-pinene and benzaldehyde, had a negative effect on the growth on Gram-positive and Gram-negative bacteria, as well as on yeast, *in vitro* (166, 191).
H. axyridis are known to be infected by intracellular *Wolbachia*, *Rickettsia* and *Spiroplasma* symbionts (192-194), whose transovarial transmission (195) should prevent them from being directly exposed to the putative antimicrobial cloud. Furthermore, *H. axyridis* harbours a microbial gut community (196). Egg smearing is one of the most common transmission routes of extracellular symbionts, particularly gut symbionts (105), and bacteria have in fact been found on the egg surface of *H. axyridis* (197). Thus one may infer that the gut microbiota of *H. axyridis* might also be transmitted in this manner. Since eggs are attached to plant leaves (198), and are not tended to by mothers, the risk posed by the putative antimicrobial cloud to the gut microbiota are presumably slim.

Microenvironment sanitation by volatiles may be inferred in the gregarious bed bug *Cimex lectularius* (Hemiptera: Cimicidae), whose glandular secretion comprises the aldehydes (*E*)-2-hexenal and (*E*)-2-octenal (167). Fumigation with synthetic (*E*)-2-hexenal and (*E*)-2-octenal inhibited fungal growth *in vitro*, and bed bugs challenged with an entomopathogenic fungus displayed a much higher survival rate in the presence of (*E*)-2-octenal (167).
Wolbachia, a gammaproteobacterial BEV-like symbiont (199), and *Rickettsia* (200) have been reported to intracellularly infect *Ci. lectularis*. Assuming that *Ci. lectularius* may be surrounded by an antimicrobial cloud, these symbionts should not be directly exposed to it, since they are maternally transmitted (199-201). A study investigating the gut content of *Ci. lectularius* did not find any gut residents, but this may have been due to its cultivation-dependent approach (202). In the congeneric *Ci. hemipterus*, Proteobacteria account for >99% of gut microbes (203, 204). It is unknown whether these gut residents provide benefits to their host – given the hematophagous lifestyle of bed bugs, one may speculate that the might be involved in blood meal digestion, as has been hypothesized for mosquitoes (see 5.7). Regardless, extracellular gut symbionts in hematophagous insects are usually re-acquired from the environment in every generation, and convincing reports on their stable vertical transmission are lacking to date (205).

Finally, earwigs may reduce the risk of pathogen infection associated with living in sub-social groups and residing in moist subterranean nests via fumigation of the microenvironment (162). Natural *Forficula auricularia* earwig populations (Dermaptera: Forficulidae) are engulfed by volatile benzoquinones (162), which are components of the defensive excretions from the abdominal glands of adults (206, 207). Similar benzoquinones are also part of the defensive excretions of *Apterygida media* and *Chelidurella guentheri* (Dermaptera: Forficulidae) (162). Inhibition zone assays with one of

the comprised benzoquinones, and the complete secretion of all three species revealed their antibacterial and antifungal activity. The *Fo. auricularia* secretion additionally displayed nematicidal activity in various bioassays (162).
Fo. auricularia has been described to harbour a gut microbiome, whose experimental disturbance caused irregularities in host digestion and in the weight of eggs and nymphs (208). In another earwig species, *Anisolabis maritima* (Dermaptera: Anisolabididae), a core microbiome is transferred from the mother to the egg, presumably from salivary glands, during the prolonged period of maternal care (209). Members of the core microbiome potentially have antifungal properties, which may serve to ensure the protection of the egg in conjunction with mechanical cleaning and the application of saliva by the mother (209). The transfer of a core microbiome from salivary glands to the egg has also been suggested for *Fo. auricularia* (208, 210). Under this assumption, one may speculate that these microbes are exposed to the volatile antimicrobial benzoquinones during the prolonged period of maternal care, a trait shared between *An. maritima* and *Fo. auricularia* (211, 212). The putative threat from benzoquinone from the close proximity to the mother during maternal care may be exacerbated by the fact that exposure presumably takes place in the confined space of the subterranean nest. Interestingly, one may speculate that maternal care might be both the source and the solution to the hypothesized threat of volatile benzoquinones. This is based on the fact that extended maternal care is necessary for maintaining the appropriate bacterial composition on the egg in *An. maritima*, since experimental removal of mothers from egg clutches resulted in a profoundly different microbial community on the eggs (209). If that is the case, the mother may either continuously replace microbes afflicted by the defensive volatiles, or steadily provide protective compounds onto the egg surface.

In conclusion, the threat of host-applied defensive volatiles to their symbionts, and consequently the potential for a host protective adaptation, can generally be considered low, based on the current knowledge of the systems in which such volatiles have been described or inferred to date. Earwigs may embody an exception to this observation.

5.9 General potential for host protective adaptation during external transmission of extracellular symbionts across insects

Vertical transmission of extracellular symbionts entailing (extended) periods outside the stable host environment is a common occurrence across insects in the order Coleoptera, Hemiptera, Diptera, Hymenoptera, as well as in a few other orders (105). While outside the host, the symbionts are exposed to environmental pressures, such as UV radiation, unfavorable temperatures and humidity, oxidative stress, microbial competition, to name but a few. It has been suggested that host adaptations to protect the symbionts should be selectively favoured given the benefits provided by the symbionts to the host (6). Which systems can be considered promising systems to unveil such host adaptations protecting the symbionts from environmental stressors during external transmission?
Based on the two systems in which a distinct host adaptation for symbiont protection has been described, namely the beewolf-*Streptomyces* and the Plataspid-*Ishikawaella* symbiosis, we can infer multiple characteristic traits coinciding with the presence of such an adaptation. Attributes shared between both exemplary systems are maternally provided conspicuous structures surrounding the symbionts, forming a matrix of organic macromolecules (12, 17); crucial benefits provided by the symbiont to the host (defensive/putatively nutritional)(31, 90, 213), symbiont compartmentalization in specialized organs coupled with vertical transmission (15, 143, 214, 215) (resulting in host-symbiont

co-cladogenesis in the Plataspid-*Ishikawaella* symbiosis (14)), a solitary lifestyle and the absence of maternal care, and symbiont genome erosion (to differential extents)(13, 14, 26). The latter may serve as a helpful indicator especially once it results in impaired symbiont cellular integrity, specifically due to the loss of cell wall-related genes, as exemplified by *I. capsulata* (13). While all of these reference points may be useful in identifying systems with potential host adaptations, it is important to note that not all of them might apply to one system at the same time. In addition, a specific threat during external transmission may be indicative of a host adaptation, such as the NO defense of beewolf eggs, but – to the best of my knowledge – has rarely been identified (see also 5.8).

Taking these reference points into account, various systems of interest can be identified. On the one hand, the symbiosis between the stinkbug *Urostylis westwoodii* (Hemiptera: Urostylididae) and '*Candidatus* Tachikawaea gelatinosa' (henceforth *T. gelatinosa*) may qualify as a promising system. In this association, *T. gelatinosa* likely provides its host with essential amino acids lacking in their diet (216). The association between urostylidid bugs and *T. gelatinosa* is characterized by host-symbiont co-cladogenesis (216). The symbiont possesses a reduced genome of 0.7 Mb (216), thus symbiont instability and/or limited metabolic capabilities may constitute major challenges for transgenerational transfer. During external transmission, the host embeds the symbiont in a gelatinous matrix, with which it subsequently provisions its egg masses (216). The production of this jelly-like substance is linked to specialized host structures. The matrix is made from 60% water, 26% sugars and 8% proteins, with galactans accounting for over 90% of the total amount of sugars (216). Notably, it also contains essential and non-essential amino acids (216). It has been experimentally demonstrated that the nutrients present in the jelly are vital for the nymphs to reach the 3rd instar, supposedly because *T. gelatinosa* may not be capable of efficient amino acid biosynthesis due to the low temperatures, as the nymphs hatch in the winter time (216). However, it has not been shown whether one or more components of the gelatinous matrix are required for a successful transmission of *T. gelatinosa*. Due to their abundance in the gelatinous matrix, it is tempting to speculate that galactans counteract symbiont instability during transmission in *U. westwoodii*.

On the other hand, host adaptations for symbiont protection may be found in semi-aquatic Donaciinae reed beetles (Coleoptera: Chrysomelidae). In these beetles, gammaproteobacterial symbionts are localized intracellularly, but also extracellularly in modified Malpighian tubules (217-219). They likely supply riboflavin and essential amino acids to the larvae feeding on plant sap, while they aid in pectin degradation in the folivorous adults (33). The association exhibits strict co-cladogenesis (219). Females attach their eggs to their aquatic host plants, usually below the water surface (217). The symbionts' genome is very reduced (~0.5 Mb) (33), highlighting symbiont instability as a putative challenge for external transmission. The symbionts are embedded in a jelly coat of host origin during transmission (217-219). However, it is unknown to date of what the jelly coat is composed, and whether one or more constituents stabilize the gammaproteobacterial symbionts during transmission.

Furthermore, the features of the obligate association between Cassidinae tortoise beetles (Coleoptera: Chrysomelidae) and '*Candidatus* Stammera capleta' (henceforth *Sta. capleta*) warrant a closer look at potential host adaptations. *Sta. capleta* putatively aids its host in the breakdown of pectin in their herbivorous diet, and is localized in specialized organs linked to the ovipositor (220). Its small genome (~0.27 Mb), which in fact represents the smallest known genome of an extracellular organism to date (220), and the resulting putative symbiont instability may embody a challenge for

vertical transmission. The process of vertical transmission entails the deposition of caplets to the anterior egg pole, and *Sta. capleta* is embedded in spherical secretions of maternal origin contained in these caplets (220, 221). While it has been shown that the surrounding caplet is not essential for symbiont transmission (221), it is not known in how far the spherical secretions execute a symbiont-protecting function during transmission.

The shield bug *Parastrachia japonensis* (Hemiptera: Parastrachiidae) is associated with the putatively uric acid-recycling, nutrient- and protective enzyme-supplementing symbiont 'Candidatus *Benitsuchiphilus tojoi*' (222, 223). Similar to the symbionts mentioned above, it possesses a small genome of ~0.8 Mb (223). Females provide maternal care to their egg masses (224). Interestingly, the symbionts are not transferred to the egg upon oviposition, but shortly before hatching: The female excretes copious amounts of white symbiont-containing mucus on the egg surface for around 40 min, immediately followed by hatching and ingestion of the mucus by the offspring (225). It is tempting to speculate that in addition to hypothetical stabilizing molecules in the mucus, this specific transmission timepoint may present a behavioural peculiarity in the framework of the provided maternal care (224) to reduce the exposure of symbionts to environmental stressors.

Systems with host-provided matrices containing the symbionts, such as caplets or jelly, may be intuitive starting points for the discovery of symbiont-protecting host adaptations due to their conspicuousness. Nevertheless, one should not overlook the potential for host adaptation in structures and media for vertical transmission perceived as less elaborate, such as egg-smearing with 'simpler' gland secretions (105). This has been observed e.g. in the fruit flies *Bactrocera dorsalis* and *Bactrocera oleae* (Diptera: Tephritidae) (105). Even in the absence of caplets or jelly, it is conceivable that these 'simpler' secretions still contain substances that are actively produced and secreted by the host to improve a reliable symbiont transmission to preserve the symbiont benefit in the next generation. For instance, putative nutritional '*Candidatus* Rosenkranzia clausaccus' (henceforth: *Ro. clausaccus*) symbionts of shield bugs (Hemiptera: Acanthosomatidae) are transferred to the egg in a gland secretion (226). This secretion stems from a pair of lubrication organs connected to the ovipositor (226). The system exhibits strict co-cladogenesis, and *Ro. clausaccus* possesses a reduced genome of ~0.94 Mb with elevated molecular evolutionary rates (226), suggesting symbiont instability as an important challenge for transmission.
Similarly, *Lagria* beetles (Coleoptera: Tenebrionidae) vertically transmit their extracellular *Burkholderia gladioli* symbionts onto the egg surface in a secretion originating from two accessory glands linked to the ovipositor (227). On the egg surface, the symbionts protect the egg from fungal infestation by producing different antibiotics (32, 227). More than one *Bu. gladioli* strain can co-exist in a host (228), and they differ in genome size and in the antibiotics they produce. Among them, the strain *Bu. gladioli*-LvStB, which has a small genome (~2.3 Mb) in comparison to co-occuring strains (e.g. *Bu. gladioli*-LvStB: ~8.5 Mb) (229, 230), is the most abundant strain in *Lagria* field populations (228, 229), and it produces the antifungal substance lagriamide on the egg (229). Given its reduced genome, it may be relatively vulnerable to abiotic pressures experienced on the egg surface for ca. 7 days (227).
In both systems, it is to date unknown whether the respective gland secretion contains host-derived components stabilizing the symbiont during transmission.

In contrast to symbiont-containing host secretions, whose composition is postulated to be the result of evolutionary adaptation, some symbionts are transferred to the egg via the excretion of fecal matter, as exemplified in firebugs (Hemiptera: Pyrrhocoridae)(105, 231). The symbionts of the latter spend 6-24 days on the egg surface before being taken up by the offspring, depending on the temperature (232). While defecation primarily serves the excretion of waste products, feces may circumstantially mediate symbiont adherence to the egg – a function that should be universally shared among all media containing symbionts during transmission. Furthermore, macromolecules excreted in feces could still be randomly oxidized by free radicals, which may offer sufficient protection from general oxidative stress to a symbiont which is not confronted with a specific and pronounced threat of ROS during external transmission (as opposed to *S. philanthi* in beewolves). Other general functions of the transmission medium, regardless of its degree of specialization and complexity, may include providing desiccation resistance and protection from microbial competitors. As the symbiotic partners evolve to become more tightly associated (resulting in a destabilized symbiont genome), the transmission medium should also evolve more specific adaptations. Starting from feces, a simple transitional step from such a circumstantial to a slightly more directed protective effect could entail host-induced protein modifications of waste products before excretion, rendering the excreted molecule more adept at scavenging free radicals. Other more specialized functions may consist of providing nutrients to the symbionts, as well as triggers for antibiotic production on the egg surface in defensive symbiosis, which may be presumed e.g. in the Lagriinae-*Burkholderia* system (see above). Upon tighter integration of host and symbionts, specialized host glands for the secretion of protective substances into the egg-smearing medium may evolve, e.g. in the form of accessory glands near the ovipositor. This development may then culminate in the evolution of intricate compositions, such as conspicuous jelly or caplets.

In addition to confirming hypothesized host adaptations in symbioses with external symbiont transmission, examining their diversity, as well as their mechanistic basis would be particularly interesting. In the Plataspid-*Ishikawaella* symbiosis, it is currently unknown how exactly PMDP achieves symbiont stabilization, although it has been speculated that it binds to the surface of the symbiont cell (12). Since symbiont instability appears to be a widespread challenge during external transmission, it would be interesting to explore whether stabilization is achieved by a similar, or several different molecular mechanisms across various systems. Studies on the brown-winged green stinkbug *Plautia stali* appear to indicate the latter, since a specific host-derived mucin-like protein is supposedly not essential for vertical transmission of its *Pantoea*-like symbiont (genome size: 2.4-5.5 Mb) (233, 234). Given that symbiont-derived molecules appear to also play a role in successful symbiont transmission (10, 12), it would further be interesting to see whether this is the case in other systems as well, and to disentangle the molecular interplay of host- and symbiont contributions to symbiont stabilization. The same would apply to symbiont transmission in less complex secretions: Do one or more compounds significantly improve successful symbiont transmission? Are there synergistic effects between the compounds?
In order to answer these questions, the first step would be to characterize the composition of conspicuous organic matrices and less complex secretions, and then test their effect on symbiont transmission. A comparison with the composition of feces may aid in identifying specific host-associated compounds that have evolved to protect the symbiont.

5.10 Conclusions

Microbial symbionts of insects commonly face prolonged periods of time outside the host during vertical transmission, which entails exposure to a diverse array of environmental stressors. Despite external transmission being widespread across insects, the mechanisms protecting the symbionts from these environmental stressors – thereby stabilizing the association over evolutionary timescales - remain poorly understood. The beewolf symbiont *S. philanthi* is embedded in a host-derived antennal gland secretion during vertical transmission, and exposed to toxic NO concentrations produced by the beewolf egg for brood cell sanitation. I demonstrate that *S. philanthi* is protected by host-produced protective enzymes and the host-derived secretion of long-chain hydrocarbons, which forms an effective NO diffusion barrier around the symbiont. My findings present one of the few examples of a host adaptation protecting a microbial symbiont during external transmission. Given the widespread distribution of external transmission across insects, and the importance of a reliable symbiont transmission for the long-term stability of the association, my findings provide an initial step towards a general understanding of how symbioses can remain stable over long evolutionary timescales.

5.11 References

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CURRICULUM VITAE

(...)

EIDESSTATTLICHE VERSICHERUNG

Hiermit versichere ich gemäß §11, Abs. 3d der Promotionsordnung der Johannes Gutenberg-Universität Mainz vom 22.12.2003, dass ich die als Dissertation vorgelegte Arbeit selbst angefertigt und alle benutzten Hilfsmittel (Literatur, Apparaturen, Material) in der Arbeit angegeben habe. Ich habe oder hatte die als Dissertation vorgelegte Arbeit nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Ich hatte weder die als Dissertation vorgelegte Arbeit noch Teile einer Abhandlung bei einer anderen Fakultät bzw. einem anderen Fachbereich als Dissertation eingereicht.

Mainz, den 17.05.2024

Chantal Selina Ingham