Ecology and evolution of symbiont management in ambrosia beetles

Ökologie und Evolution des Symbiontenmanagements bei Ambrosiakäfern

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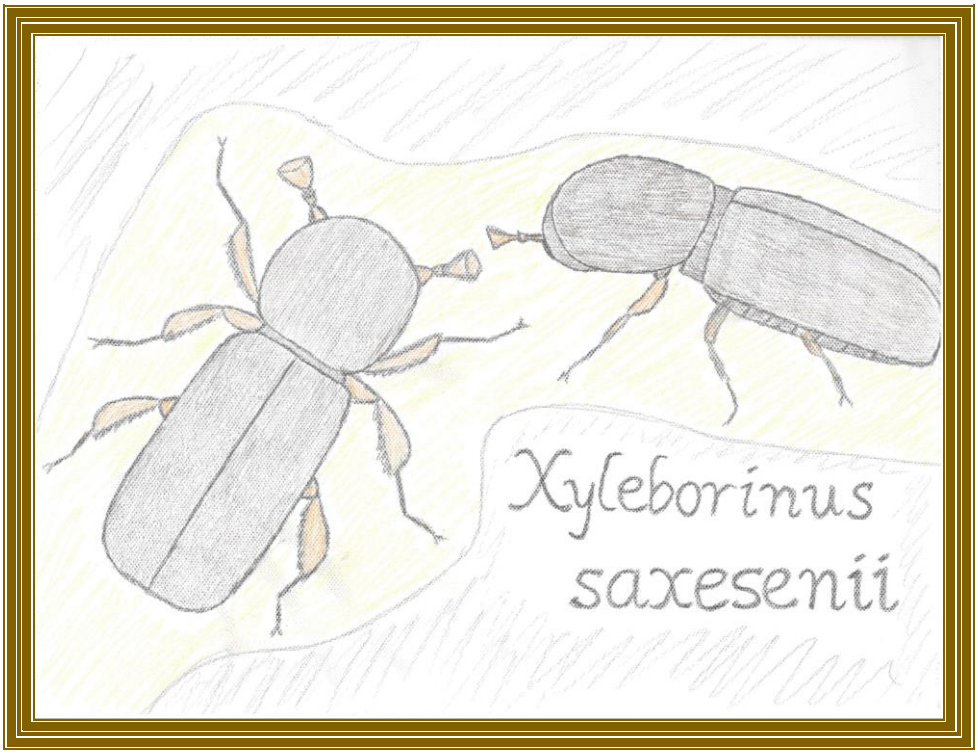
To my grandpa Joe

A great role model to whom I owe my personality, loyalty and passion.

You will always be a part of my heart.



„Ein dankbares Herz ist der Anfang jeder Größe. Es ist ein Ausdruck von Demut und eine Grundlage für Werte wie Glaube, Mut, Glück, Liebe und Zufriedenheit.“

(James E. Faust)

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SUMMARY

The relationship between a farmer and their cultivated crops in agriculture is multifaceted, with pathogens affecting both the farmer and crop, and weeds that take advantage of resources provided by farmers. For my doctoral thesis, I aimed to gain a comprehensive understanding of the ecology and symbiosis of fungus farming ambrosia beetles.

Through my research, I discovered that the microbial composition of fungus gardens, particularly the mutualists, is significantly influenced by the presence of both adults and larvae. The recognition of both beneficial and harmful symbionts is crucial for the success of ambrosia beetles, who respond differently depending on their life stage and the microbial species they encounter, which can contribute to the division of labour among family groups. The presence of antagonists and pathogens in the fungus garden depends on habitat and substrate quality, and beetle response to their introduction results in behavioural and developmental changes. Individual and social immunity measures, as well as changes in bacterial and fungal communities, were detected as a result of pathogen introduction. Additionally, the ability of ambrosia beetles to establish two nutritional fungal species depends on several factors. These insects must strike a balance between their essential functions and adapt to the constantly changing ecological and social conditions, which demonstrates their adaptive flexibility. However, interpreting data from laboratory studies should be approached with caution, as the natural environment allows for more flexibility and the potential for other beneficial symbionts to become more prominent if required.

To aid in my research, I designed primers that use the ‘fungal large subunit’ (LSU) as genetic marker to identify and differentiate mutualistic and antagonistic fungi in *X. saxesenii*. The primers were able to distinguish closely related species of the Ophiostomataceae and other fungal symbionts. This allowed me to associate the abundance of key fungal taxa with factors such as the presence of beetles, the nest's age and condition, and the various developmental stages present. My primers are a valuable tool for understanding fungal communities, including their composition and the identification of previously unknown functional symbionts. However, some aspects should be approached with caution due to the exclusion of non-amplified taxa in the relative fungal community compositions.

ZUSAMMENFASSUNG

Die Beziehung zwischen einem Landwirt und der von ihm angebauten Nahrung in der Landwirtschaft ist vielschichtig: Pathogene, die sowohl den Landwirt als auch die Pflanzen befallen, und Unkräuter, die sich die von Landwirten bereitgestellten Ressourcen zunutzen machen. In meiner Doktorarbeit wollte ich ein umfassendes Verständnis der Ökologie und der Symbiose von pilzzüchtenden Ambrosiakäfern erlangen.

Im Rahmen meiner Forschung fand ich heraus, dass die mikrobielle Zusammensetzung von Pilzgärten, insbesondere der Mutualisten, durch die Anwesenheit sowohl der erwachsenen Tiere als auch der Larven erheblich beeinflusst wird. Die Erkennung sowohl nützlicher als auch schädlicher Symbionten ist entscheidend für den Erfolg der Ambrosiakäfer, die je nach Lebensstadium und den angetroffenen Mikrobenarten unterschiedlich reagieren, was zur Arbeitsteilung zwischen Familiengruppen beitragen kann. Das Vorhandensein von Antagonisten und Krankheitserregern im Pilzgarten hängt von der Qualität des Lebensraums und des Substrats ab, und die Reaktion der Käfer auf ihre Einschleppung führt zu Veränderungen im Verhalten und in der Entwicklung. Individuelle und soziale Immunitätsmaßnahmen sowie Veränderungen der Bakterien- und Pilzgemeinschaften wurden als Folge der Einführung von Krankheitserregern festgestellt. Darüber hinaus hängt die Fähigkeit von Ambrosiakäfern, zwei Nährpilzarten zu etablieren, von mehreren Faktoren ab. Diese Insekten müssen ein Gleichgewicht zwischen ihren lebenswichtigen Funktionen herstellen und sich an die ständig ändernden ökologischen und sozialen Bedingungen anpassen, was ihre Anpassungsfähigkeit zeigt. Bei der Interpretation von Daten aus Laborstudien ist jedoch Vorsicht geboten, da die natürliche Umgebung mehr Flexibilität zulässt und die Möglichkeit bietet, dass andere nützliche Symbionten bei Bedarf stärker in Erscheinung treten.

Um meine Forschung zu unterstützen, habe ich Primer entwickelt, die die ‘fungal large subunit‘ (LSU) als genetischen Marker verwenden, um mutualistische und antagonistische Pilze in *X. saxesenii* zu identifizieren und zu unterscheiden. Die Primer waren in der Lage, eng verwandte Arten der Ophiostomataceae und andere Pilzsymbionten zu unterscheiden. Auf diese Weise konnte ich die Häufigkeit der wichtigsten Pilztaxa mit Faktoren wie dem Vorhandensein von Käfern, dem Alter und Zustand des Nests und den verschiedenen Entwicklungsstadien in Verbindung bringen. Meine Primer sind ein wertvolles Instrument für das Verständnis von Pilzgemeinschaften, einschließlich ihrer Zusammensetzung und der Identifizierung von bisher

unbekannten funktionellen Symbionten. Einige Aspekte sind jedoch mit Vorsicht zu genießen, da nicht amplifizierte Taxa in den relativen Zusammensetzungen der Pilzgemeinschaften nicht berücksichtigt werden.

GENERAL INTRODUCTION

The cultivation of fungi for nutrition is an ecological feature that has evolved only a few times in insects. Here, ectosymbionts are propagated in gardens and are consumed directly by their hosts. There are several insect groups, including ants, termites and ambrosia beetles, that exhibit this behaviour (Mueller et al., 2005). Within these task-sharing societies, microbial gardens are sequestered and thus separated from the surrounding environment. Advanced fungiculture involves insects actively farming their fungal crops and providing nutrients and protection to the fungus (Birkemoe et al., 2018). The insects may benefit directly by feeding on the fungus for amino acids and sterols or indirectly through the fungus' detoxification of defensive plant compounds (phenolics, terpenoids) and degradation of plant polymers (lignin, cellulose, pectin) (Martin, 1979, 1992; Kukor and Martin, 1983; Dowd, 1992; De Fine Licht and Biedermann, 2012; Thompson et al., 2014; Krokene, 2015). Further, insects have been observed using fungal volatiles to attract conspecifics or potential mating partners, as well as utilizing antibiotics produced by fungi to defend themselves against pathogens or competing fungi (Nakashima et al., 1982; Flórez et al., 2015; Kasson et al., 2016). A lifestyle with advanced fungiculture can even lead to several overlapping generations of offspring per nest (e.g. the ambrosia beetle *Austroplatypus incompertus*), similar to what is observed in all ants and termites (Mueller et al., 2005).

Farming behaviour provides the host with access to a previously inaccessible food source. In return, the cultivar is provided with a competitor-reduced environment. This results in a vast niche expansion for both partners (Mueller et al., 2005; Joy, 2013). However, other symbionts with beneficial, competitive or even detrimental interactions may also grow in these fungal gardens (Batra and Batra, 1979; Wood and Thomas, 1989; Currie, 2001; Poulsen et al., 2002; Scott et al., 2008). Therefore, farming societies have evolved mechanisms to maintain their crops through optimal disease surveillance and quarantine schedules, creating an evolutionarily stable foundation over tens of millions of years (Mueller et al., 2005).

One group of fungus-farming insects, and the focus of this thesis, are the ambrosia beetles (Curculionidae: Scolytinae), belonging to the larger framework of bark beetles. The term "bark beetles" has multiple meanings, making it confusing in literature. Kirkendall et al. (2015) suggested to avoid the use of it as a taxonomic term and instead using "phloeophagous" to refer to Scolytinae that breed in inner bark or using "bark and ambrosia beetles" as a collective term for Scolytinae and Platypodinae. Meanwhile, the term ambrosia beetle refers to those Scolytinae and Platypodinae whose larvae feed on symbiotic "ambrosia fungi" (xylomycetophagous) that adult females cultivate in tunnel systems in woody tissues (Kirkendall et al., 2015). The tunnels or galleries are usually built in the xylem of trees (typically weakened or recently dead trees, rarely living trees; e.g. *Austroplatypus incompertus*, Schedl 1968). The associated ambrosia fungi are in most cases the sole food source and provide their hosts with essential vitamins, amino acids and sterols (Kok et al., 1970; Beaver, 1989). Both foundress and offspring feed on their cultivar growing on the walls of their galleries (Peer and Taborsky, 2004). The mutualistic fungi are only known from these beetle farms. The apparent stability of these obligatory associations with fungi is emphasized by the fact that they have been maintained over a range of ages (Farrell et al., 2001). According to Lutzoni and Pagel (1997), initial stages of an obligate fungal mutualism can be found in the most recent origin of *Ambrosiella* and *Ophiostoma* fungi, which are associated with the conifer phloem-feeding genus *Ips* and *Hylurgops*. During dispersal, the main mutualistic fungi, commonly known as ambrosia fungi (Microascales and Ophiostomatales), are taken from the natal nests and stored by the beetles in special spore carrying organs called mycetangia. The newly excavated galleries are then inoculated with spores released from this organ (Biedermann et al., 2009, 2013).

Although the mode of fungal management in ambrosia beetles is not yet fully understood, there is some evidence to suggest that these beetles actively control the fungal crop and the composition of other symbionts (French and Roeper, 1972; Kingsolver and Norris, 1977; Roeper et al., 1980a; Biedermann, 2020; Ibarra-Juarez et al., 2020). Once the foundress dies and the offspring have dispersed, ambrosia fungus gardens transition rapidly from a crop-dominated environment to one dominated by contaminating/antagonistic fungi

(Baker, 1963; Norris, 1979; Borden, 1988). To date, there is a lack of clear experimental evidence that confirms the active contribution of beetles to the protection and promotion of the fungus garden. Similarly, the link between observed behaviours and microbial symbionts and their potential or already speculated functions is missing. Understanding the mechanism underlying ambrosia beetle fungiculture therefore deserves further study (see **Chapter 1**).

In terms of sociality, the subtribe Xyleborini is considered to be the most advanced fungiculturists among the ambrosia beetles, with some species even exhibiting cooperative breeding behaviour (Farrell et al., 2001; Jordal, 2002; Biedermann et al., 2009). Cooperative breeding in ambrosia beetles is characterized by adult female offspring staying in the natal nest and carrying out hygienic brood care and fungus-farming duties while their mother is still present and reproducing, which results in the delay of their own dispersal (Peer and Taborsky, 2007; Biedermann and Taborsky, 2011; Nuotclà et al., 2019). Occasionally, these females produce a second generation within the parental nest (Peer and Taborsky, 2007). The philopatric behaviour of adult offspring facilitates divergent tasks such as waste removal, cropping of the food fungus, and allo-grooming of brood (Roeper et al., 1980b; Bischoff, 2004; Biedermann, 2007; Biedermann et al., 2009). Thereby, females remain fully capable of founding their own nest and start reproducing at any time (Biedermann et al., 2011). It has been suggested that by prolonging their stay in the natal nest and promoting the production of closely related nestmates, individuals can increase their inclusive fitness (Bischoff, 2004; Biedermann, 2007; Peer and Taborsky, 2007). The cooperative effort and dispersal of the adult females in the group is guided by the number of siblings, depending on brood care (larvae and pupae) and the number of adult workers present (Biedermann and Taborsky, 2011). Correlative data suggest that galleries tended by more adult females produce more offspring and possibly give a hint for multiple generations in one nest (Peer and Taborsky, 2007; Biedermann et al., 2009). Dispersal, on the other hand, is costly and dangerous due to a high mortality risk (Milne and Giese, 1970; Dahlsten, 1982) and a high failure rate of establishing a fungal culture in the new nest (only 20% are successful; Peer and Taborsky 2007; Biedermann et al. 2009). A crucial factor in the decision to stay or leave would therefore be to

weigh up the benefits and drawbacks, while considering the prevailing circumstances.

Interestingly, in this model system, the beetle larvae can be seen as a distinct ‘worker caste’ cooperating with the adult offspring in brood care, gallery maintenance and fungus gardening (Biedermann and Taborsky, 2011; Biedermann, 2020), which is remarkable. This division of labour between adult and immature stages of these social insects is exceptional in holometabolous species. By contrast, in other social insects, such as certain Coleopterans and Hymenopterans, the worker caste generally consists of adults. Here, for example, ant, wasp and bee larvae are mostly immobile and fed by adults (Wilson, 1971; Choe and Crespi, 1997). Some ambrosia beetles, like *Xyleborinus saxesenii* Ratzeburg, have larvae that are predisposed to certain tasks due to their body morphology and the frequent renewal of mandibles by moulting. The complete metamorphosis between developmental stages dramatically reorganises the morphology of the insect during ontogeny (Yang, 2001), suggesting a stage dependent diversification of tasks, such as balling of frass and gallery enlargement in the larvae (Biedermann and Taborsky, 2011). Further, *X. saxesenii* larvae are capable of producing wood-degrading enzymes (hemicellulases) that are not present in the adult beetles (De Fine Licht and Biedermann, 2012). Their valuable contribution to the galleries makes them effective workers, which is consistent with the division of labour observed among the different developmental stages.

Many studies to date have focused on eusocial insects and their interactions with their cultivated counterparts, trying to disentangle their life history and social evolutionary traits in order to learn more about insect farm management (Poulsen and Boomsma, 2005; Aanen, 2006). This leaves questions that can only be addressed by going back to an earlier evolutionary stage of sociality and farming. Only then can the predispositions and selective pressure for the evolution of specific social and farming behaviours be identified. Ambrosia beetles differ from eusocial ants and termites in several ways. While fungus farming in ants and termites evolved after the evolution of eusociality with division of labour (Mueller et al., 2005), ambrosia beetles originate from sub-social or communally breeding ancestors. Their agricultural practice co-evolved

alongside their sociality (Kirkendall et al., 1997; Mueller et al., 2005; Peer and Taborsky, 2007; Biedermann and Taborsky, 2011). Targeted experiments utilizing various beetle species with different levels of social behaviour will offer valuable insights into diverse interactions related to fungus farming or sociality, and may provide answers to unresolved evolutionary questions.

Experimental Evolution

Through experimental manipulation, specific factors that contribute to host fitness in a host-microbe interaction can be tested. The microbiome has an important active role here, affecting host development, physiology and systemic defences (Huttenhower et al., 2012; Goh et al., 2013; Pieterse et al., 2014). It may enable toxin production and disease resistance (Gerardo and Parker, 2014; Flórez et al., 2015), increase stress and drought tolerance (Bresson et al., 2013; Coleman-Derr and Tringe, 2014; Lucas et al., 2014), modify niche breadth (Redman et al., 2002), and change fitness outcomes in host interactions with competitors, predators and pathogens (Friesen et al., 2011). Due to their vast number of genes and their ability to vary over space and time (Zilber-Rosenberg and Rosenberg, 2008; Morowitz et al., 2011; Vandenkoornhuyse et al., 2015), microbiomes can function as a phenotypically plastic buffer between the host's genotype and the environmental factors that shape host phenotypes. As a result, virtually any host phenotype is influenced to some extent by the presence and taxonomic composition of host-associated microbes (cf. Mueller and Sachs, 2015). By experimentally manipulating microbiomes, researchers can determine the causal role they play in influencing host performance, thereby overcoming the inferential limitations of, for example, correlational analyses (cf. Mueller and Sachs, 2015).

Artificial selection can be used to shape both the microbiome and host simultaneously in a process called “Two-Sided Host-Microbiome Selection” (Mueller and Sachs, 2015). This method is more complex and requires the hosts to be grown long enough to propagate to the next generation. However, it has the potential to yield co-evolved host-microbiome associations, which may lead to modifications that increase host-microbe fidelity between generations,

such as reducing dissociation and turnover of beneficial microbes, or improving host control and capture of beneficial microbes (Mueller and Sachs, 2015). The process of selection can be applied to both the host and microbiomes simultaneously, combining the separate selective processes that are typically applied in one-sided selection. Therefore, this approach is utilized in the host-mediated selection on the microbiome, as well as in selection upon hosts in diverging microbiomes to select for changes in host organisms (Mueller and Sachs, 2015). Fungus-growing insects apply this two-sided co-propagation scheme to grow their fungal cultivars (Mueller et al., 2005). However, analyzing the changes that occur due to two-sided selection can be challenging, because both the evolving hosts and microbiomes must be examined.

Ambrosia beetles are an excellent model system for understanding the origin and evolution of sociality, as they are a group with totipotent adult individuals that are flexible in their ability to disperse, reproduce and help, depending on the conditions in the nest (Costa, 2006; Nuotclà et al., 2019; Biedermann and Nuotclà, 2020). The evolution of complex social behaviours can be ecologically constrained by habitat instability or population structure. Insects such as ants and termites have managed the transition to eusociality by creating their own habitats, allowing them to disengage from external constraints, as noted by Hughes et al. (2008). In ambrosia beetles, the limiting factor for social evolution is habitat deterioration. Since a lot of subsocial species typically breed in small wood resources (e.g. twigs, small diameter trees) and facultative eusocial species inhabit dead trees of larger diameters, the longevity of the resource is confined (Kirkendall et al., 2015). Only one species (*Australoplatypus incompertus*), which breeds in living Eucalyptus trees, can produce multiple generations in one nest, leading to long-lived colonies with obligate eusociality (Kent and Simpson, 1992).

Through experimental manipulations and artificial selection, it is possible to identify factors that are important for the evolution of sociality. Laboratory experiments can shed light on evolutionary interactions that are otherwise difficult to study. By repeatedly selecting ambrosia beetles for late and early dispersal, Biedermann & Taborsky (unpubl. data) were able to breed two lines with different dispersal timing within six generations, with the late dispersal line

correlating with higher numbers of offspring and more adults remaining in the nest (cooperative strategy). By conducting an experiment that incorporates the two-way selection approach between host and microbiome, it is possible to explore the extent of a manipulation on both sides and disentangle functional traits of microbes that feed back into habitat properties, selecting for responses in (social) behaviour (see **Chapter 3**).

Pathogens as Drivers of Social Evolution

Pathogen pressure is expected to be an important driver of sociality (Biedermann and Rohlfs, 2017). Fungus-farming insects have developed a number of strategies to cope with pathogen pressure and keep themselves and their crop in full vigour (Mueller et al., 2005). Behavioural measures, such as garden surveillance, weeding and the application of antimicrobial secretion, are considered to keep competitors and pathogens in check (Batra and Batra, 1979; Currie and Stuart, 2001; Bot et al., 2002). Additionally, associated secondary microbes can contribute to the health and productivity of the garden by producing antimicrobial metabolites (Craven et al., 1970; Currie et al., 1999b; Grubbs et al., 2020). As is the case for eusocial Hymenoptera, similar social immune responses can be found in facultative eusocial ambrosia beetles like the fruit-tree pinhole borer, *X. saxesenii* (Nuotclà et al., 2019), where hygienic behaviours are expected to be the main response to pathogens (Biedermann and Taborsky, 2011; Meunier, 2015). Collective behaviour may strengthen the efficiency of individual strategies to suppress harmful microbes, and consequently, individuals could be selected to remain in the community and help (Biedermann and Rohlfs, 2017). Therefore, social interactions might provide ambrosia beetles with powerful means to construct high-quality microbial environments, where positive evolutionary feedback processes influence the beetle fitness and strengthen the insect-microbe mutualism (Biedermann and Rohlfs, 2017). Although eusocial insects are already well studied and many functions have been elucidated, the role of pathogens in ambrosia beetle gardens remains poorly understood and deserves further study (see **Chapter 3**).

Recognition of Symbionts

All Insect farmers are able to detect the presence of fungal symbionts in their fungus gardens (Mueller et al., 2005). This ability has been demonstrated by the behavioural response of these insects following confrontation. The response to symbionts can be expressed by defence mechanisms, such as pathogen alarm behaviour (Rosengaus et al., 1999), avoidance (De Roode and Lefèvre, 2012; Tranter et al., 2015; Rocha et al., 2017; Bodawatta et al., 2019), separation of infected areas (Milner et al., 1998), delayed rejection (Herz et al., 2008; Saverschek et al., 2010; Saverschek and Roces, 2011; Arenas and Roces, 2017) or active removal measures (Rosengaus et al., 1998; Myles, 2002; Yanagawa and Shimizu, 2007; Griffiths and Hughes, 2010; Yanagawa et al., 2012). Mutualists, on the other hand, create a targeted attraction/selection in their partners (Bot et al., 2001; Viana et al., 2001; Mueller et al., 2004; Richard et al., 2007; Hulcr et al., 2011). These differentiated behaviours demonstrate the ability of farming insects to make individual decisions about the symbiont they are confronted with. Not only are they able to discriminate between crop and pathogenic fungi (Katariya et al., 2017), but several factors, such as the fungal concentration, fungal species, or environment, also play a role in the response.

The perception of symbionts in fungus-growing insects is mostly associated with their odour. Chemical blends such as the overall odour or specific volatile organic compounds (VOCs) appear to be used by the insects to detect and respond to the symbionts (Bot et al., 2001; Viana et al., 2001; Mueller et al., 2004; Richard et al., 2007; Hulcr et al., 2011). In the case of delayed avoidance, semiochemical signals produced by the pathogen-damaged fungus garden trigger the response (Knapp et al., 1990; Ridley et al., 1996; North et al., 1999; Green and Kooij, 2018).

Among ambrosia beetles, some species of the tribe of Xyleborini have a specialized olfactory-perception in addition to their symbiont specificity. Hulcr et al. (2011) suggested that these traits may be correlated and act as a mechanism to locate already established fungal gardens of conspecifics and even aid in orientation within a gallery. The volatile profiles of bark and ambrosia beetle associated fungal species are unique and can vary qualitatively

and quantitatively during fungal development and between different genotypes (Mburu et al., 2013; Weikl et al., 2016; Kandasamy et al., 2019). Influences such as temperature, substrate and season affecting the growth conditions, can change the volatile blend (Kandasamy et al., 2016). This sensory ability is possible thanks to the beetles' olfactory receptor neurons (OSNs), which are specialized in fungal volatiles (Kandasamy et al., 2019). And, as seen in other fungus farmers, a "specific removal" or a "specific hygienic response" to the presence, species and threat posed (Currie and Stuart, 2001; Tranter et al., 2015; Mighell and Van Bael, 2016) may be possible through a well-differentiated chemical profile of the fungi in these (e.g. Nuotclà et al., 2019). Different reactions between the developmental stages provide additional information that is helpful in clarifying the potential for danger, as well as the role of the stages in the nest community (c.f. Luna et al., 2014).

However, to fully understand the interactions between insects and their symbiotic fungi, a comprehensive view of all factors is needed. Thus, not only the odour of the microorganisms should be considered, but also the presence of physical structures (e.g. spores, mycelia, bacterial cells) (Goes et al., 2020). Additionally, modifying the experimental set-up by either controlling or introducing environmental factors such as temperature, humidity, nutritional availability, or competing symbionts, can enhance our understanding of the recognition capacity and determine the conditions that affect decision-making processes (see **Chapter 2**).

The Influence of Habitat Quality on the Ambrosia Beetle Fungus Garden Microbiome and Beetle Behaviour

Important information for any organism is whether or not it is positioned in a suitable habitat. Consequently, the most beneficial behaviour for an individual in an unsuitable habitat would be to emigrate, if better options are available. The dispersal strategy depends on many different factors, including habitat quality and environmental conditions (Lin et al., 2006; Best et al., 2007; Hughes et al., 2007). Variation in the quality of the habitat is common. To reach a high standard, individuals need to gain and evaluate information. In general,

information can be obtained by exploring the surroundings of a potential new habitat (Norberg et al., 2002; Haughland and Larsen, 2004) using visual or olfactory cues (Schooley et al., 2003; Merckx and Van Dyck, 2007). The decision process thereby underlies individual experiences of natal and neighbouring habitats and the risks of emigration (e.g. mortality) may outweigh the benefits, leading the individual to stay. Overall, the level of information gained by the organism is important for the evolution of dispersal (Enfjäll and Leimar, 2009).

The tendency to assist in the care of others' offspring is an attribute of inclusive fitness benefits and correlated payoffs in social insects and cooperative breeders (Hamilton, 1964; Bourke, 2011; Taborsky et al., 2016). Harsh environments, high predation and saturation of the habitat cause dispersal delays in favour of offspring and set the foundation for the evolution of alloparental brood care in philopatric individuals (Stacey, 1979; Koenig et al., 1992; Heg et al., 2004; Mullon et al., 2018). Moreover, it is believed that once an individual ambrosia beetle leaves its natal gallery, it is unable to return. Depending on the conditions in the nest, it would therefore be advantageous for some females to stay and possibly start their own generation in the natal galleries, assuming the nest/fungus garden is still in good condition (cf. Peer and Taborsky, 2007).

Microbes in fungus gardens need a suitable environment to survive and function. Extreme conditions in terms of humidity, temperature and oxygen content are decisive for the occurrence and spread of the species. Conditions of the fungal gardens of ambrosia beetles within the wood can also change and thus influence the quality of the substrate (c.f. Ulyshen, 2016). Compared to other plant tissues (e.g. leaves, flowers, seed, etc.), wood is the substrate with the lowest nutrient content (Käärik, 1974; Woodwell et al., 1975). Therefore, ambrosia beetles depend on their mutualists for the use of this niche (c.f. Mueller et al., 2005; Joy, 2013). The facultative eusocial beetle, *X. saxesenii*, is known to excavate tunnels in relatively fresh dead wood in a variety of tree species (Fischer, 1954), which may differ in humidity by season and degree of decay. The limiting factor for nest longevity, and thus for sociality, is the progressive loss of nutrients in dead trees (Ulyshen, 2016) and the

requirements of the ambrosia fungal garden. The humidity content of the wood substrate plays a central role and is crucial for the successful establishment of the fungus garden by the beetles (Hosking, 1972; Zimmermann and Butin, 1973; Biedermann et al., 2009; Ulyshen, 2016). The impact of a healthy and fertile fungus garden is significant for the productivity, nest longevity and the fitness of the beetles. Beetle strategies such as closing the nest entrance could counteract habitat degradation and slow down the desiccation process (Kirkendall et al., 1997). However, even in the best-case scenario, these measures are only temporary. Ultimately, the devastation of the nest is unavoidable. Unlike other ambrosia beetle species, *X. saxesenii* forms an association with two mutualistic fungal species, *Dryadomyces sulphureus* (*Raffaelea sulphurea*) and *Raffaelea canadensis*. The reason for this dual association is not fully understood, but it could be related to specific nutritional (for both the fungus or a beetle life stage) or substrate-dependent requirements (see **Chapter 4**).

The Succession of Symbionts in Ambrosia Beetle Fungus Gardens

As with other model organisms, knowledge of the fungus garden microbiome of ambrosia beetles is obtained from field collections of beetles and galleries (Kajimura and Hijii, 1992; Skelton et al., 2019b; Morales-Rodríguez et al., 2021). Since these insects live cryptically and samples can only be collected by destroying the entire nest, factors such as developmental status, environmental conditions, etc. are difficult to measure and have mostly been neglected. In addition, studies typically examine either the associated bacterial or fungal community. The only known attempt to test the whole microbiome of both ambrosia galleries and beetles during development is a study by Ibarra-Juarez et al. (2020), otherwise, culture-independent quantifications of symbiont abundances are rare.

A culture-dependent study by Kajimura and Hijii (1992) showed that the fungal community of *Xylosandrus mutilates* is mainly represented by three groups of fungi (*Ambrosiella sp.*, yeasts and *Paecilomyces sp.*) that change their relative abundance and dominance largely depending on the developmental stages of the beetle. Kajimura and Hikii (1992) found the isolation rates of fungi on the

gallery walls increased gradually from the boring and oviposition, to the larval and pupal stages, followed by a steep increase at the eclosion stage. However, the dominance of *Ambrosiella sp.* decreased rapidly after the emergence of the new adults, while yeasts and *Paecilomyces sp.* became co-dominant. During the overwintering and dispersal period, species such as *Ceratocystis sp.* and other moulds appeared (Kajimura and Hijii, 1992). Other isolation studies revealed that some ambrosia beetle species rely on multiple nutritional cultivars changing in abundance over time (Cruz et al., 2019; Ibarra-Juarez et al., 2020). Two cultivars from gardens of *X. saxesenii*, which showed a changing pattern of abundance from one cultivar to another as nest age increased, were detected by Francke-Grosmann (1975) and later by Biedermann et al. (unpublished data). A succession of cultivars could benefit *X. saxesenii* beetles by optimising substrate use through different enzymatic capabilities (De Fine Licht and Biedermann, 2012; Ibarra-Juarez et al., 2020). Currently, there is inadequate information available to describe the succession process in detail, including the temporal aspect, developmental stages, and the entire microbiome (see **Chapter 5**).

Comparison: Field vs. Laboratory

Most studies on fungus gardens or insect microbiomes are carried out either on specimens collected in the field or on samples taken from laboratory nests. Both methods have advantages and disadvantages. Some researchers have found that laboratory-bred insect strains behave similarly to field strains, indicating that even after several years of breeding under artificial conditions, lab-raised strains can be representative of the species' populations (Kölliker-Ott et al., 2003; Jong et al., 2017). However, in other species, traits can be significantly affected by extremely rapid changes in the laboratory. As a result, such strains no longer reflect natural populations (Meats et al., 2004; Liedo et al., 2007). Adaptation to artificial breeding conditions is most likely when the new environment exerts a selective pressure that is absent or weak in nature. As invertebrates are predominantly maintained in discrete cultures, traits such as developmental speed and reproduction are positively selected for (Leppla et al., 1976; Matos et al., 2000; Diamantidis et al., 2011). The habitat's lack of

variation and complexity negatively affects the tolerance to stressors (e.g. temperature, desiccation, starvation) or substrate use (Bravo and Zucoloto, 1998; Lamberty et al., 2001).

Field studies provide more realistic conditions, but less control over environmental factors. Furthermore, replication of specific conditions with high replicate numbers and controls is only possible in the laboratory (Calisi and Bentley, 2009). Nevertheless, the ecological relevance of traits and habitat for understanding the biology and ecology of species can be reliably demonstrated in the field, and thus make an important contribution to the study of such questions (Wiggins et al., 2018). An important aspect to consider is the influence of laboratory breeding on the development and structure of the fungus garden. Laboratory nests usually have a much shorter development time (~ 1 month), compared to field tests. The exact development time is difficult to estimate in the field, as the beetles live in a cryptic lifestyle and it is almost impossible and very labour intensive to follow the foundation and dispersal of the offspring. To gain more information about the specific differences between these two contrasting environments, it is necessary to sample fungus gardens from both laboratory and field nests of the same population. However, this information is lacking. A study comparing field and laboratory galleries would help identify problems and offer possible improvements in artificial breeding studies (see **Chapter 5**).

Choice of a suitable Genetic Marker for Next Generation Sequencing of Ambrosia Beetle related Fungi

Over the past few decades, amplicon sequencing has become increasingly important in ecological studies. It is not surprising that the use of this technique has become popular among researchers, as it offers the possibility to identify and understand complex structures. Many microbiome analyses examine the domain of bacterial and fungal communities and are also becoming increasingly attractive for ecologists. The metabarcoding method enables the estimation of the microbial diversity of environmental samples (e.g. soil, plant roots, water) on an individual sample scale, further facilitating global-scale comparisons (Taberlet et al., 2012; Tedersoo et al., 2014; Sun et al., 2020). Fungus-farming

insects represent an excellent model to study tripartite interactions between fungi, bacteria and their hosts by applying the next generation sequencing technique.

Typically, ITS primers are used for metabarcoding of fungal communities (Tedersoo et al., 2022). However, these primers come with the disadvantage that they amplify the bark beetle associated Ophiostomataceae fungi poorly or not at all (Kostovcik et al., 2014; Malacrinò et al., 2017). The choice of appropriate genetic markers and primers should always be based on the research question and the information required to answer it. For questions that focus on the presence and relative abundance of the food fungi and important partners of ambrosia beetles, there is a great need for one or more suitable primer sets that can be used during high-throughput sequencing (HTS). Suitable primers would not only ensure easy detection of the mutualists, but also allow conclusions to be drawn about possible interactions and processes within the fungus garden community by covering a wide range of microbial taxa (depending on the marker gene).

One obstacle concerning HTS platforms (e.g. 454 pyrosequencing, Illumina sequencing and Ion Torrent) and choosing a suitable genetic marker is that only short (<550 bases) fragments of the genetic markers can be sequenced, resulting in a loss of taxonomic resolution and phylogenetic information (Tedersoo et al., 2022). Identifying and distinguishing technical artefacts is also more difficult than with the much longer Sanger reads (Tedersoo et al., 2022). Obtaining high-quality amplicons is therefore one of the most important steps in metabarcoding analyses. Achieving this can be done by selecting the proper genetic marker, polymerase, as well as, relevant primers and appropriate thermocycling conditions (Tedersoo et al., 2022). Ecologists tend to mostly use primers designed for Sanger sequencing analyses (White et al., 1990), which lack an optimal coverage of many fungal groups. However, the performance of original primers can be optimized by incorporating degenerate positions to reduce primer bias (Tedersoo and Lindahl, 2016) and promote the quantitative performance (Piñol et al., 2019). Adding multiple degeneracies may demand further adjustment of the 1:1 primer ratio and additional PCR cycles, as not all

primer variants are guaranteed to match with the templates (Tedersoo et al., 2022).

Research aims

Within my PhD project “The Ecology and Evolution of Symbiont Management in Ambrosia Beetles” I worked with both key players for fungiculture and sociality – the ambrosia beetles and their fungus garden. Long-standing questions on the beetles’ role on fungus garden vitality, the microbial succession during nest development and the role of pathogens in the evolution of beetle sociality were the focus of this project. I hoped to gain a better understanding of host-symbiont interactions and learn more about the underlying mechanisms determining fungus and beetle productivity. Therefore, I addressed major unsolved issues in social evolution and mutualism, using *X. saxesenii* as my model system:

Chapter 1) Ambrosia beetles are currently believed to belong to the advanced fungus-farming insects, but experimental evidence of whether beetle activity effectively promotes the food fungi and suppresses contaminants, is lacking. Therefore, I aimed to examine if ambrosia beetles can actively control their fungus garden communities by investigating the microbial fungus garden community in the experimental presence or absence of their beetle hosts. I focused on the two main fungal mutualists *Raffaelea sulphurea* and *R. canadensis,* as well as on their opponent fungal contaminant in laboratory reared nests, *Chaetomium globosum*. To allow for the identification and discrimination of the Ophiostomataceae in my study system, I designed target-specific primers for next generation sequencing (NGS) of the fungal communities that I also used for subsequent questions.

Chapter 2) I then concentrated on the ability of ambrosia beetles to detect the presence of ubiquitous fungal pathogens. This would allow them to detect and eliminate pathogens. My aim was to explore the potential of adult *X. saxesenii* beetles and their larvae to detect

potential threats both to the fungus garden, as well as the beetles themselves (entomopathogenic fungi). Further, determining individual responses of the developmental stages towards the volatiles of different fungi may help to identify whether larvae or adults are mostly responsible for pathogen defence.

The next two questions addressed the investigation whether social evolution in ambrosia beetles may be driven by microbial management. In particular, I focused on the influence of pathogens and the impact of habitat quality on social behaviour of the beetles:

Chapter 3) In the first step, my aim was to find evidence whether the hypothesis that pathogens are drivers of social evolution holds true for ambrosia beetles. I conducted experimental selection by exposing artificial ambrosia beetle nests with high pathogen pressure over multiple generations, in order to test the potential for selection on microbial communities and social (hygienic) behaviour.

Chapter 4) In the second step, I focused on the impact of the habitat. Here, I addressed whether the beneficial microbes have a similar connection to the social lifestyle similar to the harmful microbes. Using wood as the substrate to grow their fungus gardens comes with the problem of a constant change in its properties over time. One property is the humidity, which is only in the optimal range for successful growth of the food fungi for a short period of time. This ultimately limits ambrosia beetles in the extent of their social evolution, though it requires prolonged philopatry of individuals in the nest to increase reproductive success. I tested the beetles' behavioural response under changing humidity, complementing my point of view by including the presence and relative abundance of their microbial mutualists with NGS.

Chapter 5) It is well known that, under natural conditions, microbial communities go through temporal succession depending on

multiple factors (Shigo, 1967; Buresova et al., 2019; Carrias et al., 2020). The fungus gardens of the ambrosia beetles are no exception (Ibarra-Juarez et al., 2020). Recent research poorly investigated the exact extent (Ibarra-Juarez et al., 2020). For *X. saxesenii*, the fungal succession of the two nutritional mutualists is expected, but could not be linked to processes in the beetle nest. Therefore, my aim was to gain an understanding of the microbial succession, including both the fungal and bacterial alpha and beta diversity across the various developmental stages in ambrosia beetle nests. To allow for a detailed comparison between natural and artificial circumstances and to define the core fungus garden community of *X. saxesenii*, I sampled from both field and laboratory nests.

CHAPTER 1

Diehl Janina M. C., Kowallik V., Keller A., Biedermann P.H.W. 2022.

First experimental evidence for active farming in ambrosia beetles and strong heredity of garden microbiomes.

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First experimental evidence for active farming in ambrosia beetles and strong heredity of garden microbiomes

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Fungal cultivation is a defining feature for advanced agriculture in fungus-farming ants and termites. In a third supposedly fungus-farming group, wood-colonizing ambrosia beetles, an experimental proof for the effectiveness of beetle activity for selective promotion of their food fungi over others is lacking and farming has only been assumed based on observations of social and hygienic behaviours. Here, we experimentally removed mothers and their offspring from young nests of the fruit-tree pinhole borer, *Xyleborinus saxesenii*. By amplicon sequencing of bacterial and fungal communities of nests with and without beetles we could show that beetles are indeed able to actively shift symbiont communities. Although being consumed, the *Raffaelea* food fungi were more abundant when beetles were present while a weed fungus (*Chaetomium* sp.) as well as overall bacterial diversity were reduced in comparison to nests without beetles. Core symbiont communities were generally of low diversity and there were strong signs for vertical transmission not only for the cultivars, but also for secondary symbionts. Our findings verify the existence of active farming, even though the exact mechanisms underlying the selective promotion and/or suppression of symbionts need further investigation.

1. Introduction

The cultivation of crops for food is a rare ecological feature, which has evolved only a few times in animals. Apart from humans, the most prominent farmers are fungus-cultivating insect groups specifically some lineages of ants (220 species) and one lineage of termites (330 species) [1–3]. Although farming insects are clearly biologically distinct from humans, their farming techniques are remarkably similar, suggesting convergent evolution in response to similar ecological challenges [4]. An important example of these shared challenges is the ubiquitous threat of weeds and pathogens for the long-term cultivation of crops. Here, insect farmers evolved a wide variety of strategies to selectively facilitate the growth of their cultivars [1,2,5]. These include the sequestration and compartmentalization of gardens [2], usage of antibiotic-producing bacteria [6–8] and the active monitoring and behavioural management of fungus-garden communities [9–11].

While ants and termites are famous for their pronounced farming practices, this phenomenon likely also evolved in other insect groups. Ambrosia beetles (Coleoptera: Curculionidae: Scolytidae) are a polyphyletic group composed of at least 12 lineages (greater than 3400 species) of wood-dwelling weevils in the subfamilies Scolytinae and Platypodinae, which are closely associated with symbiotic fungi. Ambrosia beetles carry and introduce these fungal

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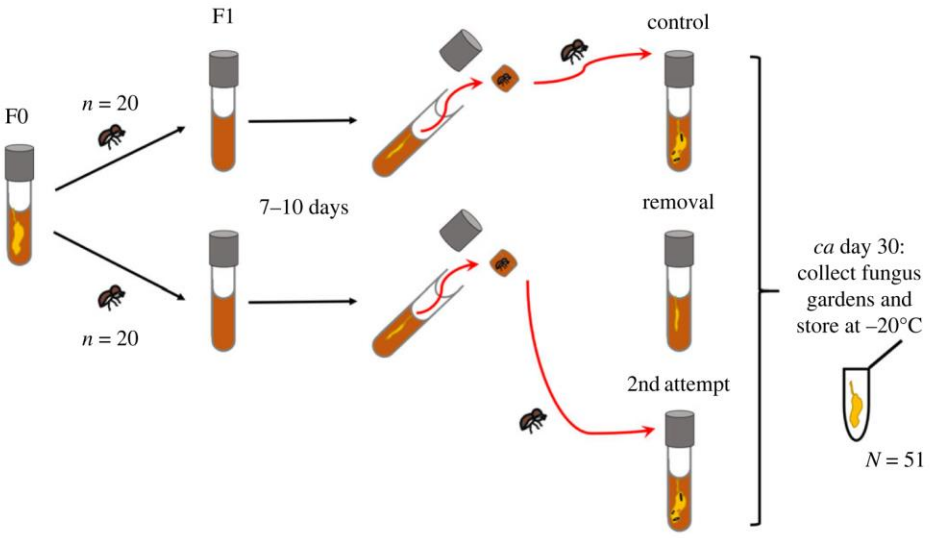

Figure 1. Experimental set-up for the treatment groups reared in semi-natural media within tubes. Adult sisters from 11 different nests (F0) were equally spread among *control* and *removal* group (F1; each $n = 20$). Seven to 10 days later 1 cm of the medium including the foundress was removed and she was either returned immediately to the original nest (*control* group) or was permanently removed from the tube (*removal* group) and introduced immediately into a new rearing tube (2nd *attempt* group). Eleven of these 20 re-introduced females bred successfully. At around day 30, when first beetles matured, fungus-garden samples of all F1 nests were collected and stored at −20°C for bacterial and fungal community sequencing. (Online version in colour.)

characterization of ambrosia beetle fungal symbionts as they amplify Ophiostomatales ambrosia fungi, which are typically not picked up by standard internal transcribed spacer (ITS) barcoding primers (cf. [43]).

2. Material and methods

(a) Beetle rearing and experimental treatments

Xyleborinus saxesenii females were collected in the Steinbachtal near Wuerzburg, Germany (49.767500, 9.896770/49°46′03.0″ N 9°53′48.4″ E) using ethanol-baited traps (70% EtOH) in May 2018. After bringing them to the laboratory they were reared in a sawdust composed 'standard medium' in transparent plastic tubes following Biedermann *et al.* [38]. More precisely, these wild-caught adult females (=F0) (that are already mated with brothers before dispersal) were introduced individually into the tubes after rinsing them briefly with 70% EtOH followed by tap water and letting them dry on tissue paper. These founder females immediately start tunnelling when put inside the tubes and 4–7 days later, symbiont growth starts covering the tunnel walls (i.e. 'fungus garden'). Fourty dispersing adult female offspring of 11 family groups were used for starting the treatments described in the following.

After all 40 females of the F1 generation successfully established their fungus gardens (7–10 days after nest foundation) three treatment groups were created (figure 1), whereby nests from the same original families were split to *removal* and *control* treatment to control for between-family differences in symbiont communities: (i) *removal group:* nests with fungus gardens but no beetles present ($N = 20$ nests). The solid media was shaken out of the tube and females were removed from the tunnel with a flame-sterilized dissecting needle; if females were not seen from the outside they were removed together with the upmost centimetre of the medium, where they normally reside until the first larvae hatch [35]. (ii) *2nd attempt group:* subsequently, these females were transferred to the remaining sterile rearing tubes for a second nest foundation ($N = 11$ nests). Repeated nest founding also occurs in nature [44], but still only 11 out of the 20 2nd-foundation attempts succeeded and could be used for our analyses. (iii) *Control group:* nests were treated as in the *removal group* to control for experimental disturbance, but females were put back ($N = 20$ nests). The first two treatments can be compared in a pairwise manner since founding females are the same in both. The *control group* allows to test for possible differences in symbiont communities vectored with 1st and 2nd founding attempts. Additionally, informative metadata, such as family lineage, nest origin and the exact dates of (i) first introduction to medium, (ii) removal of foundress and (iii) collection of nest material for amplicon sequencing were recorded.

(b) Fungus-garden sampling and DNA extraction

When the first females eclosed, nests of all three groups were opened and fungus-garden samples were collected. For this, nests were knocked out of the tube under a sterile hood and garden material growing along the gallery walls was sampled with a flame-sterilized spatula (mean ± s.d. = 96.47 mg ± 34.34). Sampling time did vary between day 19 and day 40 due to variation of development time between nests (mean ± s.d. = 30.88 days ± 3.09). All samples were mechanically ground to break up tissues and cell walls using a ceramic bead and mixer mill (Retsch MM400), followed by another step with glass beads (0.1 and 0.5 mm) vortexed on a Vortex Genie 2 (see supplementary material in [32]). Afterwards, DNA of all samples was extracted using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Germany) according to the manufacturer's instructions. The isolated DNA was stored at −20°C until further processing.

(c) Library preparation and amplicon sequencing

PCRs were performed in triplicate reactions (each 10 µl) in order to avoid PCR bias. Bacterial 16S rRNA gene libraries were constructed using the dual-indexing strategy described in Kozich *et al.* [45] using the 515f and 806r primers to amplify amplicon sequences of a mean merged length of 238.18 bp, encompassing the full V4 region (modified from [46]). Conditions for the PCR were as follows: initial denaturation at 95°C for 4 min, 30 cycles of denaturation at 95°C for 40 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min; followed by a final extension step at 72°C for

5 min. Sample-specific labelling was achieved by assigning each sample to a different forward/reverse index combination.

ITS primers typically used for metabarcoding of fungal communities of insects [47] do not amplify the bark beetle symbionts in the Ophiostomataceae [31,48]. Therefore, we used the LSU (28S) rRNA region for designing our primers. Gene libraries (mean merged length of 276.74 bp) were constructed similarly from the same samples to amplify the LSU region. Again, adapters and dual-indices were incorporated directly into the PCR primers. Conditions for this PCR with the self-designed, dual-index primers of LIC15R and nu-LSU-355–3′ (described in electronic supplementary material in [32]) were: initial denaturation at 98°C for 30 s, 35 cycles of denaturation at 98°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 15 s; followed by a final extension step at 72°C for 10 min.

After PCRs, triplicate reactions of each sample were combined per marker and further processed as described in Kozich *et al*. [45], including between-sample normalization using the SequalPrep Normalization Plate Kit (Invitrogen GmbH, Darmstadt, Germany) and pooling of 96 samples. The pools were cleaned-up with the AMPure Beads Purification (Agilent Technologies, Inc. Santa Clara, CA, USA), quality controlled using a Bioanalyzer High Sensitivity DNA Chip (Agilent Technologies) and quantified with the dsDNA High Sensitivity Assay (Life Technologies GmbH, Darmstadt, Germany). Afterwards, pools were combined to a single library pool containing 384 samples in total. This library was diluted to 8 pM, denatured and spiked with 5% Phix Control Kit v3 (Illumina Inc., San Diego, CA, USA) according to the Sample Preparation Guide (llumina Inc. 2013). Sequencing was performed on an Illumina MiSeq using 2 × 250 cycles v2 chemistry with each marker on a separate chip (Illumina Inc.). See electronic supplementary material for further methodology of sequencing controls and details on bioinformatics processing.

(d) Statistical analysis of molecular data

All statistical analyses and visualization of the sequence output were performed in RStudio (v.1.4.1106) with R v.4.0.5 [49] using the phyloseq package ([50]; see GitHub repository for information on the bioinformatic processing and R-script).

After filtering chloroplast genes and amplicon sequence variants that were only identified to domain level, we ran a contaminant removal method ('decontam' [43]) and visualized the taxonomic composition of the negative and Mock control samples, which were then excluded from the sample set, as well as samples with a read number less than 500 (see electronic supplementary material). For the final analysis, 28 samples with an average of 15 011.72 reads for 16S sequences (min. 793 reads; max. 44 924 reads) and 69 ASVs (amplicon sequence variants, [51]) were included. Microbial composition of the bacteria was studied up to genus level. For the LSU, 51 samples with an average of 17 344.02 reads (min. 1087 reads; max. 39 932 reads) and 202 ASVs were included in the analyses. Fungal composition was studied up to species level.

(i) Testing for active farming between sisters: *removal* group versus *control* group

To test for the influence of the beetle's presence on the fungus-garden microbiome, we compared the microbial community between nests in the *control* and the *removal* group. Alpha diversity of the rarefied samples (2000 reads/sample removing six samples and 15 ASVs from the 16S dataset and two samples and 23 ASVs from the LSU dataset) was explored by plotting observed species richness (OR) and Shannon's diversity index (SDI) ('microbiome' package: [52]). For the bacterial alpha diversity, we ran a generalized linear model (GLM) with gamma family and log link function on the SDI to test the influence of the treatment (*control* versus *removal*) and 'family lineage'. A GLM with normal distribution best fitted the fungal diversity data. For OR (number of observed ASVs) we applied the same model. The package 'ggplot2' [53] was used to build the figures of alpha diversity (SDI/OR).

To visualize differences in composition (beta diversity), non-metric multidimensional scaling (NMDS, 'phyloseq' package: [50]) was used on Bray–Curtis dissimilarity matrices derived from proportion transformed data, which consider presence/absence as well as abundance of ASVs [54]. To compare the microbial communities between treatments and 'family lineage', we performed a permutational ANOVA test (PERMANOVA) on Bray–Curtis distance matrices of the proportion data using the R package 'vegan' [55]. The homogeneity of multivariate dispersions was tested with betadisper() and distance structures of the bacterial and fungal data ('vegan' package: [55]) were applied on each the 'treatment' and 'family lineage' variables. Taxa composition barplots (agglomerated to 'genus' (bacteria) or 'species' level (fungi)) faceted by lineage and heatmaps of fungal and bacterial communities were built for visualization. As ASVs can represent biological variance between microbial strains of the same species, we plotted an additional bar graph of the highly abundant strains of core species (ASVs greater than 0.5%) from the *control* and *removal* group.

(ii) Testing for active farming by controlling foundress identity: *removal* group versus *2nd attempt* group

Comparing the *2nd attempt* with the *removal* group gave us the opportunity to directly study the effect of the beetle's presence, by controlling for between-individual differences in microbial symbionts of founding females (because the same females were used in both groups). Eleven of 20 females succeeded in their 2nd founding attempt. In addition to the GLMs, NMDSs and PERMANOVAs and figures we used in the previous analysis, we ran a linear model (LM) applying logistic transformation on the SDI of the bacteria to test the influence of the treatment (*2nd attempt* versus *removal*) by controlling for 'family lineage'.

(iii) The responses of core bacterial and fungal taxa

For gaining deeper insights into differences/changes of abundant core taxa, we agglomerated the same taxa ('genus' level for bacteria and 'species' level for fungi) of the compositional phyloseq object and extracted the individual taxa. We ran LMs to test whether relative abundances of core taxa differed between treatments and 'family lineage'. Precisely, we compared the logistic transformed relative abundances of the two ambrosia fungi, *R. sulphurea* and *R. canadensis*, the commensal fungus *Chaetomium globosum* and the bacterium *Pseudoxanthomonas* between the treatments and familiar lineages. The abundance data of *Wolbachia* was subjected to a Tukey transformation to transform the response variable more towards a normal distribution before being used in an LM with the same variables ('rcompanion' package: [56]). Relative abundance boxplots of core taxa were built for both bacteria and fungi.

(iv) Effects of the microbial community of nests in the *removal group* on the success rate of the same foundresses during the 2nd foundation attempt

The *removal* treatment subset provided us with the possibility to compare nests of foundresses being successful in their *2nd attempt* (*successful*) with those that failed to found a 2nd brood (*failed*). To examine this, we performed two PERMANOVAs on Bray–Curtis distance matrices of the relative abundances data for each bacterial and fungal community and tested the homogeneity as previously described. Since core taxa were of particular interest to us, we compared the logistically transformed relative abundances of core taxa

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The *removal* treatment subset provided us with the possibility to compare nests of foundresses being successful in their *2nd attempt* (*successful*) with those that failed to found a 2nd brood (*failed*). To examine this, we performed two PERMANOVAs on Bray–Curtis distance matrices of the relative abundances data for each bacterial and fungal community and tested the homogeneity as previously described. Since core taxa were of particular interest to us, we compared the logistically transformed relative abundances of core taxa

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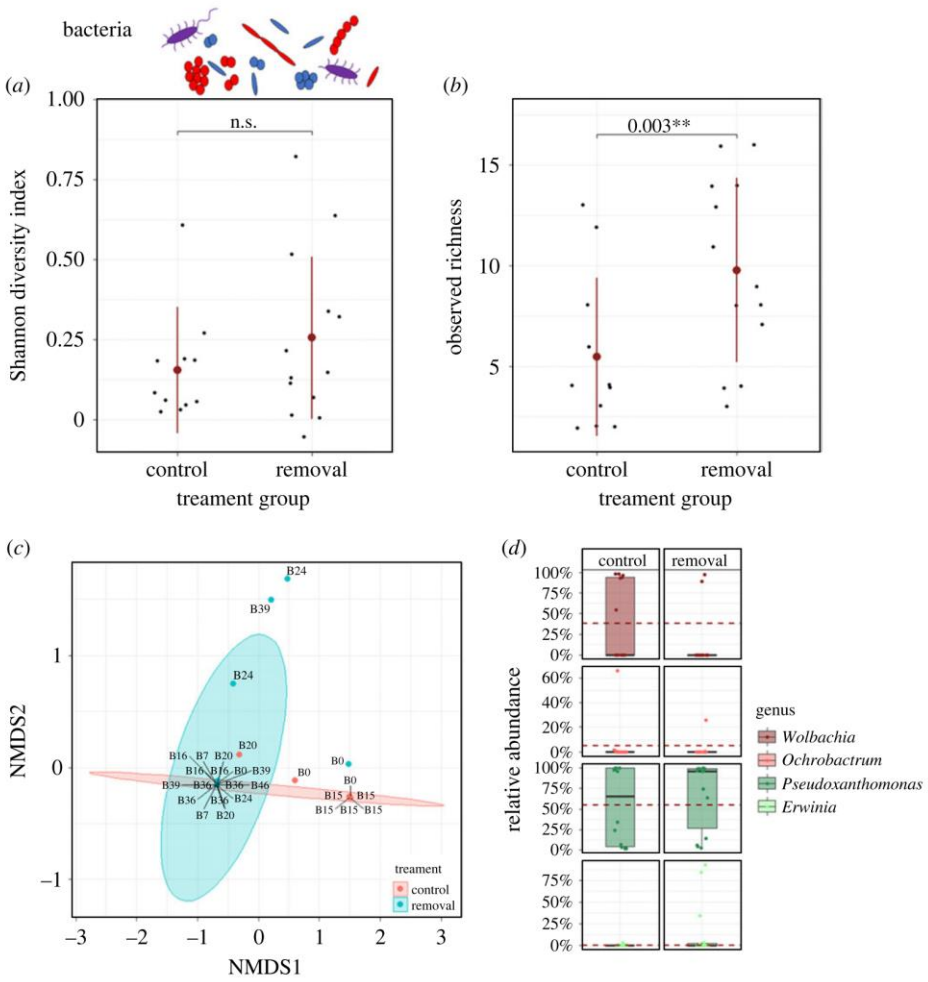

Figure 2. Effect of beetle removal on bacterial diversity, composition and relative abundance. (*a*) Bacterial SDI did not differ between *removal* and *control* group (each $n = 20$ nests; GLM: $\chi^2 = 1.57$, $p = 0.210$; plot shows mean and s.d.). (*b*) Bacterial observed richness (OR) was higher when beetles were removed ($\chi^2 = 8.81$, $p = 0.003$; plot shows mean and s.d.). (*c*) Visualization of compositional differences between bacterial communities of nests in *removal* and *control* group (NMDS on Bray–Curtis dissimilarity: stress = 0.004; ellipses represent 95% CIs; labels represent family lineage of samples). PERMANOVA verified that bacterial communities are significantly affected by beetle presence ($R^2 = 0.066$, $p = 0.038$). (*d*) Comparisons of relative abundances of core bacterial taxa did not reveal any significant differences between treatments (boxplots represent median with its interquartile range and whiskers; red dashed line shows the mean relative abundance of the '*control* group'). (Online version in colour.)

between failed and successful 2nd attempts using an LM and plotted them with boxplots.

3. Results

(a) Detected taxa in bacterial and fungal datasets

Altogether 10 bacterial phyla were detected across samples. Among these, Gammaproteobacteria, Alphaproteobacteria and Actinobacteria were most abundant and accounted for approximately 90% of total sequences (figure 2; electronic supplementary material, table S1). Gammaproteobacteria comprised ASVs of *Pseudoxanthomonas* (mean + s.d. = 59.93% ± 44.28 RA), *Erwinia* (7.83% ± 22.86) and *Acinetobacter* (0.14% ± 0.85). Alphaproteobacteria were dominated by *Wolbachia* (Alphaproteobacteria) (28.11% ± 43.28) and *Ochrobactrum* (Alphaproteobacteria) (2.67% ± 11.62) and Actinobacteria by *Microbacterium* (0.63% ± 1.16). All abundant bacteria showed to be relatively equally distributed across samples while the endosymbiont *Wolbachia* was only highly dominant in few samples/ lineages. Bacteroidetes, Firmicutes, Acidobacteria, Chloroflexi, Verrucomicrobia, Planctomycetes and Fusobacteria were detected in relative abundances less than 0.5% of total reads.

The analyses of the fungal dataset yielded exclusively ASVs of the phylum Ascomycota. The most abundant species was *C. globosum* (Chaetomiaceae) with a relative abundance of 24.19% ± 34.18 (mean ± s.d.) followed by the Ophiostomatales ambrosia fungi of *X. saxesenii*, *R. sulphurea* (18.46% ± 26.89) and *R. canadensis* (4.17% ± 8.23). Other species with a relative abundance of greater than 0.5% in some samples were *Acremonium biseptum* (Bionectriaceae; 2.48% ± 13.75) and *Penicillium commune* (Trichocomaceae, 0.96% ± 5.23) (electronic supplementary material, table S2). A closer look on our own fungal mock communities revealed that the newly designed primers could distinguish between the two ambrosia fungi *R. canadensis* and *R. sulphurea*. Moreover, additional fungi in the orders Eurotiales, Sordariales, Hypocreales, Capnodiales, Helotiales and Xylariales were successfully amplified, but yeasts in the Saccharomycetales order (e.g. *Pichia* sp., *Candida* sp.) could not be differentiated

by our approach (cf. supplementary figure 5 in [32]; electronic supplementary material, figure S8).

(b) Testing for active farming

(i) Bacterial symbiont communities

Comparisons of alpha diversity of bacterial communities between treatment groups showed observed bacterial richness (OR) was significantly higher in the ambrosia gardens without beetles (*control* versus *removal:* GLM: $\chi^2 = 8.81$, $p = 0.003$; figure 2*b*, table 1; *removal* versus *2nd attempt*: GLM: $\chi^2 = 4.51$, $p = 0.034$; electronic supplementary material, figure S3, table 1), but SDI did not reveal differences (*control* versus *removal:* GLM: $\chi^2 = 1.57$, $p = 0.210$; figure 2*a*, table 1; *removal* versus *2nd attempt*: LM: $F = 0.406$, $p = 0.538$; electronic supplementary material, figure S3, table 1). There was also a significant difference in bacterial beta diversity (i.e. the turnover of taxa) between groups (*control* versus *removal:* PERMANOVA: $R^2 = 0.066$, $p = 0.038$, Betadisper: $F = 0.771$, $p = 0.388$; table 1; *removal* versus *2nd attempt*: PERMANOVA: $R^2 = 0.042$, $p = 0.169$, Betadisper: $F = 0.478$, $p = 0.497$; table 1), which was also visible in the NMDS plot (figure 2*c*, electronic supplementary material, figure S3). Comparing the relative abundances of core bacterial taxa, neither the two most abundant taxa *Wolbachia* (*control* versus *removal:* LM: $F = 2.41$, $p = 0.138$; *removal* versus *2nd attempt*: LM: $F = 0.409$, $p = 0.534$) and *Pseudoxanthomonas* (*control* versus *removal:* LM: $F = 0.177$, $p = 0.679$; *removal* versus *2nd attempt*: LM: $F = 0.163$, $p = 0.693$) nor any of the other core bacterial ASVs showed a significant response to beetle presence (figure 2*d*, electronic supplementary material, figure S3 and table S3).

(ii) Fungal symbiont communities

Comparisons of fungal alpha diversity between treatment groups did not reveal differences neither for SDI (GLM: $\chi^2 = 1.28$, $p = 0.258$; figure 3*a*) nor OR ($\chi^2 = 0.639$, $p = 0.424$; figure 3*b*, table 1) when comparing *control* with *removal* group. However, when comparing *removal* with *2nd attempt* group directly, SDI was significantly higher in the nests without beetles ($\chi^2 = 4.51$, $p = 0.034$; electronic supplementary material, figure S3; table 1) and also OR showed this tendency ($\chi^2 = 3.17$, $p = 0.075$; electronic supplementary material, figure S3, table 1). As observed in bacteria, there was also a significant difference in fungal beta diversity between *control* and *removal* groups (PERMANOVA: $R^2 = 0.083$, $p = 0.032$; Betadisper: $F = 0.771$, $p = 0.388$; table 1), but not between *removal* and *2nd attempt* groups (PERMANOVA: $R^2 = 0.016$, $p = 0.641$; Betadisper: $F = 0.150$, $p = 0.702$; table 1). The NMDS plots displayed strong overlap of the fungal communities (figure 3*c*, electronic supplementary material, figure S3).

Comparing the relative abundances of core taxa between treatments revealed strong responses to beetle presence (figure 3*d*; electronic supplementary material, table S3). While *C. globosum* tended to be more abundant in the absence of beetles (LM: $F = 3.37$, $p = 0.077$), both ambrosia fungi, *R. sulphurea* and *R. canadensis*, were significantly more abundant in the presence of beetles (*R. canadensis*: $F = 5.18$, $p = 0.031$; *R. sulphurea*: $F = 4.97$, $p = 0.034$). There was no significant difference between *removal* and *2nd attempt* groups for *C. globosum* ($F = 0.092$, $p = 0.765$) and *R. sulphurea* ($F = 1.46$, $p = 0.241$), but *R. canadensis* tended to have a higher relative abundance when beetles were present ($F = 4.30$, $p = 0.051$).

(iii) The effect of the microbial community on the success rate of a 2nd foundation attempt

We found no significant effect of neither the overall bacterial community (PERMANOVA: $R^2 = 0.071$, $p = 0.573$, Betadisper: $F = 0.021$, $p = 0.889$) nor the relative abundance of the core bacterial taxa (*Erwinia*: LM: $F = 0.641$, $p = 0.439$; *Pseudoxanthomonas*: $F = 0.044$, $p = 0.837$; *Wolbachia*: $F = 2.46$, $p = 0.143$) of *removal* nests on the success rate of 2nd founding attempts (electronic supplementary material, figure S2). There was also only a trend for an effect of the overall fungal community (PERMANOVA: $R^2 = 0.091$, $p = 0.164$, Betadisper: $F = 0.211$, $p = 0.164$) of *removal* nests on the success rate of 2nd founding attempts. However, when testing the effect of the relative abundance of core fungal taxa, *C. globosum* turned out to be significantly less abundant in *removal* nests of successful foundresses (LM: $F = 5.51$, $p = 0.031$). On the other hand, at least one ambrosia fungus tended to be more abundant in these nests (*R. canadensis*: $F = 2.85$, $p = 0.108$; *R. sulphurea*: $F = 2.10$, $p = 0.164$), indicating effects of at least *C. globosum* and possibly also *R. canadensis* on the success rate of foundresses.

(iv) Heredity of the symbiont community

Overall there were strong signatures for heredity (i.e. vertical transmission) of symbiont communities over generations as seen by the effect of 'family lineage' in almost every model (table 1). By including the variable 'family lineage' in our models we were able to control for this factor (electronic supplementary material, figure S6, table 1). Interestingly, we found two strains of *R. canadensis* in our dataset, which never appeared together in one sample or within samples of the same family lineage (electronic supplementary material, figure S7).

4. Discussion

Farming is a complex behaviour that evolved several times across the animal kingdom and strongly affects the ecology and evolution of the farmers and their cultivars [4]. This study aimed to test if farming (i.e. the active behavioural management of symbiont communities) is present in ambrosia beetles. The latter is known from other fungus-farming insects [1,2] and has been assumed since the first descriptions of ambrosia beetles without proper proof (e.g. [13]). By investigating the effect of beetle presence or removal on the composition of microbial communities, we were able to show that active farming is indeed present in our study species, the fruit-tree pinhole borer *X. saxesenii* (Scolytinae, Xyleborini), even if their managing abilities are not as effective as in fungus-farming ants and termites [2].

(a) To farm or not to farm—the debate of active fungus farming in ambrosia beetles

Apart from humans, fungus-farming ants, termites and ambrosia beetles were defined as agriculturists even though active farming, as one defining feature of agriculture, has never been proven for ambrosia beetles [2]. Our findings show that active behavioural farming of cultivars is present in *X. saxesenii*, supporting this premature claim. Firstly, relative abundances of the nutritional ambrosia fungi (two *Raffaelea* spp.) significantly increased in the presence of *X.*

Table 1. Statistical output of alpha and beta diversity analyses in both bacterial and fungal communities. Both comparisons of *control* versus *removal* group and *removal* versus *2nd attempt* group are given. Statistically significant results are highlighted ($p < 0.05$).

	control versus removal								removal versus 2nd attempt							
	bacteria				fungi				bacteria				fungi			
Shannon diversity index		χ^2	**d.f.**	p		χ^2	**d.f.**	p		F	**d.f.**	p		χ^2	**d.f.**	p
	treatment	1.57	1	0.21	treatment	1.28	1	0.258	treatment	0.406	1	0.538	treatment	4.51	1	**0.034**
	lineage	19.26	8	**0.014**	lineage	22.55	10	**0.013**	lineage	3.81	7	**0.028**	lineage	14.14	9	0.118
observed richness		χ^2	**d.f.**	p		χ^2	**d.f.**	p		F	**d.f.**	p		χ^2	**d.f.**	p
	treatment	8.81	1	**0.003**	treatment	0.639	1	0.424	treatment	5.19	1	**0.023**	treatment	3.17	1	0.075
	lineage	19.36	8	**0.013**	lineage	21.94	10	**0.015**	lineage	29.25	7	**<0.001**	lineage	18.84	9	**0.027**
PERMANOVA		**d.f.**	R^2	F	p		**d.f.**	R^2	F	p		**d.f.**	R^2	F	p		**d.f.**	R^2	F	p
	treatment	1	0.066	4.3	**0.038**	treatment	1	0.083	3.94	**0.032**	treatment	1	0.042	1.88	0.169	treatment	1	0.016	0.544	0.641
	lineage	8	0.66	5.41	**0.001**	lineage	10	0.325	1.54	0.107	lineage	7	0.664	4.19	**0.003**	lineage	9	0.39	1.46	0.132
Betadisper		**d.f.**		F	p		**d.f.**		F	p		**d.f.**		F	p		**d.f.**		F	p
	treatment	1		0.771	0.388	treatment	1		1.744	0.195	treatment	1		0.478	0.497	treatment	1		0.15	0.702
	lineage	8		1.16	0.374	lineage	10		0.392	0.94	lineage	7		2.13	0.109	lineage	9		0.625	0.763

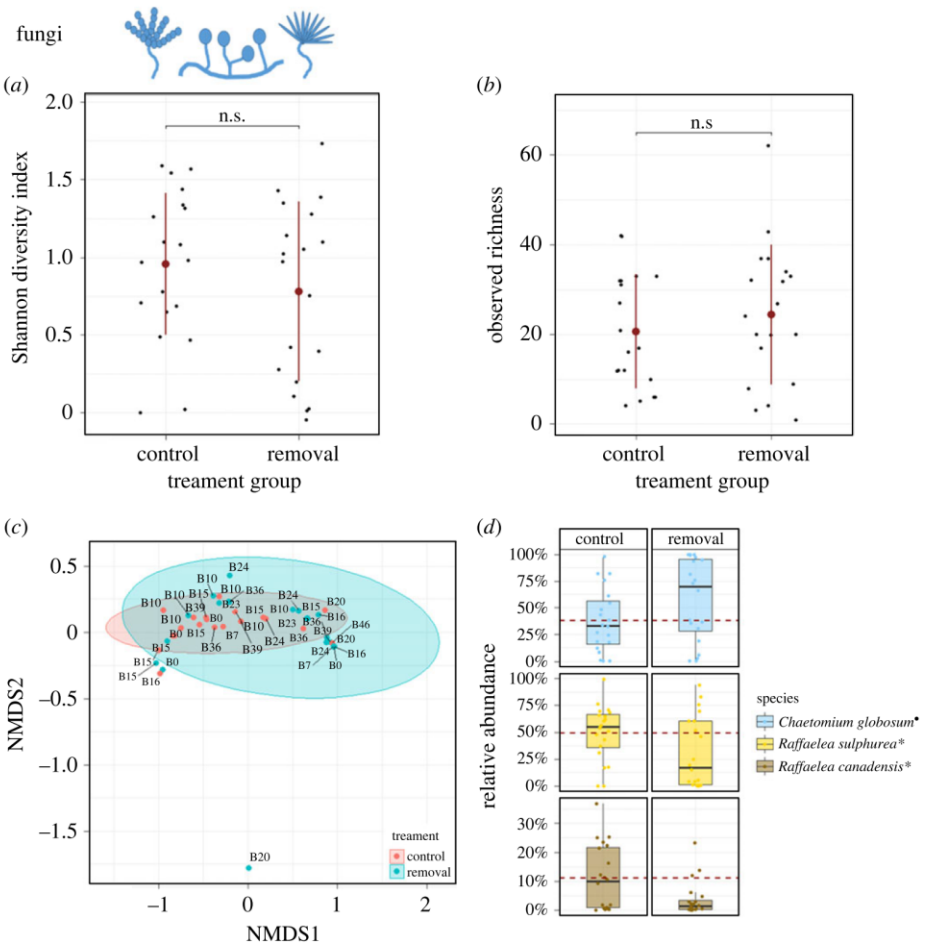

Figure 3. Effect of beetle removal on fungal diversity, composition and relative abundance. (*a*,*b*) Fungal SDI and OR did not differ between *removal* and *control* group (each $n = 20$ nests; SDI: GLM: $\chi^2 = 1.28$, $p = 0.258$; OR: $\chi^2 = 0.639$, $p = 0.424$; plot shows mean and s.d.). (*c*) Visualization of compositional differences between fungal communities of nests in *removal* and *control* group (NMDS: stress = 0.034; ellipses represent 95% CIs; labels represent family lineage of samples). PERMANOVA verified that fungal communities are significantly affected by beetle presence ($R^2 = 0.083$, $p = 0.032$). (*d*) Comparisons of relative abundances of core fungal taxa revealed significant reduction of food fungi, *R. canadensis* (LM: $F = 5.18$, $p = 0.031$) and *R. sulphurea* ($F = 4.97$, $p = 0.034$), in nests without beetles, whereas relative abundance of *C. globosum* tended to increase in these nests ($F = 3.37$, $p = 0.077$; $^*p < 0.05$; $^\bullet p < 0.1$ (boxplots represent median with its interquartile range and whiskers; red dashed line shows the mean relative abundance of the '*control* group'). (Online version in colour.)

saxesenii foundresses and their first immature/larval offspring. Secondly, bacterial alpha diversity and the relative abundance of a fungal competitor of the ambrosia fungi (*Chaetomium* sp.) were both significantly reduced by the presence of foundresses and their immature offspring. Together this confirms old assumptions by Hubbard [13] and many others (e.g. [2,12,24]) that ambrosia beetle species (or at least *X. saxesenii*) can actively manage its fungus-garden communities to some degree.

(b) The presence of beetles affects symbiont communities

An important finding of our study was that feeding mothers and immatures were not reducing the amount of their *Raffaelea* food fungi, but instead relative fungal abundances increased in their presence (figure 3*d*). Fungus-farming ants and termites are known to directly influence growth of their cultivars, for example, by inducing nutritional structures, which has also been shown for one ambrosia beetle species (*Anisandrus dispar* with its *Ambrosiella* symbiont; [2,21]). Furthermore, there is tremendous evidence in all insect farmers for indirect promotion of cultivars by direct suppression of fungal competitors and pathogens. In ambrosia beetles, *X. saxesenii* larvae can suppress the growth of a fungal pathogen [35]. Moreover, adult females of the same species upregulated allogrooming and cannibalism (i.e. removal of corpses) after experimental injection of a pathogenic *Aspergillus* sp. in their nests, which effectively reduced its spore loads [36]. Such effects may explain the reduced diversity and relative abundances of secondary symbionts (bacteria and *Chaetomium* sp.) in the presence of mothers and immatures we found here.

Known hygienic behaviours of ambrosia beetles such as the compartmentalization of fungus gardens, allogrooming and removal of infected nest-mates have all been observed before in *X. saxesenii* (and other ambrosia beetles), but their effects on symbiont communities have so far been unknown [1,12,15]. Our results clearly show that their effectiveness are by no means comparable to the ones of farming ants and termites that maintain more or less monocultures of their fungal cultivars over several years and include behaviours such as pathogen alarm [2,57]. Ambrosia beetles, on the other hand, typically live in small subsocial to cooperatively breeding

societies (with a maximum of 100 individuals in *X. saxesenii*), with relatively short durability (max. 2 years in *X. saxesenii*) [2]. Nevertheless, it is possible that the effectiveness of farming was underestimated by our study because we only tested the effect of mothers and their larvae. A stronger effect can be expected when beetle galleries are sampled at a later stage, during the presence of mature daughters that delay dispersal and display various hygienic behaviours [35].

(c) Putative mechanisms for regulating symbiont communities

The exact mechanisms underlying the suppression of secondary symbionts remain unknown, but it is likely that beetle secretions and/or antibiotic-producing bacteria are playing a role [2,6]. Yet unknown selective secretions nurture fungal spores during hibernation and dispersal within ambrosia beetle's cuticular mycetangia [58] and it is possible that these selective secretions are also released in the nest environment. Against this hypothesis speaks the observation that the mycetangial glands are only active before and during adult female dispersal, but are reduced soon after ejection of fungal spores and establishment of a fungus garden [58,59]. *X. saxesenii* may release selective secretions through the gut as adult females in this species use the gut as a second mycetangium for transmitting *R. sulphurea* [28]. Similarly, both termite faeces and saliva have anti-microbial/-fungal activity and have been implicated to reduce the microbial load when applied on dead nest-mates [60,61].

Another already proven source of selective compounds are symbiotic bacteria. Fungus-growing ants and termites are known to use antibiotic-producing bacteria to protect their fungal gardens from invaders [8,62]. Workers in both groups can perceive pathogen presence and apply chemical defences locally [9,17]. A *Streptomyces griseus* (Actinobacteria) strain with selective inhibition of secondary fungi (but not the cultivars) has been isolated from both *X. saxesenii* and *X. affinis* [7]. Here, we could not detect this species, but possibly other symbionts provide similar functions, given that screens for antibiotic-producing bacteria in related bark beetles revealed many taxa of which some genera are present in our community (e.g. *Microbacterium*, *Pseudoxanthomonas*; [6,63–65]. However, due to the well-known functional variability within and between bacterial taxa any specific function would need to be proven experimentally.

(d) The bacterial community and potential functions

In ambrosia beetles, so far, most descriptive and experimental studies focused on the fungal symbionts (but see [32,66–68]). However, given that ambrosia beetle communities resemble those of fungus-farming ants and termites [66], where bacteria are known to play essential roles (e.g. [8,69–71]), more functional studies on these symbionts are crucially needed.

The overall number of core bacteria was surprisingly small and common taxa belonged to the Actinobacteria, the Alphaproteobacteria and the Gammaproteobacteria, with *Pseudoxanthomonas*, *Acinetobacter*, *Erwinia*, *Ochrobactrum*, *Microbacterium* and *Wolbachia* being the most abundant phylotypes. This result closely resembles communities found by other studies for *X. saxesenii* and other ambrosia beetles [32,66,68]. One of the most abundant genera in our samples was *Pseudoxanthomonas*. This bacterium had been detected in the gut of *X. saxesenii* by Fabig [72] before, but only in low relative abundance (1.2%). Other studies repeatedly isolated this bacterium from gut samples of bark beetles and profiled it as a cellulolytic bacterium, similar to *Ochrobactrum*, able to produce an array of cellulolytic–xylanolytic enzymes [67,73,74]. *Erwinia*, on the other hand, may be able to fix atmospheric nitrogen [75,76], which may profit the farmers in this nitrogen-deficient wood substrate.

Wolbachia infections were found in four of our 11 different family lineages and were also passed on between F0 and F1 generations. This and our previous study [32] are the first to show *Wolbachia* infections in *X. saxesenii* [77]. Here, we also detected *Wolbachia* from abandoned fungus gardens, which is surprising as these bacteria are obligate endosymbionts of insects [77]. So, either we were able to report a case of plant-mediated horizontal transmission, as it was already found for whiteflies [78] or the *Wolbachia* DNA originated from dead cells in the beetle's faeces. The second hypothesis is supported by the lower relative abundances in the *removal* treatment for these lineages. The role of *Wolbachia* for *X. saxesenii* remains enigmatic as no obvious effects on beetle fitness or sex ratio could be detected in the few infected families. However, our bacterial communities were either dominated by *Pseudoxanthomonas* or *Wolbachia*, which could either be caused by an indirect effect through the host (e.g. host genome differences or an upregulation of the immunity response) or just sequencing dominance by *Wolbachia*.

(e) The fungal community and potential functions

The core community of fungi detected by our primers is small and made up of *C. globosum* (Chaetomiaceae), *R. sulphurea*, *R. canadensis* (Ophiostomaceae), *P. commune* (Trichocomaceae) and *A. biseptum* (Bionectriaceae). Among the secondary symbionts, *C. globosum* was the only species present in all nests. It is a saprophyte that is in obvious competition with the two *Raffaelea* fungi. The same nature of competing interaction may be the case for *A. biseptum* as indicated by the replacement of the food fungi in some nests (electronic supplementary material, figure S2). *Chaetomium globosum* is commonly isolated from wood and wood-boring insects, and it is also known to be toxic to insects [79]. Its negative effect on beetles is further illustrated by the reduced success rate of 2nd founding attempts when it was common in *removal* nests (electronic supplementary material, figure S6B). However, beetles seem to have some strategies to control the spread of this fungus as demonstrated by the reduced relative abundance when mothers and immatures are present (figure 3*d*). *Penicillium* and *Acremonium* species may be regarded as weak competitors of the food fungi as they especially dominate old nests [34,80]. All of these secondary fungi are ubiquitous saprophytes within wood, do not infect beetles and are among the most common secondary symbionts of bark and ambrosia beetles [81].

(f) Strong heredity of beetle microbiome

Apart from the effect of farming there were strong signatures of family lineage on microbial symbiont communities between generations demonstrating transgenerational inheritance. This was expected for the *Raffaelea* cultivars, given that *X. saxesenii* females vertically transmit spores of their *Raffaelea* cultivars in elytral mycetangia and the gut [28]. However, it is fascinating that this strong signature of family even prevails when females are removed from the nest and symbiont

communities grow without the beetle's presence (figure 3*d*, electronic supplementary material, figure S2). The latter finding is a strong indication for the competitive abilities of the *Raffaelea* cultivars, which are apparently able to maintain their growth, possibly by producing anti-microbial compounds (e.g. ethanol; [80]). The detected family-specific strain variation within *R. canadensis* in our data (electronic supplementary material, figure S7) is another clear sign for a strong heredity of symbionts. On the other hand, vertical transmission is also strong for the secondary symbionts. This may be surprising given that most of these taxa are likely competitors and pathogens of the mutualism. A closer look at these taxa reveals, however, that the majority are not only ubiquitous in bark and ambrosia beetles, but also many other saproxylic insects [82,83]. Therefore, while we do not know much about the mechanisms, it seems that they are specialized for hitch-hiking and dispersal by insects.

(g) Usage of special primers for fungal metabarcoding helps to distinguish between food fungal species

Up to now there has been no satisfactory method for metabarcoding of the fungal communities of bark and ambrosia beetles, as the universal, highly variable ITS primers used for species identification of fungi do not amplify Ophiostomataceae, their primary group of symbionts [31,48]. In line with Skelton *et al*. [84] and Ibarra-Juarez *et al*. [30], our work is another attempt to use self-designed non-ITS primers for sequencing the fungal symbionts of an ambrosia beetle. Originally designed for this study, our LSU primer pair LIC15R and nu-LSU-355-3′ has already been used in another recently published article by Nuotclà *et al*. [32]. Both of our studies successfully amplified the ambrosia fungi in the Ophiostomataceae. Despite a mean amplicon length of 276.74 bp, we achieved a reliable discrimination of closely related species, which is specifically important for the two *Raffealea* ambrosia fungi of *X. saxesenii* (*R. sulphurea* and *R. canadensis*). Likewise, it was possible to identify the majority of other fungal symbionts, which included the following ascomycete orders: Ophiostomatales, Eurotiales, Sordariales, Hypocreales, Capnodiales, Helotiales and Xylariales. Yeasts of the order Saccharomycetales, however, that were successfully amplified by the SSU primers used by Ibarra-Juarez *et al*. [30] failed to be detected by our primers as we see in the mock community output (electronic supplementary material, figure S10). Also, Basidiomycetes could not be detected, which together demonstrates the bias of all fungal metabarcoding primers. Amplicon sequencing does not provide information on absolute abundances of phylotypes, it sets the amplified taxa in relation [85] which is important to keep in mind especially when some taxa could not be amplified as their appearance may shift relative abundance pictures. For future studies we propose to carefully choose metabarcoding primers that amplify the fungal groups of main interest for testing the key hypothesis, while acknowledging their limitations [47,86].

5. Conclusion

In this study we tested the presence of active farming behaviour in ambrosia beetles. A significant effect of mothers and their larvae on the microbial composition of their fungus gardens could be proven and even stronger farming effects of adult daughters at a later developmental stage of nests may be expected. However, the exact mechanisms underlying the defence against weeds and the potential promotion of ambrosia fungi still remains unknown. Most studies investigating microbial symbionts of ambrosia beetles are surveys and we need more experimental studies on symbiont communities to understand the roles of specific symbionts in these ecosystems. Our new amplicon primers, which amplify Ophiostomatacea symbionts of bark and ambrosia beetles could distinguish the two ambrosia fungi in this study and therefore provide a useful tool for this purpose. In combination with more advanced techniques like quantitative real-time PCR this should help to understand symbiont shifts under different conditions. Also, we can move on to closely investigating some of the core taxa that we found (*Pseudoxanthomonas*, *Wolbachia*, *Penicillium*) in bioassays to find out more about their potential functions in the ambrosia beetle–fungus mutualism.

Data accessibility. The data and scripts that support the findings of this study are openly available in our GitHub Repository at https://github.com/janinad88/ambrosia-beetle-fungus-farming. Raw sequence data are uploaded on the NCBI database under the BioProject ID: PRJNA884345.

The data are provided in electronic supplementary material [87].

Authors' contributions. J.M.C.D.: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing—original draft, writing—review and editing; V.K.: validation, writing—original draft, writing—review and editing; A.K.: methodology, resources, writing—review and editing; P.H.W.B.: conceptualization, funding acquisition, methodology, project administration, resources, supervision, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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References

1. Biedermann PHW, Vega FE. 2020 Ecology and evolution of insect–fungus mutualisms. *Annu. Rev. Entomol.* **65**, 431–455. (doi:10.1146/annurev-ento-011019-024910)
2. Mueller UG, Gerardo NM, Aanen DK, Six DL, Schultz TR. 2005 The evolution of agriculture in insects. *Annu. Rev. Ecol. Evol. Syst.* **36**, 563–595. (doi:10.1146/annurev.ecolsys.36.102003.152626)
3. Mayer VE, Nepel M, Blatrix R, Oberhauser FB, Fiedler K, Schönenberger J, Voglmayr H. 2018 Transmission of fungal partners to incipient *Cecropia*-tree ant colonies. *PLoS ONE* **13**, e0192207. (doi:10.1371/journal.pone.0192207)
4. Schultz T, Gawne R, Peregrine P. 2022 *The convergent evolution of agriculture in humans and fungus-farming ants. The convergent evolution of agriculture in humans and insects*. Cambridge, Massachusetts: MIT Press.
5. Schultz TR. 2022 The convergent evolution of agriculture in humans and fungus-farming ants. In *The convergent evolution of agriculture in humans and insects* (eds TR Schultz, R Gawne, PN Peregrine), pp. 281–313. Cambridge, Massachusetts: MIT Press1st edn.

6. Scott JJ, Oh D-C, Yuceer MC, Klepzig KD, Clardy J, Currie CR. 2008 Bacterial protection of beetle-fungus mutualism. *Science* **322**, 63. (doi:10.1126/science.1160423)
7. Grubbs KJ, Surup F, Biedermann PHW, McDonald BR, Klassen JL, Carlson CM, Clardy J, Currie CR*m*2020 Cycloheximide-producing *Streptomyces* associated with *Xyleborinus saxesenii* and *Xyleborus affinis* fungus-farming ambrosia beetles. *Front. Microbiol.* **11**, 1–12. (doi:10.3389/fmicb.2020.562140)
8. Currie CR, Scott JA, Summerbell RC, Malloch D. 1999 Fungus-growing ants use antibiotic producing bacteria to control garden parasites. *Nature* **398**, 701–704. (doi:10.1038/19519)
9. Currie CR, Stuart AE. 2001 Weeding and grooming of pathogens in agriculture by ants. *Proc. R. Soc. Lond. B* **268**, 1033–1039. (doi:10.1098/rspb.2001.1605)
10. Aanen DK. 2006 As you reap, so shall you sow: coupling of harvesting and inoculating stabilizes the mutualism between termites and fungi. *Biol. Lett.* **2**, 209–212. (doi:10.1098/rsbl.2005.0424)
11. Little AEF, Murakami T, Mueller UG, Currie CR. 2006 Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens. *Biol. Lett.* **2**, 12–16. (doi:10.1098/rsbl.2005.0371)
12. Kirkendall LR, Biedermann PHW, Jordal BH. 2015 Evolution and diversity of bark and ambrosia beetles. In *Bark beetles: biology and ecology of native and invasive species* (eds FE Vega, RW Hofstetter), pp. 85–156. Cambridge, Massachusetts: Elsevier Academic Press.
13. Hubbard HG. 1897 The ambrosia beetles of the United States. In *Bulletin*, Vol. **7** (ed. LQ Howard). Washington, DC: US Department of Agriculture Bureau of Entomology.
14. Neger FW. 1908 Die Pilzkulturen der Nutzholzborkenkäfer. *Zentralblatt für Bakteriol Jena Abt II.* **20**, 279–282.
15. Biedermann PHW, Rohlfs M. 2017 Evolutionary feedbacks between insect sociality and microbial management. *Curr. Opin. Insect. Sci.* **22**, 92–100. (doi:10.1016/j.cois.2017.06.003)
16. Aanen DK, Eggleton P, Rouland-Lefevre C, Guldberg-Froslev T, Rosendahl S, Boomsma JJ. 2002 The evolution of fungus-growing termites and their mutualistic fungal symbionts. *Proc Natl. Acad. Sci. USA* **99**, 14 887–14 892. (doi:10.1073/pnas.222313099)
17. Katariya L, Ramesh PB, Gopalappa T, Desireddy S, Bessière JM, Borges RM. 2017 Fungus-farming termites selectively bury weedy fungi that smell different from crop fungi. *J. Chem. Ecol.* **43**, 986–995. (doi:10.1007/s10886-017-0902-4)
18. Batra LRR. 1966 Ambrosia fungi: extent of specificity to ambrosia beetles. *Science* **153**, 193–195. (doi:10.1126/science.153.3732.193)
19. Skelton J, Johnson AJ, Jusino MA, Bateman CC, Li Y, Hulcr J. 2019 A selective fungal transport organ (mycangium) maintains coarse phylogenetic congruence between fungus-farming ambrosia beetles and their symbionts. *Proc. R. Soc. B* **286**, 20182127. (doi:10.1098/rspb.2018.2127)
20. Harrington TC. 2005 Ecology and evolution of mycophagous bark beetles and their fungal partners. In *Ecological and evolutionary advances in insect-fungal associations* (eds FE Vega, M Blackwell), pp. 257–291. Oxford, England: Oxford University Press.
21. Batra LR, Michie MD. 1963 Pleomorphism in some ambrosia and related fungi. *Trans. Kans. Acad. Sci.* **66**, 470–481. (doi:10.2307/3626545)
22. Francke-Grosmann H. 1967 Ectosymbiosis in wood-inhabiting insects. In *Symbiosis* (ed. SM Henry), pp. 141–205. New York, NY: Academic Press.
23. Kok LT, Norris DM, Chu HM. 1970 Sterol metabolism as a basis for a mutualistic symbiosis. *Nature* **225**, 661–662. (doi:10.1038/225661b0)
24. Beaver RA. 1989 Insect–fungus relationships in the bark and ambrosia beetles. In *Insect–fungus interactions* (eds N Wilding, NM Collins, PM Hammond, JF Webber), pp. 121–143. London, UK: Academic Press.
25. Lehenberger M, Foh N, Göttlein A, Six D, Biedermann PHW. 2021 Nutrient-poor breeding substrates of ambrosia beetles are enriched with biologically important elements. *Front. Microbiol.* **12**, 1–15. (doi:10.3389/fmicb.2021.664542)
26. Francke-Grosmann H. 1956 Hautdrüsen als Träger der Pilzsymbiose bei Ambrosiakäfern. *Z. Morphol Ökol. Tiere* **45**, 275–308. (doi:10.1007/BF00430256)
27. Batra LR. 1963 Ecology of ambrosia fungi and their dissemination by beetles. *Trans Kans. Acad. Sci.* **66**, 213–236. (doi:10.2307/3626562)
28. Francke-Grosmann H. 1975 Zur epizoischen und endozoischen Übertragung der symbiotischen Pilze des Ambrosiakäfers *Xyleborus saxeseni* (Coleoptera: Scolytidae). *Entomol. Ger.* **1**, 279–292.
29. Haanstad JO, Norris DM. 1985 Microbial symbiotes of the ambrosia beetle *Xyloterinus politus*. *Microb. Ecol.* **11**, 267–276. (doi:10.1007/BF02010605)
30. Ibarra-Juarez LA *et al.* 2020 Evidence for succession and putative metabolic roles of fungi and bacteria in the farming mutualism of the ambrosia beetle *Xyleborus affinis*. *mSystems* **5**, e00541-520.
31. Kostovcik M, Bateman CC, Kolarik M, Stelinski LL, Jordal BH, Hulcr J. 2014 The ambrosia symbiosis is specific in some species and promiscuous in others: evidence from community pyrosequencing. *ISME J.* **9**, 126–138. (doi:10.1038/ismej.2014.115)
32. Nuotclà JA, Diehl JMC, Taborsky M. 2021 Habitat quality determines dispersal decisions and fitness in a beetle–fungus mutualism. *Front. Ecol. Evol.* **9**, 1–15. (doi:10.3389/fevo.2021.602672)
33. Biedermann PHW. 2020 Cooperative breeding in the ambrosia beetle *Xyleborus affinis* and management of its fungal symbionts. *Front. Ecol. Evol.* **8**, 1–12. (doi:10.3389/fevo.2020.00001)
34. Biedermann PHW, Klepzig KD, Taborsky M, Six DL. 2013 Abundance and dynamics of filamentous fungi in the complex ambrosia gardens of the primitively eusocial beetle *Xyleborinus saxesenii* Ratzeburg (Coleoptera: Curculionidae. Scolytinae). *FEMS Microbiol. Ecol.* **83**, 711–723. (doi:10.1111/1574-6941.12026)
35. Biedermann PHW, Taborsky M. 2011 Larval helpers and age polyethism in ambrosia beetles. *Proc. Natl Acad. Sci. USA* **108**, 17 064–17 069. (doi:10.1073/pnas.1107758108)
36. Nuotclà JA, Biedermann PHW, Taborsky M. 2019 Pathogen defence is a potential driver of social evolution in ambrosia beetles. *Proc. R. Soc. B* **286**, 1–9.
37. De Fine Licht HH, Biedermann PHW. 2012 Patterns of functional enzyme activity in fungus farming ambrosia beetles. *Front. Zool.* **9**, 1–11.
38. Biedermann PHW, Klepzig KD, Taborsky M. 2009 Fungus cultivation by ambrosia beetles: behavior and laboratory breeding success in three Xyleborine species. *Environ. Entomol.* **38**, 1096–1105. (doi:10.1603/022.038.0417)
39. Fischer M. 1954 Untersuchungen über den kleinen Holzbohrer (*Xyleborus saxeseni*). *Pflanzenschutzberichte* **12**, 137–180.
40. Biedermann PHW. 2010 Observations on sex ratio and behavior of males in *Xyleborinus saxesenii* Ratzeburg (*Scolytinae.* Coleoptera). *Zookeys* **56**, 253–267. (doi:10.3897/zookeys.56.530)
41. Nuotclà JA, Taborsky M, Biedermann PHW. 2014 The importance of blocking the gallery entrance in the ambrosia beetle *Xyleborinus saxesenii* Ratzeburg (Coleoptera; Scolytinae). *Mitt. Dtsch. Ges. Allg. Angew. Entomol.* **19**, 203–210.
42. Peer K, Taborsky M. 2007 Delayed dispersal as a potential route to cooperative breeding in ambrosia beetles. *Behav. Ecol. Sociobiol.* **61**, 729–739. (doi:10.1007/s00265-006-0303-0)
43. Davis NM, DiM P, Holmes SP, Relman DA, Callahan BJ. 2018 Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* **6**, 1–4. (doi:10.1186/s40168-018-0605-2)
44. Biedermann PHW. 2012 *Evolution of cooperation in ambrosia beetles*. Doctoral dissertation, University of Bern: Institute of Ecology and Evolution.
45. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013 Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* **79**, 5112–5120. (doi:10.1128/AEM.01043-13)
46. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. 2011 Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl Acad. Sci. USA* **108**, 4516–4522. (doi:10.1073/pnas.1000080107)
47. Tedersoo L, Bahram M, Zinger L, Nilsson RH, Kennedy PG, Yang T, Anslan S, Mikryukov V. 2022 Best practices in metabarcoding of fungi: from experimental design to results. *Mol. Ecol.* **31**, 2769–2795. (doi:10.1111/mec.16460)
48. Malacrinò A, Rassati D, Schena L, Mehzabin R, Battisti A, Palmeri V. 2017 Fungal communities associated with bark and ambrosia beetles trapped

at international harbours. *Fungal Ecol.* **28**, 44–52. (doi:10.1016/j.funeco.2017.04.007)

49. R Core Team. 2021 *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
50. McMurdie PJ, Holmes S. 2013 Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **8**, e61217. (doi:10.1371/journal.pone.0061217)
51. Edgar RC. 2018 Updating the 97% identity threshold for 16S ribosomal RNA OTUs. *Bioinformatics* **34**, 2371–2375. (doi:10.1093/bioinformatics/bty113)
52. Lahti L, Shetty S. 2019 microbiome R package.
53. Wickham H. 2016 *Ggplot2: elegant graphics for data analysis*. New York, NY: Springer-Verlag New York.
54. Clarke KR, Somerfield PJ, Chapman MG. 2006 On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray–Curtis coefficient for denuded assemblages. *J. Exp. Mar. Biol. Ecol.* **330**, 55–80. (doi:10.1016/j.jembe.2005.12.017)
55. Oksanen J *et al.* 2020 vegan: Community ecology package.
56. Mangiafico S. 2021 rcompanion: Functions to support extension education program evaluation.
57. Rosengaus RB, Jordan C, Lefebvre ML, Traniello JFA. 1999 Pathogen alarm behavior in a termite: a new form of communication in social insects. *Naturwissenschaften* **86**, 544–548. (doi:10.1007/s001140050672)
58. Mayers CG, Harrington TC, Biedermann PHW. 2022 Mycangia define the diverse ambrosia beetle–fungus symbioses. In *The convergent evolution of agriculture in humans and insects*. Cambridge, Massachusetts: The MIT Press.
59. Schneider I. 1991 Einige ökologische Aspekte der Ambrosiasymbiose. *Anz. Schädlingskd. Pflanzenschutz Umweltschutz* **64**, 41–45. (doi:10.1007/BF01909741)
60. Lamberty M, Zachary D, Lanot R, Bordereau C, Robert A, Hoffmann JA, Bulet P. 2001 Insect immunity: constitutive expression of a cysteine-rich antifungal and a linear antibacterial peptide in a termite insect. *J. Biol. Chem.* **276**, 4085–4092. (doi:10.1074/jbc.M002998200)
61. Chouvenc T, Su N-Y. 2012 When subterranean termites challenge the rules of fungal epizootics. *PLoS ONE* **7**, e34484. (doi:10.1371/journal.pone.0034484)
62. Um S, Fraimout A, Sapountzis P, Oh D-C, Poulsen M. 2013 The fungus-growing termite *Macrotermes natalensis* harbors bacillaene-producing *Bacillus* sp. that inhibit potentially antagonistic fungi. *Sci. Rep.* **3**, 1–7.
63. Cardoza YJ, Klepzig KD, Raffa KF. 2006 Bacteria in oral secretions of an endophytic insect inhibit antagonistic fungi. *Ecol. Entomol.* **31**, 636–645. (doi:10.1111/j.1365-2311.2006.00829.x)
64. Adams ASAS, Currie CRCR, Cardoza YC, Klepzig KDKD, Raffa KFRF. 2009 Effects of symbiotic bacteria and tree chemistry on the growth and reproduction of bark beetle fungal symbionts. *Can. J. For. Res.* **39**, 1133–1147. (doi:10.1139/X09-034)
65. Hulcr J, Adams AS, Raffa K, Hofstetter RW, Klepzig KD, Currie CR. 2011 Presence and diversity of *Streptomyces* in *Dendroctonus* and sympatric bark beetle galleries across North America. *Microb. Ecol.* **61**, 759–768. (doi:10.1007/s00248-010-9797-0)
66. Aylward FO *et al.* 2014 Convergent bacterial microbiotas in the fungal agricultural systems of insects. *MBio* **5**, e02077-14. (doi:10.1128/mBio.02077-14)
67. Ibarra-Juarez LA *et al.* 2018 Impact of rearing conditions on the ambrosia beetle's microbiome. *Life* **8**, 63.
68. Nones S, Fernandes C, Duarte L, Cruz L, Sousa E. 2022 Bacterial community associated with the ambrosia beetle *Platypus cylindrus* on declining *Quercus suber* trees in the Alentejo region of Portugal. *Plant Pathol.* **71**, 966–979. (doi:10.1111/ppa.13536)
69. Pinto-Tomás AA, Anderson MA, Suen G, Stevenson DM, Chu FST, Wallace Cleland W, Weimer PJ, Currie CR. 2009 Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. *Science* **326**, 1120–1123. (doi:10.1126/science.1173036)
70. Visser AA, Nobre T, Currie CR, Aanen DK, Poulsen M. 2012 Exploring the potential for Actinobacteria as defensive symbionts in fungus-growing termites. *Microb. Ecol.* **63**, 975–985. (doi:10.1007/s00248-011-9987-4)
71. Murphy R, Benndorf R, de Beer ZW, Vollmers J, Kaster A, Beemelmanns C, Poulsen M. 2021 Comparative genomics reveals prophylactic and catabolic capabilities of Actinobacteria within the fungus-farming termite symbiosis. *mSphere* **6**, 1–12.
72. Fabig W. 2011 *The microbial community associated with the ambrosia beetle Xyleborinus saxesenii (Coleoptera: Curculionidae) and its influence on the growth of the mutualistic fungus*. Master thesis, University of Bayreuth, Germany.
73. Hu X, Yu J, Wang C, Chen H. 2014 Cellulolytic bacteria associated with the gut of *Dendroctonus armandi* larvae (Coleoptera: Curculionidae: Scolytinae). *Forests* **5**, 455–465. (doi:10.3390/f5030455)
74. Okeke BC, Lu J. 2011 Characterization of a defined cellulolytic and xylanolytic bacterial consortium for bioprocessing of cellulose and hemicelluloses. *Appl. Biochem. Biotechnol.* **163**, 869–881. (doi:10.1007/s12010-010-9091-0)
75. Papen H, Werner D. 1979 N2-fixation in *Erwinia herbicola*. *Arch. Microbiol.* **120**, 25–30. (doi:10.1007/BF00413267)
76. Morales-Jiménez J, Vera-Ponce de León A, García-Domínguez A, Martínez-Romero E, Zúñiga G, Hernández-Rodríguez C. 2013 Nitrogen-fixing and uricolytic bacteria associated with the gut of *Dendroctonus rhizophagus* and *Dendroctonus valens* (Curculionidae: Scolytinae). *Microb. Ecol.* **66**, 200–210. (doi:10.1007/s00248-013-0206-3)
77. Kawasaki Y, Schuler H, Stauffer C, Lakatos F, Kajimura H. 2016 *Wolbachia* endosymbionts in haplodiploid and diploid scolytine beetles (Coleoptera: Curculionidae: Scolytinae). *Environ. Microbiol. Rep.* **8**, 680–688. (doi:10.1111/1758-2229.12425)
78. Li SJ, Ahmed MZ, Lv N, Shi PQ, Wang XM, Huang JL, Qiu BL. 2017 Plantmediated horizontal transmission of *Wolbachia* between whiteflies. *ISME J.* **11**, 1019–1028. (doi:10.1038/ismej.2016.164)
79. Wicklow DT, Dowd PF, Gloer JB. 1999 *Chaetomium* mycotoxins with antiinsectan or antifungal activity. *Mycotoxins* **1999**, 267–271. (doi:10.2520/myco1975.1999.Suppl2_267)
80. Ranger CM *et al.* 2018 Symbiont selection via alcohol benefits fungus farming by ambrosia beetles. *Proc. Natl Acad. Sci. USA* **115**, 4447–4452. (doi:10.1073/pnas.1716852115)
81. Hulcr J *et al.* 2020 Bark beetle mycobiome: collaboratively defined research priorities on a widespread insect–fungus symbiosis. *Symbiosis* **81**, 101–113. (doi:10.1007/s13199-020-00686-9)
82. Seibold S, Müller J, Baldrian P, Cadotte MW, Šťursová M, Biedermann PHW, Krah FS, Bässler C. 2019 Fungi associated with beetles dispersing from dead wood – let's take the beetle bus! *Fungal Ecol.* **39**, 100–108. (doi:10.1016/j.funeco.2018.11.016)
83. Birkemoe T, Jacobsen RM, Sverdrup-Thygeson A, Biedermann PHW. 2018 Insect–fungus interactions in dead wood systems. In *Saproxylic insects*, pp. 377–427. Berlin, Germany: Springer.
84. Skelton J, Jusino MA, Carlson PS, Smith K, Banik MT, Lindner DL, Palmer JM, Hulcr J. 2019 Relationships among wood-boring beetles, fungi, and the decomposition of forest biomass. *Mol. Ecol.* **28**, 4971–4986. (doi:10.1111/mec.15263)
85. Gloor GB, Macklaim JM, Pawlowsky-Glahn V, Egozcue JJ. 2017 Microbiome datasets are compositional: and this is not optional. *Front. Microbiol.* **8**, 2224. (doi:10.3389/fmicb.2017.02224)
86. Ceballos-Escalera A, Richards J, Arias MB, Inward DJG, Vogler AP. 2022 Metabarcoding of insect-associated fungal communities: a comparison of internal transcribed spacer (ITS) and large-subunit (LSU) rRNA markers. *MycoKeys* **88**, 1. (doi:10.3897/mycokeys.88.77106)
87. Diehl JMC, Kowallik V, Keller A, Biedermann PHW. 2022 First experimental evidence for active farming in ambrosia beetles and strong heredity of garden microbiomes. Figshare. (doi:10.6084/m9.figshare.c.6251419)

ANNEX - CHAPTER 1

Supplementary Material

Supplementary Material to publication "First experimental evidence for active farming in ambrosia beetles and strong heredity of garden microbiomes" by J. M. C. Diehl, V. Kowallik, A. Keller & P. H. W. Biedermann; Proc. R. Soc. B DOI: 10.1098/rspb.2022.1458

GitHub Repository: https://github.com/janinad88/ambrosia-beetle-fungus-farming

Raw Sequence Data available in NCBI SRA under BioProjectID: PRJNA884345

Sequencing controls

To ensure the reliability of our symbiont community results, we included bacterial mock communities, fungal mock communities and negative controls. The "Microbial Community Standard" from ZymoBIOMICS was used as a bacterial mock community. Our fungal mock community consisted of five known fungal associates of *X. saxesenii*. The two primary symbionts, *R. sulphurea* and *R. canadensis*, the contaminant *Chaetomium globosum* as well as *Sporothrix stenocerans* and the yeast *Pichia sp.*.

To further enhance the quality of our data, we ran the contaminant removal method with the R package 'decontam' (1), taking into account the 'negative' control samples (*n* = 4; autoclaved rearing medium for beetle breeding and PCR water control) which had overall a very low read number of bacterial and fungal ASVs. This filtering process reduces the complexity of microbiome data while preserving their integrity in downstream analysis. A reduction of the classification methods' sensitivity and of technical variability, allows the generation of more reproducible and comparable results in microbiome data analysis (2).

Bioinformatic processing and reference databases

The raw sequence reads were obtained from the Illumina MiSeq output directly (sample reads already demultiplexed by the MiSeq Reporter v. 2.5.1.3 with perfect index matches only). We merged the forward and reverse reads using the *-fastq_mergepairs* command in USEARCH v11.0-2.667 (3). A first quality filtering (sequence length >200 and maximum differences in the alignment = 30) was performed and overall quality was assessed with the command *–fastq_filter* specifying a high expected error threshold of 1 (*fastq_maxee*). Unique sequences in our FASTq file were identified with the command *–fastx_uniques* and sorted by decreasing

size annotation with a minimum size of 4 (*-sortbysize*). Before clustering, we denoised the amplicon reads with *–unoise3* of the UNOISE algorithm. Instead of the traditional Operational Taxonomic Units (OTUs) our attempt produced ASVs (amplicon sequence variants, (4)) as a higher resolution option identifying biological sequences after denoising. With the *–usearch_global* command we set the identity threshold for the 16S taxa first to 99% identity. ASVs were taxonomically classified in three steps with RDP classifier and the rdp_16s_v16_sp.fa reference database (5). In the first step, we used the reference database with manually added sequences of *Pseudomonas fluorescens* isolated from *X. saxesenii* in our laboratory. In a second step, all unclassified ASVs gathered in a "no hit"-file were classified with the rdp_16s_v16.fa database at 99% identity threshold and afterwards the remaining unclassified taxa with a sintax cut off from 0.8. Two reference databases were used for the classification of the LSU ribosomal RNA sequences (see also (6)): Firstly, we used a LSU rRNA sequences database of a fungal stock culture at the Chair of Forest Entomology and Protection (University of Freiburg, Germany). This database from known fungal symbionts of ambrosia beetles included eighteen reference sequences of twelve unique fungal species. Since this database comprises all the sequences of known fungal symbionts of *X. saxesenii*, we lowered the identity threshold to 97% identity. Secondly, ASVs not receiving a hit were classified with a custom reference database of fungi from NCBI, created with BCdatabaser v.1.1.1. (7), using the usual identity threshold of 99% identity and if still classifiable afterwards hierarchically with USEARCH Sintax using a cut off of 0.8. The newly created reference database from NCBI sequence data included 85,250 sequences fungal species (https://zenodo.org/record/5109344#.YPFDjEBCSUk). Finally, all tables, each for 16S and LSU taxonomic data, were combined into a common table. For a full script on parameter settings of all steps above, see the supplemented Shell-scripts in the GitHub repository.

Overall symbiont communities

In total we obtained 36,625,164 raw 16S reads and 24,009,500 raw LSU reads, which accounted for an average of 95,378 reads for 16S and 62,525 reads for LSU per sample in our two sequencing runs including several other projects. The total raw read size for this specific project was 1,175,496 raw 16S reads and 2,008,472 raw LSU reads, which accounted for an average of either 20,623 or 35,236 reads per sample. After data processing (merging, low

quality <Q20, short reads <200 bp, ambiguous base-pairs), a mean of 18,056 reads per 16S sample and 16,995 per LSU sample remained. After read processing, and removing nonbacterial/fungal and rare sequences (<500 reads across sample set), the bacterial samples without community standard and negative controls contained on average 15,012 reads (range, 793 to 44,924) and 69 amplicon ASVs. The fungal samples contained on average 17,344 reads (range, 1087 to 39,932) and 202 amplicon ASVs.

Negative controls (autoclaved rearing medium for beetle breeding and PCR water control) showed very few bacterial (< 80 reads/sample) and fungal reads (< 220 reads/sample). In addition, the first controls of these samples with gel electrophoresis ahead to sequencing revealed no visible bands and rarefaction curves as well as richness estimates suggest a low number of single sequences due to possible cross-contamination (Supplementary Figures 1).

Positive mock controls for both bacteria (ZymoResearch, ZymoBIOMICS Microbial Community Standard D6300) and fungi data (self-designed community consisting of equal ratios of cultivated *C. globosum, R. sulphurea, R. canadensis, Sporothrix stenocerans* and *Pichia sp.*) confirmed successful sequencing of the contained taxa. However, the yeasts (*Pichia sp., Saccharomyces cerevisiae* and *Cryptococcus neoformans*) from the ZymoBIOMICS and fungal mock control could not be detected with the LSU primers we used. Bacterial species from the ZYMO mock community rarely appeared in other samples than the mock communities representing neglectable cross-contamination (<15 reads/sample).

The contaminant removal method identified 178 of the bacterial 16S ribosomal RNA and two of the fungal LSU ribosomal RNA ASVs as external contaminants, which were excluded from the data. Species accumulation curves of the final data sets showed that most samples were sequenced to saturation after approximately 20,000 high quality reads for 16S (Fig. S1a). The rarefaction curve for LSU sequence data reached the plateau after approximately 5,000-6,000 reads (Fig. S1b).

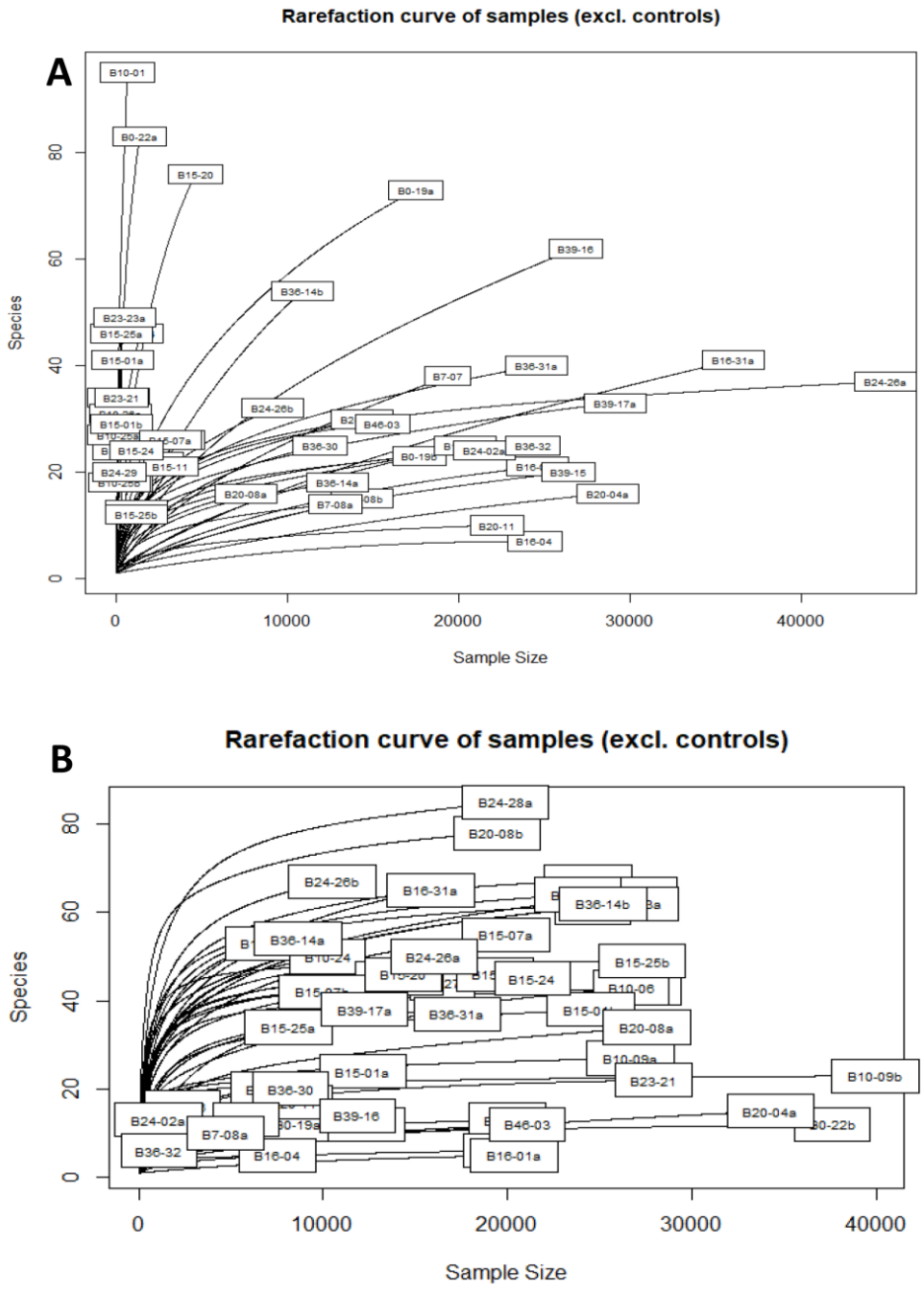

Fig. S1: rarefaction curves for 16S (A) and 28S (B) amplicon sequence variants in the final datasets.

4

Tab. S1: Relative abundance of the most abundant bacterial taxa in the three treatments (mean ± SD).

Genus	Treatment	Mean RA (%)
Acinetobacter	control	0.57 ± 1.43
	removal	0.16 ± 0.57
	2nd attempt	0.02 ± 0.03
Erwinia	control	0.29 ± 0.78
	removal	15.41 ± 32.43
	2nd attempt	7.57 ± 20.75
Ochrobactrum	control	4.51 ± 16.90
	removal	1.88 ± 6.90
	2nd attempt	0.25 ± 0.68
Pseudomonas	control	0.97 ± 3.35
	removal	0.28 ± 1.00
	2nd attempt	0.08 ± 0.10
Pseudoxanthomonas	control	50.94 ± 47.03
	removal	67.85 ± 41.61
	2nd attempt	54.78 ± 48.03
Streptomyces	control	0.80 ± 2.77
	removal	0.05 ± 0.14
	2nd attempt	0.02 ± 0.05
Wolbachia	control	36.88 ± 44.08
	removal	11.55 ± 29.90
	2nd attempt	36.15 ± 49.15

Tab. S2: Relative abundance of the most abundant fungal species (>0.5%) in the three treatments (mean ± SD).

Species	Treatment	Mean RA (%)
Chaetomium globosum	control	18.98 ± 28.11
	removal	28.75 ± 39.06
	2nd attempt	25.37 ± 34.99
Raffaelea sulphurea	control	24.74 ± 29.65
	removal	15.37 ± 26.34
	2nd attempt	12.66 ± 20.63
Raffaelea canadensis	control	5.52 ± 9.29
	removal	1.88 ± 4.37
	2nd attempt	5.88 ± 10.64
Penicillium commune	control	0.001± 0.004
	removal	0.71 ± 3.17
	2nd attempt	3.17 ± 10.51
Acremonium biseptum	control	0.20 ± 0.90
	removal	4.82 ± 21.9
	2nd attempt	2.37 ± 7.85

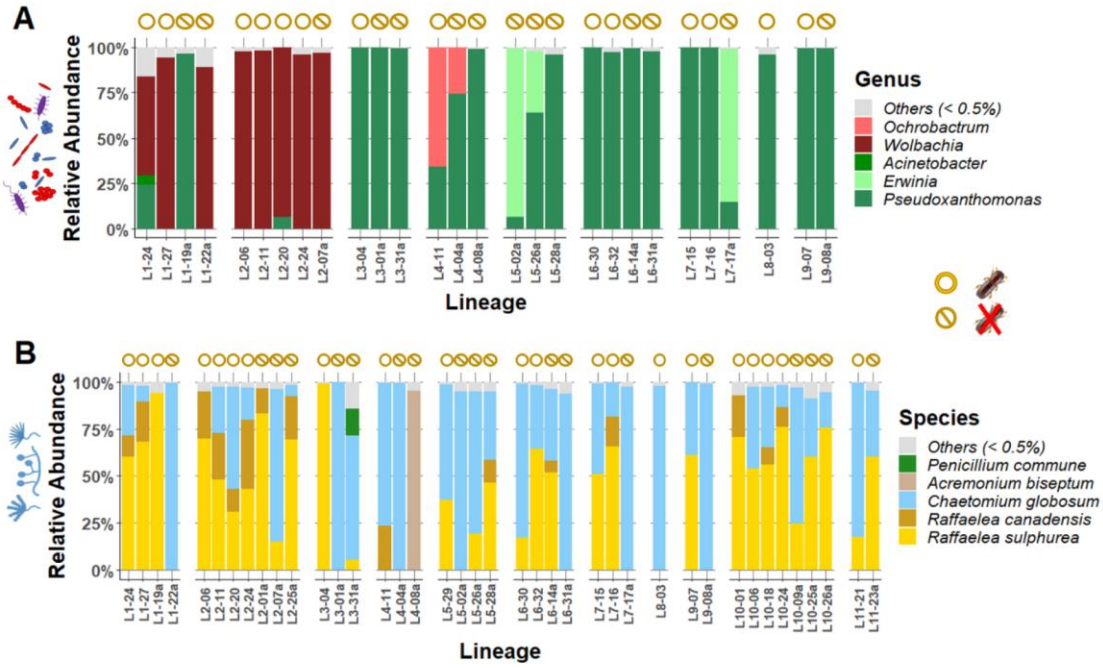

Fig. S2: Effect of family lineage on microbial communities. The core bacterial (A) and fungal community (B) of *X. saxesenii* is small. In addition to the treatment effect there is also a significant effect of family lineage on both bacterial and fungal communities (for details see Tab. 1). Only bacterial and fungal taxa with a relative abundance of >0,5% are displayed (everything else is combined in "others"). *Control* nests are marked by closed circles, *removal* nests by crossed out circles.

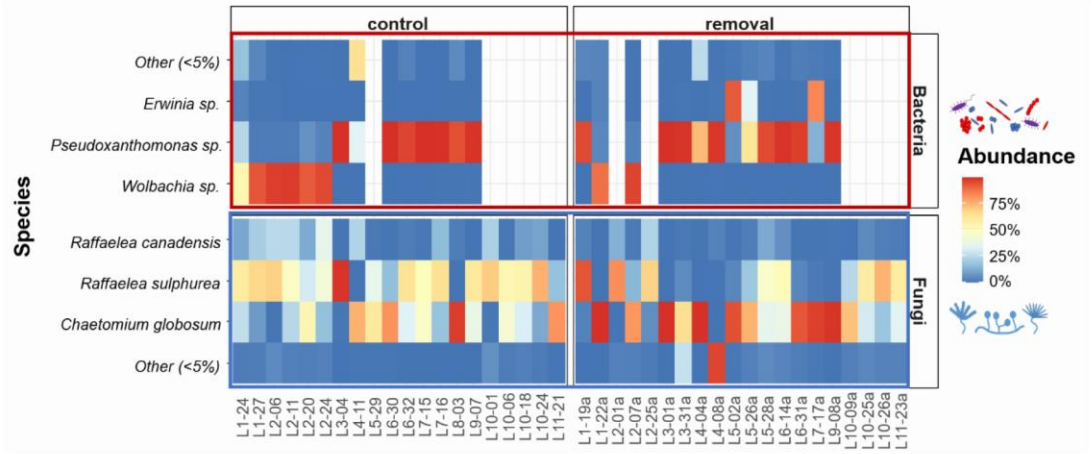

Fig. S3: Heatmap of relative abundance of core bacterial and fungal phylotypes in all samples displayed for *control* and *removal* group. Taxa under the detection threshold of 5% relative abundance are combined into "Other".

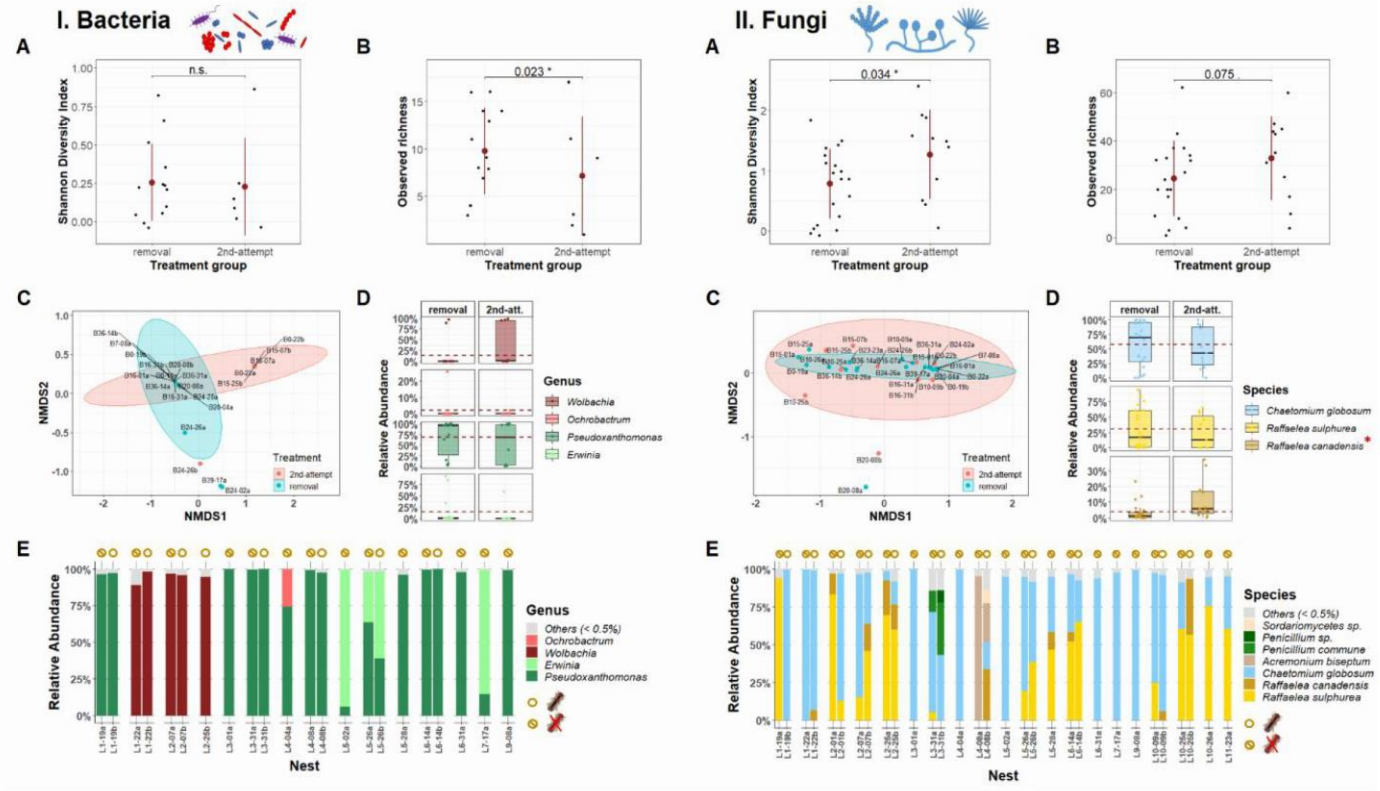

Fig. S4: Effect of beetle removal on bacterial and fungal diversity, composition and relative abundance. (A) Bacterial Shannon's diversity indices did not differ between *removal* (n = 20 nests) and *2nd attempt group* (n = 11 nests; LM: F = 0.406, p = 0.538), opposite to the fungal SDI, which was higher in the *2nd attempt group* (GLM: χ^2 = 1.28, p = 0.258; plots show mean and standard deviation). (B) Bacterial and fungal observed richnesswas higher when beetles were removed (Bacteria: GLM: χ^2 = 4.51, p = 0.034; Fungi: GLM: χ^2 = 3.17, p = 0.075; plot shows mean and standard deviation). (C) Visualization of compositional differences between bacterial and fungal communities of nests in *removal* and *2nd attempt group* (NMDS on Bray-Curtis dissimilarity: $stress_{Bacteria}$ = <0.001, $stress_{Fungi}$ = 0.032; ellipses represent 95% confidence intervals; labels represent family lineage of samples). PERMANOVA of bacterial and fungal communities showed no effect by beetle presence (Bacteria: R^2 = 0.042, p = 0.169; Fungi: R^2 = 0.016, p = 0.641). D). Comparisons of relative abundances of core bacterial taxa did not reveal any significant differences between treatments (boxplots represent median with its interquartile range and whiskers; red dashed line shows the mean relative abundance of the *`removal group`*). Comparisons of relative abundances of core fungal taxa revealed a reduction of the food fungus, *R. canadensis* (LM: F = 4.30, p = 0.051; * - p < 0.05), in nests without beetles. E) The core bacterial and fungal community of *X. saxesenii* is small. There is a significant effect of family lineage on the bacterial community (for details see Tab. 1). Only bacterial and fungal taxa with a relative abundance >0,5% are displayed (everything else is combined in "others"). *Control* nests are marked by closed circles, *removal* nests by crossed out circles.

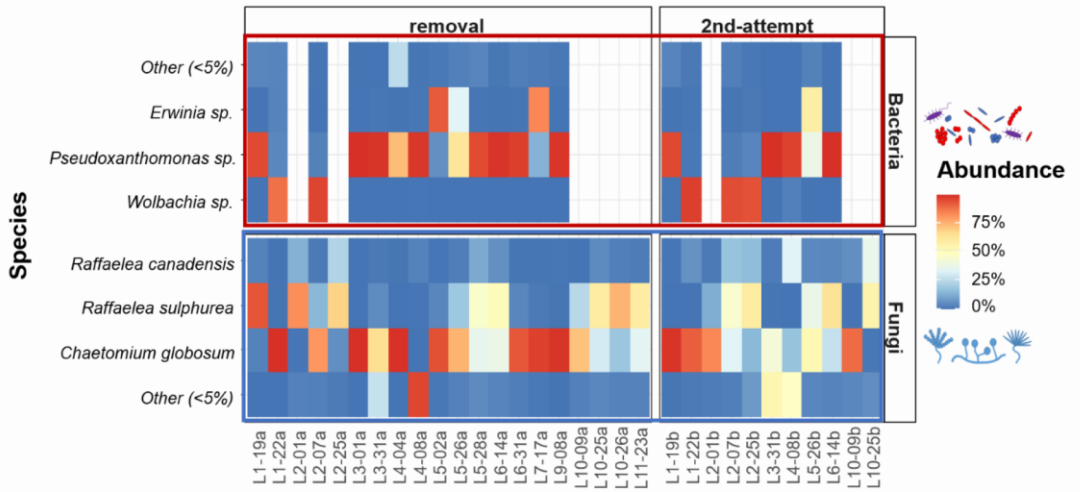

Fig. S5: Heatmap of relative abundances of core bacterial and fungal phylotypes in all samples displayed for *removal* and *2nd attempt* group. Taxa under the detection threshold of 5% relative abundance are combined into "Other".

Tab. S3: Statistical output of linear models on relative abundances of core bacterial and fungal taxa. Both comparisons of *control* vs. *removal* and *removal* vs. *2nd attempt* are shown. Statistically significant results are depicted in bold.

control* vs. *removal					
Bacteria			**Fungi**		
	p-value			*p*-value	
Wolbachia	Treatment	0.138	*Chaetomium globosum*	Treatment	0.077
	Lineage	**<0.001**		Lineage	0.618
Pseudoxanthomonas	Treatment	0.679	*Raffaelea canadensis*	Treatment	**0.031**
	Lineage	**0.001**		Lineage	**0.038**
			Raffaelea sulphurea	Treatment	**0.034**
				Lineage	**0.095**
removal* vs. *2nd attempt					
Bacteria			**Fungi**		
	p-value			*p*-value	
Wolbachia	Treatment	0.534	*Chaetomium globosum*	Treatment	0.765
	Lineage	**0.045**		Lineage	0.562
Pseudoxanthomonas	Treatment	0.693	*Raffaelea canadensis*	Treatment	**0.051**
	Lineage	**0.005**		Lineage	0.623
			Raffaelea sulphurea	Treatment	0.241
				Lineage	**0.051**

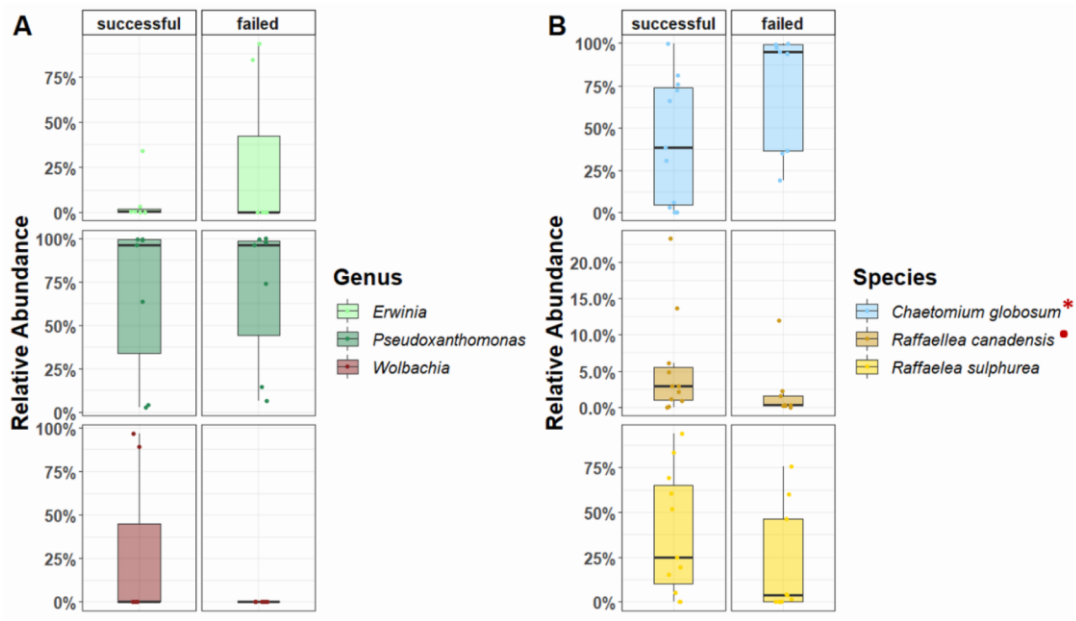

Fig. S6: Effects of the relative abundance of core taxa in *removal* nests on the success of the same foundresses in their 2nd nest-founding attempt. There was no significant effect of core bacterial taxa on founding success, but relative abundance of *C. globosum* correlated with a lower founding success (* - $p < 0.5$), while *R. canadensis* tended to correlate with a higher founding success (• - $p = 0.1$) (boxplots represent median with its interquartile range and whiskers).

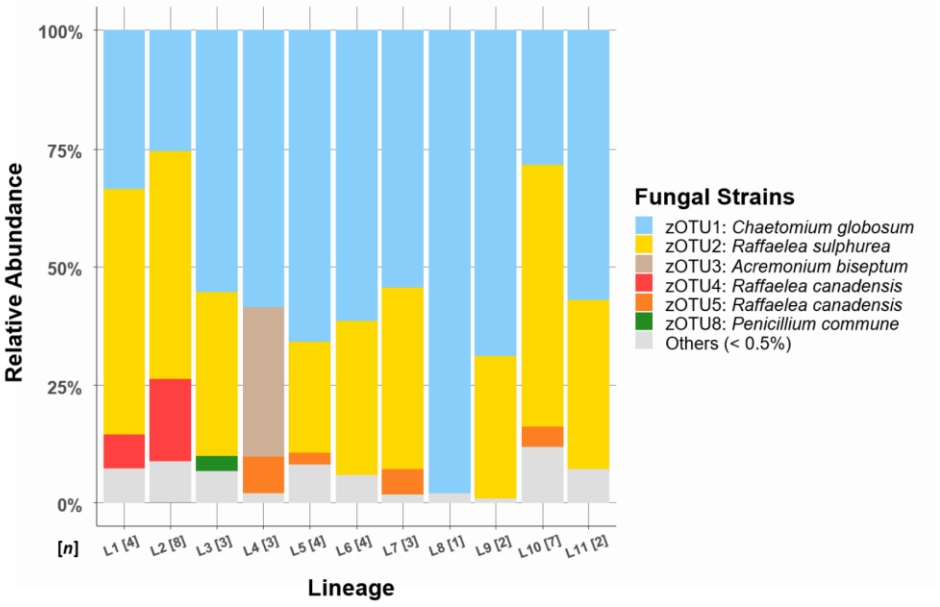

Fig. S7: Heritability of fungal communities in control nests. Visualization of highly abundant strains in ambrosia beetle nests, revealed the presence of two *R. canadensis* strains each only individually dominant per family lineage.

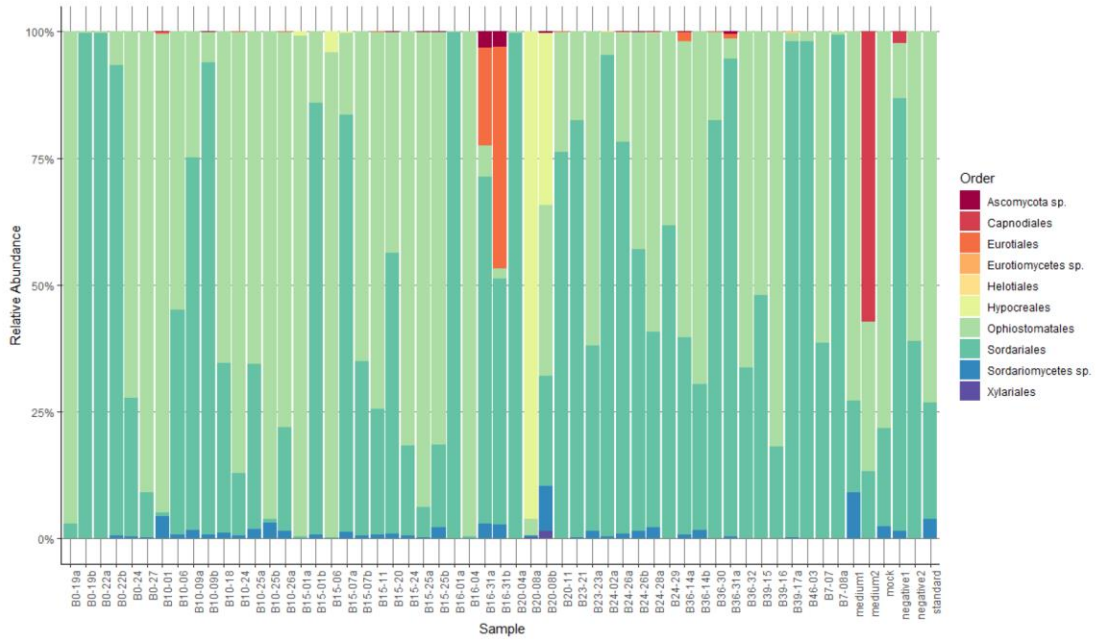

Fig. S8: Relative Abundance of detected orders in all samples (including controls) prae filtering with 'decontam'.

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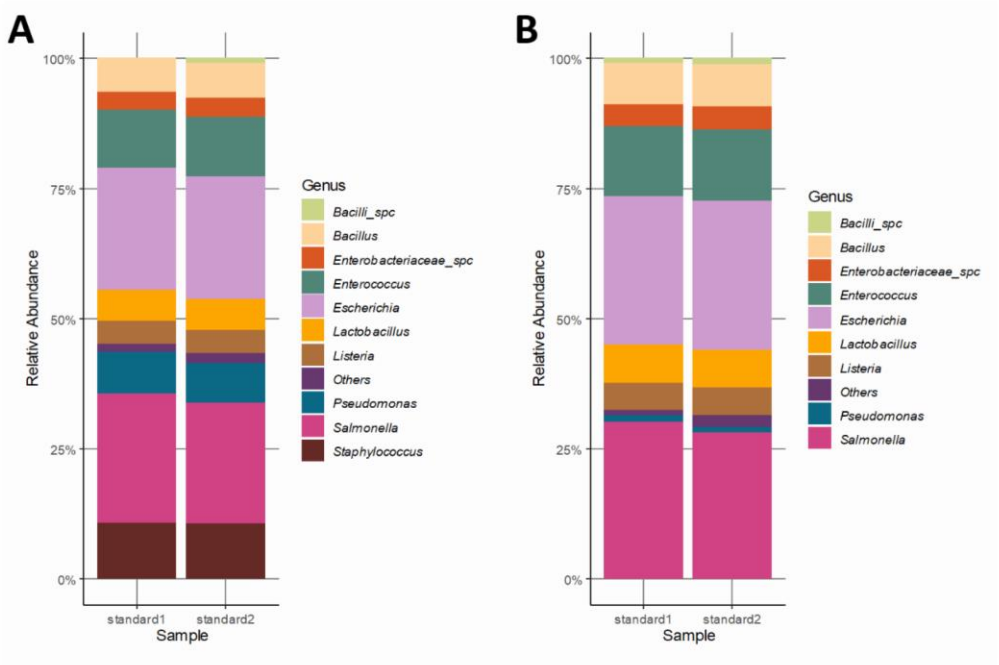

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Fig. 9: Relative Abundance of ZymoBIOMICS™ Microbial Community Standard A) prae und B) post filtering step with the 'decontam' package. Standard sample 1 had 10,263 reads prae filtering and had 8,444 reads afterwards. A similar result was found for Standard sample 2, which had 9,062 reads and after filtering was reduced to 7,477 reads. The Zymo Microbial Community Standard should contain the following eight bacterial genera: *Pseudomonas, Escherichia, Salmonella, Lactobacillus, Enterococcus, Staphylococcus, Listeria, Bacillus* (ZymoBIOMICS™ Microbial Community Standard Instruction

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Manual). These could all be detected in our sequencing, however, the decontamination step removed *Staphylococcus* as this also appeared as a contaminant in the negative controls and could not be found in our true samples.

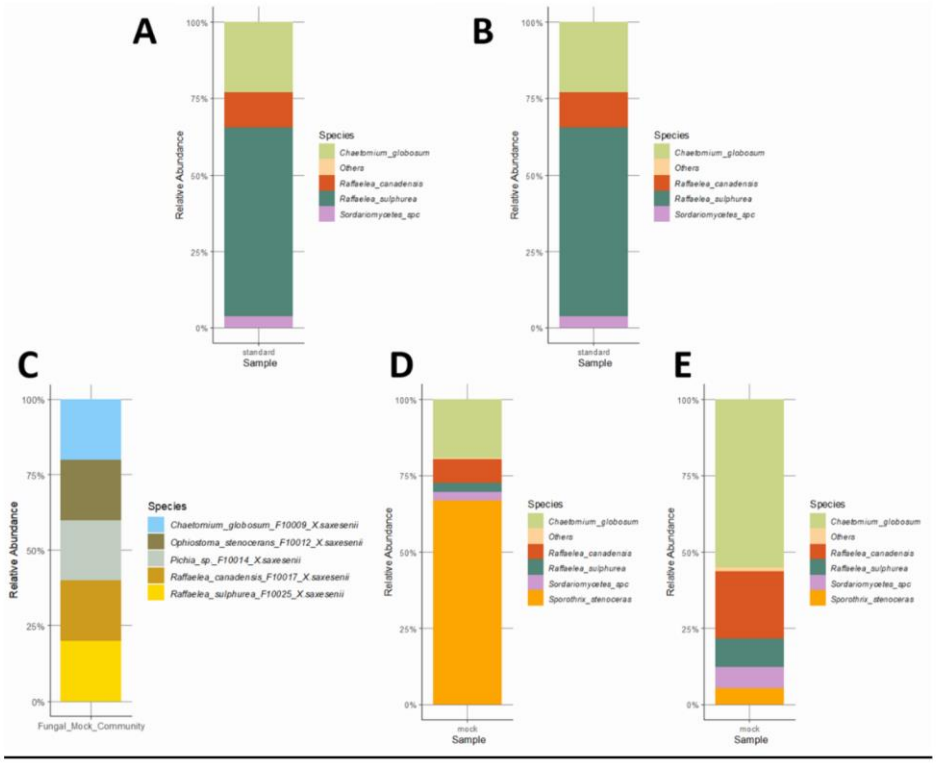

Fig. S10: Relative Abundance of detected taxa in the sequencing control samples prae und post filtering with the 'decontam' package. A) ZymoBIOMICS™ Microbial Community Standard prae removal of contaminants (26 reads) B) ZymoBIOMICS™ Microbial Community Standard post removal of contaminants (26 reads). These Mock communities should contain two fungal genera: *Cryptococcus* and *Saccharomyces* (Instruction Manual) which both could not be amplified with our primers. Instead, the Ambrosia beetle fungi appear, however the extremely low read numbers in these samples demonstrate that only neglectable cross contamination was sequenced.

C) Self-made fungal mock community, how it should be proportionally composed. Equal amounts of biomass were used to create the community. D) Mock community prae removal of contaminants (23,868 reads) E) Mock community post removal of contaminants (8,317 reads)

References

1. Davis NM, Proctor DiM, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome. 2018 Dec 17;6(1).

2. Cao Q, Sun X, Rajesh K, Chalasani N, Gelow K, Katz B, et al. Effects of rare microbiome

taxa filtering on statistical analysis. Front Microbiol. 2021 Jan 12;11:3203.

3. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26(19):2460–1. https://academic.oup.com/bioinformatics/article-lookup/doi/10.1093/bioinformatics/btq461

4. Edgar RC. Updating the 97% identity threshold for 16S ribosomal RNA OTUs. Bioinformatics. 2018;34(14):2371–5.

5. Edgar RC. SINTAX: a simple non-Bayesian taxonomy classifier for 16S and ITS sequences. bioRxiv. 2016;074161. https://www.biorxiv.org/content/10.1101/074161v1

6. Nuotclà JA, Diehl JMC, Taborsky M. Habitat quality determines dispersal decisions and fitness in a beetle – fungus mutualism. Front Ecol Evol. 2021;9(April):1–15.

7. Keller A, Hohlfeld S, Kolter A, Schultz J, Schultz J, Gemeinholzer B, et al. BCdatabaser: On-The-fly reference database creation for (meta-)barcoding. Bioinformatics. 2020;36(8):2630–1. https://ecoevorxiv.org/cmfu2/

CHAPTER 2

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Friend or Foe: Ambrosia beetle response to volatiles of common threats in their fungus gardens.

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https://www.biorxiv.org/content/10.1101/2022.12.23.521835v1.

(submitted to *Symbiosis*, December 2022, under review)

Friend or Foe: Ambrosia Beetle response to volatiles of common threats in their fungus gardens

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Abstract

Fungus farming insects encounter multiple microbial threats in their cultivar gardens. They can affect both the nutritional cultivar and the insect's health. In this study, we explored the potential of ambrosia beetles and their larvae to detect the presence of ubiquitous weed or entomopathogenic fungi. The ability to recognize a threat offers individuals a chance to react. Our study organism, the fruit-tree pinhole borer, *Xyleborinus saxesenii*, is associated with two mutualistic fungi, *Dryadomyces sulphureus* (*Raffaelea sulphurea)* and *Raffaelea canadensis*. Both symbionts were tested in combinations with two common fungus-garden weeds (*Aspergillus* sp. and *Penicillium commune*) and the entomopathogen *Beauveria bassiana* in two-choice experiments. Behavioural repellence was found in many, but not all combinations. Larvae and adult females showed an opposite reaction towards the entomopathogen, whereas for *Aspergillus* sp., neither provoked repellence nor attraction of larvae and adult females, if *R. canadensis* was used as lure. Our results validate a response of both larvae and adult ambrosia beetles towards other fungal volatiles. Their decision to confront a potential threat or preferably to avoid it could be subject to a more complex context.

Keywords:
symbiosis, insect agriculture, insect-fungus mutualism, choice-test, ambrosia beetle, repellence

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We thank Dr. Maximilian Lehenberger and Giulio Criscione for the design of the modified petri dishes applied in the adult beetle testing.

Introduction

Farming is a prominent behaviour found in several groups in the animal kingdom. Among the most prominent farmers, apart from humans, are fungus-cultivating insects (Mueller et al. 2005; Mayer et al. 2018; Biedermann and Vega 2020). A shared challenge of humans, as well as insects, is the ubiquitous threat of weeds and pathogens for the stable cultivation of crops (Schultz et al. 2022). Fungus farming insects encounter multiple threats of microbes in their cultivar gardens. These threats can be either a danger to the nutritional cultivar (e.g. competing "weeds" or pathogens (Schultz 2022)) or directly affect the health of the insects (e.g. entomopathogens (Kushiyev et al. 2018)). Ants and termites are known for their ability to detect and react to these intruders (Rosengaus et al. 1999; Griffiths and Hughes 2010; Katariya et al. 2017; Goes et al. 2020), but little is known about a third big group of fungus-farming, wood-boring weevils, the so called bark and ambrosia beetles (Curculionidae: Platypodinae and Scolytinae).

Bark and ambrosia beetles bore tunnels in phloem or xylem of trees, which they inoculate with mutualistic fungi, their sole food source (Harrington 2005; Kirkendall et al. 2015). Xylem-boring ambrosia beetles are obligately dependent on these fungi, while most bark beetles (except a few exceptions, e.g. *Dendroctonus* spp.) are facultatively associated with fungi (Six and Klepzig 2021). Whether all bark and ambrosia beetles are able to detect and react to fungal weeds is doubtful, but recent experiments on the ambrosia beetle, *Xyleborinus saxesenii* (Ratzeburg), revealed that this species can detect and selectively remove an *Aspergillus* fungal pathogen (Nuotclá et al. 2019). Furthermore, beetles can actively promote their nutritional mutualists over fungal weeds (Diehl et al. 2022). So far, it is unknown, however, how *X. saxesenii* and other farming beetles detect the presence of pathogens within nests. Volatiles of the weeds itself or changing volatile profiles of the nutritional fungi may be cues or signals, respectively. Attraction of bark and ambrosia beetles to fungal volatiles is still very sparsely documented. By now we know, that bark and ambrosia beetles can detect olfactory cues of their mutualists and are attracted to them, whereas non-associated, weedy fungi provided no sign of attraction (Hanula et al. 2008; Hulcr et al. 2011; Luna et al. 2014; Kandasamy et al. 2016; Kandasamy et al. 2019). Therefore, it is unclear if the beetles can detect the weedy fungi if lab studies just showed that they are not attracted (e.g. Hulcr et al. 2011). This requires the testing of weed fungi in combination with an attractive lure, which

would show if its attraction is reduced in the presence of a fungal weed. Here, we tested whether both life stages (larvae and adult beetles) of the fruit-tree pinhole borer, *X. saxesenii*, can detect the presence of two fungus-garden competitors (*Aspergillus* sp. and *Penicillium commune*) and one entomopathogen (*Beauveria bassiana*), if presented together with its *Raffaelea* fungal mutualists, which attraction to adult beetles has been shown previously (Hulcr et al. 2011). Repellence of beetles would be expected in all three cases.

Material/Methods

Beetle breeding

In January 2022, nests were established in tubes filled with artificial beech medium following the standard protocol (Biedermann et al, 2009). Previously sib-mated *X. saxesenii* females out of our laboratory stock (origin: EtOH-baited trap-collected dispersers from Steinbachtal/Wuerzburg, Bavaria, June-July 2019) were surface-sterilized with 70% EtOH, followed by washing with distilled water and drying on a paper towel. Females were afterwards individually introduced into the sterile rearing medium and kept under standard conditions in the climate chamber (25°C, 24h darkness, 60-70% humidity) to establish a new generation of nests.

After some weeks, adult beetles and larvae were extracted for the experimental tests in February and March 2022 (*N* = 22 nests). The beetles used in the experiment were intended to be in the same period of development (approx. 28 days after nest foundation). In this developmental phase adult individuals had not dispersed out of the nest yet, but instead are engaged in fungus farming and brood care in the maternal nest (Nuotclà et al. 2019). Therefore, it is expected that they would be sensitive for the presence of fungal pathogens.

Fungus Cultivation

Strains of the different mutualistic and antagonistic fungi used in this experiment were obtained from our laboratory's stock of the Chair of Forest Entomology and Protection (Univ. Freiburg, Germany), stored on agar slants within tubes at 5°C and previously grown on yeast extract malt agar (YEMA; Carl Roth®) with streptomycin. We cultivated the different fungi

under a sterile bench, by transferring a small sample of the fungi from the stock tubes with a sterilized scalpel onto the middle of a petri dishes with YEMA medium. Cultures were sealed with parafilm and incubated at 25°C.

Over time, several cultures of each *Dryadomyces sulphureus* (aka *Raffaelea sulphurea*; mutualist, GenBank Accession: MT880108.1), *R. canadensis* (mutualist, GenBank Accession: MT880109.1), *Penicillium commune* (antagonist, strain GenBank Accession: MT252032.1), *Aspergillus* sp. (antagonist, GenBank Accession: MT252035.1) and *Beauveria bassiana* (entomopathogen, GenBank Accession: MT159433.1) were weekly re-cultivated on new media to secure the permanent supply of fungal plugs for the experimental execution.

Two-choice experiment with *X. saxesenii* adults

We performed 210 two-choice experiment runs with modified petri dishes for adult ambrosia beetles. Here, the surface of the dishes was roughened using sandpaper to allow unhindered walking. Furthermore, we perforated the petri dish three times – in equal distance to the center and each other – with a Dremel 2001 (Dremel Deutschland) to fit 1.5 ml Eppendorf reaction tubes (Fig. 1A/B). These tubes were fixed with glue and served both as cavities for fungal test plugs and collection vials for the choosing beetles. Since this was a two-choice set-up, only two tubes were punctured with a small hole, to allow beetles to detect volatiles and access the fungi. The third tube was used only as a stand and for stability of the construction. Before each test run, modified petri dishes were washed in EtOH and distilled water and placed in the sterile bench under a UV lamp (254 nm wave length) for 30 min.

We measured repellence of adult females to volatiles of the fungal pathogens by providing the test tubes either with a sole 5 mm diameter agar plug (cork borer) of one of the mutualistic food fungi (*D. sulphureus* or *R. canadensis*) (= positive control) or a food fungus in combination with one of the other antagonistic or entomopathogenic fungi (*P. commune*, *Aspergillus* sp. or *B. bassiana*) (= repellence treatment). In total we tested six combinations with each 35 replications. Combinations applied were: i) *D. sulphureus* and *P. commune*, ii) *R. canadensis* and *P. commune*, iii) *D. sulphureus* and *Aspergillus* sp., iv) *R. canadensis* and *Aspergillus* sp. v) *D. sulphureus* and *B. bassiana*, vi) *R. canadensis* and *B. bassiana*. Test individuals (females) were directly extracted from nests and placed after a surface

sterilization in the center of the test arena. The choice-behaviour of focal beetles was observed for 30 min in 5-minute intervals and a final observation after 24 hours in our climate chamber under red light (switched on only during observations). Orientation of the arenas was changed regularly. Finally, we recorded, if beetles made a choice ('choice' by entering a tube or 'no choice') and if so, which side they chose ('food' or 'food-pathogen').

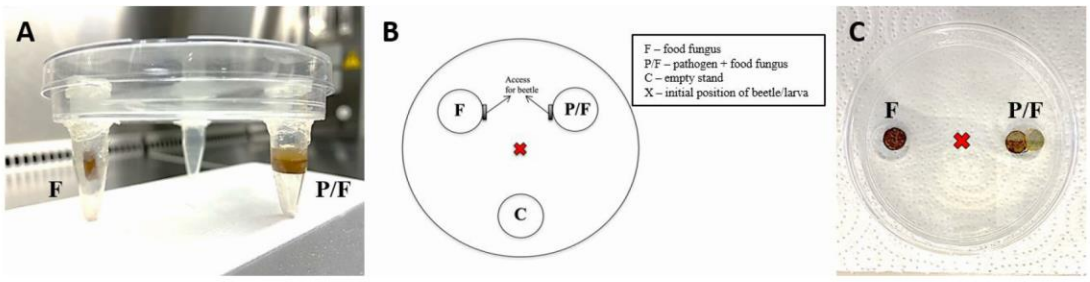

Figure 1: Experimental set-up of two-choice tests for *Xyleborinus saxesenii* females (A/B) and larvae (C). Petri dishes with a roughened surface were provided with three 1.5 ml Eppendorf tubes, two tubes included either one 5 mm plug of a food fungus or two plugs with each food fungus and pathogen to test female choice (A). Schematic representation of the experimental set-up for female choice tests (B). Petri dishes filled with solid agar-agar medium served as basis for the larval choice test. Here, two pits were punched out to place either one 5 mm plug of a food fungus or two plugs with each food fungus and pathogen (C).

Two-choice experiment on *X. saxesenii* larvae

Since larvae are slower moving and not protected by a chitinous carapace, they are more at risk of drying out during the experiment. Therefore, we used another set-up to test them (similar to Luna et al. 2014). Each arena was built of a petri dish with a bottom layer (1 cm) of solid agar-agar (14 g/800 ml, Carl Roth®) and two pits punched out with an eleven millimeter (diameter) cork borer on opposite sides. We provided each of these pits with either a plug (5 mm diameter) of the food fungus alone (= positive control) or a combination of food fungus and antagonist/entomopathogen (= repellence treatment; see above) (Fig. 1C). However, the secondary food fungus (*R. canadensis*) was not used in this series of tests, which resulted in only three combinations to be tested (*P. commune*, *Aspergillus* sp. and *B. bassiana*). We only used *D. sulphureus*, since larvae are more attracted to it, probably because it is their primary food source (Lehenberger; unpubl. data). Each combination was repeated 40 times with each a new petri dish (*N* = 120). To avoid arenas to dry out during tests, they were prepared three days ahead. We placed a single larva into the center of the plate after surface sterilization and recorded the position of it after 24 hours. If the larva was within a 1cm proximity to the pit,

this was recorded as its final choice. Again, we distinguished between 'choice' and 'no choice', as well as 'food' and 'food-pathogen' for the final analysis.

Statistics

All statistical analyses and visualizations were performed in RStudio (Version 1.4.1106) with R version 4.0.5 (R Core Team 2021) using the 'ggplot2' package (Wickham 2016) for the graphical output.

We ran a series of *chi-square* tests to examine if intended choices were made by the individuals per tested combination and life stage. To check if the sample size is good enough to perform appropriate tests, we looked at the expected values. Since all tested combinations had expected values the cells of the contingency table higher than 5, we were able to apply *chi-square* tests.

Results

The food fungi were attractive in all combinations because all tested life-stages showed a statistically significant decision for a choice, except larvae in the combination with *D. sulphureus* and *B. bassiana,* which was almost significant (p = 0.114; Tab. 1). Behavioural repellence was found in many, but not all combinations. First, there was neither repellence nor attraction found in the reaction of larvae towards *Aspergillus* sp. and in adult females, if *R. canadensis* was used as lure (Tab. 1, Fig. 2). Second and most surprisingly, we found attraction of *B. bassiana*, in both combinations when either presented with *D. sulphureus* or *R. canadensis*. By contrast, larvae are repelled by this entomopathogenous fungus.

Table 1: Outcome of two-choice tests between food fungus and food fungus with pathogen for adult females and larvae. Significant results state a preference for making a choice over staying undecided. Bold p-values denote attraction by the pathogen side. Attractivity of the lures itself is tested on the right (no choice *vs.* choice). Significant results state a preference for making a choice over staying undecided.

Life stage	Pathogen combination	Food – food & pathogen				No choice – choice			
		X^2	*df*	*p*	*N*	X^2	*df*	*p*	*N*
Adult females	*Beauveria – D. sulphureus*	4.5	1	**0.034**	32	24.03	1	< 0.001	35
	Beauveria – R. canadensis	5.14	1	**0.023**	28	12.6	1	< 0.001	35
	Aspergillus – D. sulphureus	4.8	1	0.028	30	17.86	1	< 0.001	35
	Aspergillus – R. canadensis	0.36	1	0.549	25	6.43	1	0.011	35
	Penicillium – D. sulphureus	24.5	1	< 0.001	32	24.03	1	< 0.001	35
	Penicillium – R. canadensis	4.24	1	0.040	34	31.11	1	< 0.001	35
Larvae	*Beauveria – D. sulphureus*	4.84	1	0.028	25	2.5	1	0.114	40
	Aspergillus – D. sulphureus	2.13	1	0.144	30	10	1	0.002	40
	Penicillium – D. sulphureus	6.53	1	0.011	30	10	1	0.002	40

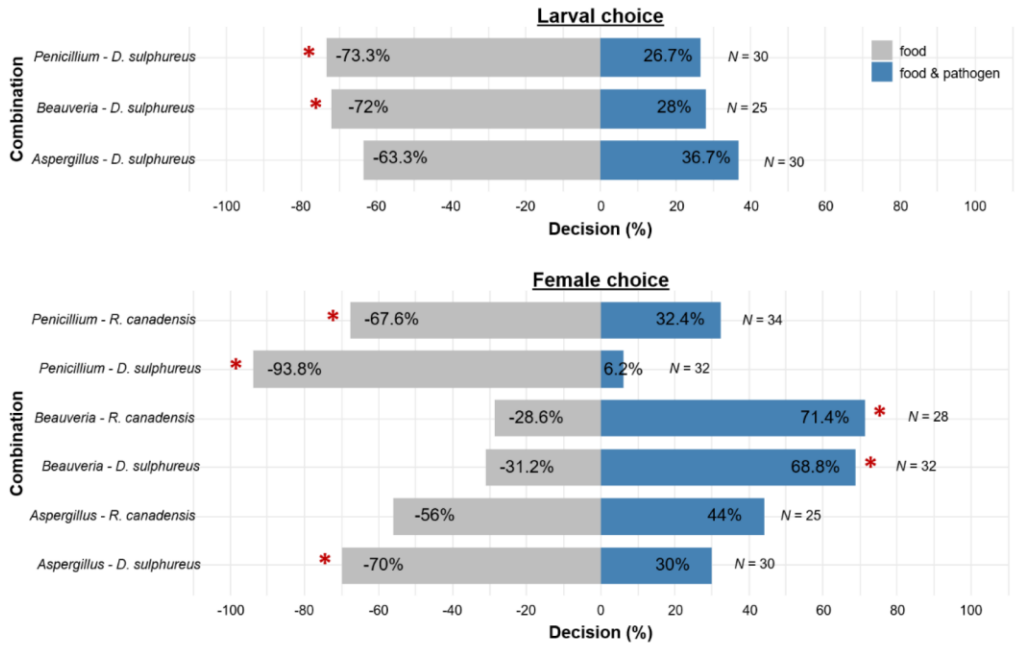

Figure 2: Decisions of larvae and adult females in the two-choice arenas. Percentage of larvae and adult females attracted to the positive control (= food fungus; left bars) or the repellence treatment (= food & pathogen; right bars) (*p < 0.05). N gives the total number of tested individuals. Non-deciding individuals were excluded here.

Discussion

Overall, we were able to show that adult beetles and larvae of *X. saxesenii* responded positively to volatiles from their symbiotic fungi and even varied in their response to pathogens depending on their life stage. Larvae seem to be aware of their physical weakness compared to adults, especially in confrontation with the entomopathogen *Beauveria*. They do the opposite than their adult sisters and avoid contact with the entomopathogen. We suggest that female preference for *Beauveria* is a hygienic and social response to a serious hazard when present in the nest community. By confronting the entomopathogen, they have the chance to fight it before causing far-reaching damage. Various behaviours to reduce the establishment of parasites and pathogens like disinfection, relocation of dead and infected nestmates or waste management have already been shown in social insects (Cremer et al. 2007) and some of them are also present in *X. saxesenii* (Biedermann & Taborsky 2011; Nuotclá et al. 2019) . Alternatively, it is possible that adult females are exploited by *Beauveria*, because it is a specialized entomopathogen that depends on infecting insects for survival. This is supported by the observation that volatiles of *B. bassiana* are apparently attractive also for other non-farming insects (Geedi et al. 2022 Nov 24).

Concerning the antagonistic fungi, both life stages were more attracted to the pure food fungus side, which can be interpreted in two ways. One would be a simple preference for a good nutritional food source, when having the choice, in particular when being away from the beetle community. This reaction might be different inside the nest. On the other hand, individuals could not see a severe hazard in the presence of the antagonists and both *P. commune* and *Aspergillus* sp. might be managed in other ways than active beetle treatment (e.g. passive application of antibiotic *Streptomyces griseus* (Grubbs et al. 2020)). Why adult individuals take no choice against *Aspergillus* when *R. canadensis* is used as a lure is unknown. It is possible that not volatiles of the pathogens themselves, but instead changed volatiles of the nutritional fungi when in presence with fungi are used as cues by the deciding beetles.

Future experiments may require a modified assay, which includes a more realistic in-nest situation with multiple conspecifics. Clearly, our results demonstrate a response of both larvae and adult ambrosia beetles towards other fungal volatiles. The decision to confront a potential threat or rather to avoid it may be based on more complex connections. Future studies should also investigate the volatiles of both fungal mutualists and pathogens and if

and how co-occurrence influence these volatiles. This will also help to disentangle the single volatile compounds that are responsible for the attractions and repellences found.

References

Biedermann PHW, Vega FE. 2020. Ecology and evolution of insect-fungus mutualisms. Annu Rev Entomol. 65:431–455. doi:10.1146/annurev-ento-011019-024910.

Cremer S, Armitage SAO, Schmid-Hempel P. 2007. Social immunity. Curr Biol CB. 17(16):R693-702. doi:10.1016/j.cub.2007.06.008. http://www.ncbi.nlm.nih.gov/pubmed/17714663.

Diehl JMC, Kowallik V, Keller A, Biedermann PHW. 2022. First experimental evidence for active farming in ambrosia beetles and strong heredity of garden microbiomes. Proc R Soc B Biol Sci. 289(1986). doi:10.1098/rspb.2022.1458. https://royalsocietypublishing.org/doi/10.1098/rspb.2022.1458.

Geedi R, Canas L, Reding ME, Ranger CM. 2022. Attraction of *Myzus persicae* (Hemiptera: Aphididae) to volatiles emitted from the entomopathogenic fungus *Beauveria bassiana*. Cory J, editor. Environ Entomol. doi:10.1093/EE/NVAC100. https://pubmed.ncbi.nlm.nih.gov/36421055/.

Goes AC, Barcoto MO, Kooij PW, Bueno OC, Rodrigues A. 2020. How do leaf-cutting ants recognize antagonistic microbes in their fungal crops? Front Ecol Evol. 8(May):1–12. doi:10.3389/fevo.2020.00095.

Griffiths HM, Hughes WOH. 2010. Hitchhiking and the removal of microbial contaminants by the leaf-cutting ant *Atta colombica*. Ecol Entomol. 35(4):529–537. doi:10.1111/J.1365-2311.2010.01212.X. https://onlinelibrary.wiley.com/doi/full/10.1111/j.1365-2311.2010.01212.x.

Grubbs KJ, Surup F, Biedermann PHW, McDonald BR, Klassen JL, Carlson CM, Clardy J, Currie CR. 2020. Cycloheximide-producing *Streptomyces* associated with *Xyleborinus saxesenii* and *Xyleborus affinis* fungus-farming ambrosia beetles. Front Microbiol. 11(September):1–12. doi:10.3389/fmicb.2020.562140.

253 Hanula JL, Mayfield AE, Fraedrich SW, Rabaglia RJ. 2008. Biology and host associations of
254 redbay ambrosia beetle (Coleoptera: Curculionidae: Scolytinae), Exotic vector of laurel wilt
255 killing redbay trees in the southeastern United States. J Econ Entomol. 101(4):1276–1286.
256 doi:10.1093/JEE/101.4.1276. https://academic.oup.com/jee/article/101/4/1276/2198983.

257 Harrington TC. 2005. Ecology and evolution of mycophagous bark beetles and their fungal
258 partners. In: Vega FE, Blackwell M, editors. Ecological and evolutionary advances in insect-
259 fungal associations. Oxford: Oxford University Press. p. 257–291.

260 Hulcr J, Mann R, Stelinski LL. 2011. The scent of a partner: Ambrosia beetles are attracted to
261 volatiles from their fungal symbionts. J Chem Ecol. 37(12):1374–1377. doi:10.1007/s10886-
262 011-0046-x.

263 Kandasamy D, Gershenzon J, Andersson MN, Hammerbacher A. 2019. Volatile organic
264 compounds influence the interaction of the Eurasian spruce bark beetle (*Ips typographus*)
265 with its fungal symbionts. ISME J. 13(7):1788–1800. doi:10.1038/s41396-019-0390-3.
266 http://dx.doi.org/10.1038/s41396-019-0390-3.

267 Kandasamy D, Gershenzon J, Hammerbacher A. 2016. Volatile organic compounds emitted
268 by fungal associates of conifer bark beetles and their potential in bark beetle control. J
269 Chem Ecol. 42(9):952–969. doi:10.1007/s10886-016-0768-x.
270 http://dx.doi.org/10.1007/s10886-016-0768-x.

271 Katariya L, Ramesh PB, Gopalappa T, Desireddy S, Bessière JM, Borges RM. 2017. Fungus-
272 farming termites selectively bury weedy fungi that smell different from crop fungi. J Chem
273 Ecol. 43(10):986–995. doi:10.1007/s10886-017-0902-4.

274 Kirkendall LR, Biedermann PHW, Jordal BH. 2015. Evolution and diversity of bark and
275 ambrosia beetles. In: Bark beetles: Biology and ecology of native and invasive species.
276 Elsevier Academic Press. p. 85–156. http://dx.doi.org/10.1016/B978-0-12-417156-5.00003-
277 4.

278 Kushiyev R, Tuncer C, Erper I, Ozdemir IO, Saruhan I. 2018. Efficacy of native
279 entomopathogenic fungus, *Isaria fumosorosea*, against bark and ambrosia beetles,
280 *Anisandrus dispar* Fabricius and *Xylosandrus germanus* Blandford (Coleoptera:
281 Curculionidae: Scolytinae). Egypt J Biol Pest Control. 28(1):55. doi:10.1186/s41938-018-

0062-z. https://ejbpc.springeropen.com/articles/10.1186/s41938-018-0062-z.

Luna E, Cranshaw W, Tisserat N. 2014. Attraction of walnut twig beetle *Pityophthorus juglandis* (Coleoptera: Curculionidae) to the fungus *Geosmithia morbida*. Plant Heal Prog. 15(3):135–140. doi:10.1094/php-rs-14-0001.

Mayer VE, Nepel M, Blatrix R, Oberhauser FB, Fiedler K, Schönenberger J, Voglmayr H. 2018. Transmission of fungal partners to incipient *Cecropia*-tree ant colonies. Borges RM, editor. PLoS One. 13(2):e0192207. doi:10.1371/journal.pone.0192207. https://dx.plos.org/10.1371/journal.pone.0192207.

Mueller UG, Gerardo NM, Aanen DK, Six DL, Schultz TR. 2005. The evolution of agriculture in insects. Annu Rev Ecol Evol Syst. 36(1):563–595. doi:10.1146/annurev.ecolsys.36.102003.152626. http://www.annualreviews.org/doi/10.1146/annurev.ecolsys.36.102003.152626.

Nuotclà JA, Biedermann PHW, Taborsky M. 2019. Pathogen defence is a potential driver of social evolution in ambrosia beetles. Proc R Soc B Biol Sci. 286(20192332):1–9. doi:10.1098/rspb.2019.2332. https://royalsocietypublishing.org/doi/abs/10.1098/rspb.2019.2332.

R Core Team. 2021. R: A language and environment for statistical computing.

Rosengaus RB, Jordan C, Lefebvre ML, Traniello JFA. 1999. Pathogen alarm behavior in a termite: A new form of communication in social insects. Naturwissenschaften 1999 8611. 86(11):544–548. doi:10.1007/S001140050672. https://link.springer.com/article/10.1007/s001140050672.

Schultz T, Gawne R, Peregrine P. 2022. The convergent evolution of agriculture in humans and fungus-farming ants. MIT Press. https://books.google.com/books?hl=de&lr=&id=aiMuEAAAQBAJ&oi=fnd&pg=PR9&dq=Schu

Schultz TR. 2022. The convergent evolution of agriculture in humans and fungus-farming ants. In: The Convergent Evolution of Agriculture in Humans and Insects. 1st Editio. p. 281–313.

Six DL, Klepzig KD. 2021. Context dependency in bark beetle-fungus mutualisms revisited:

310 Assessing potential shifts in interaction outcomes against varied genetic, ecological, and
311 evolutionary backgrounds. Front Microbiol. 12:1161.
312 doi:10.3389/FMICB.2021.682187/BIBTEX.

313 Wickham H. 2016. ggplot2: Elegant graphics for data analysis. New York: Springer-Verlag
314 New York.

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CHAPTER 3

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Working title: Ambrosia beetle response to pathogen pressure – A glance at life-history, behaviour and symbiont community over multiple generations

Ambrosia beetle response to pathogen pressure – A glance at life-history, behaviour and symbiont community over multiple generations

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Abstract

Social immunity is a collective behaviour that protects social groups from disease and parasites. Ambrosia beetles are cooperative breeders that exhibit social behaviours, in both adult and larval stages, too. Here, female beetles delay dispersal and act as temporary workers, engaging in brood care and social hygiene, which are key pathogen-induced behaviours that drive social evolution. So far, there has been limited research into the specific ways in which ambrosia beetles respond to pathogens. Our study sought to enhance our understanding of these beetles and uncover the mechanisms behind their defence against antagonistic and entomopathogenic fungal pathogens in the context of social evolution. We accomplished this by integrating data on behaviour, development, and the associated microbial community. Results showed that larvae invest a high percentage of their activity into hygienic behaviours and feeding, while adult females tend to rest more. Overall, beetles' lineages recovered from the pathogen disturbance within one generation, and the emergence of first females occurred earlier with each generation regardless of the treatment applied. In addition to the bacterial taxa that were previously identified in other studies, we observed a greater prevalence of Alphaproteobacteria. Their function and significance in relation to ambrosia beetles are yet to be determined. The study suggests that the response of ambrosia beetles to pathogens is a complex process that involves multiple factors, including life-history traits, behavioural adaptations, and microbial

defences. However, further comprehensive investigation is required to understand the interactions and microbes involved.

Introduction

Group living, a basic component of sociality, can be found in almost all animal taxa. Social complexity can range from timely limited attraction between individuals in family units, to permanent societies with reproductive division of labour (Wilson 1971; Costa 2006). Group living species thereby profit from fitness benefits like protection, increased reproductive success, foraging efficiency and increased survival (Krause and Ruxton 2002). However, frequent intimate contact can also have fitness disadvantages. It may, for instance, increase the risk of parasite and pathogen transmission (Cote and Poulin 1995; Schmid-Hempel 1998; Wilson et al. 2003; Cremer et al. 2007; Rifkin et al. 2012; Patterson and Ruckstuhl 2013). Further, genetic similarity is usually high in groups, which can render individuals more susceptible to parasite and pathogen outbreaks (Pie et al. 2004; Otterstatter and Thomson 2007; Stroeymeyt et al. 2014).

Social immunity, which involves nest members' behavioural defence against disease (Cremer et al. 2007; Cremer et al. 2018), may have evolved in two ways: either as result of increased pathogen transmission in eusocial organisms (the 'eusocial framework'; Cremer et al. 2007; Cremer et al. 2018), or in coevolution with sociality in close feedback interactions (the 'group living framework'; Meunier 2015; Biedermann and Rohlfs 2017; Van Meyel et al. 2018). Ants and termites have been extensively studied in the context of social immunity, unlike other groups. For example, termites exhibit "pathogen alarm" behaviour, warning their nest-mates to avoid or seal off infected areas of the nest when they encounter pathogens (Diehl-Fleig and Lucchese 1991; Rosengaus et al. 1999; Myles 2002). Similarly, ants and bees fortify their nests with antimicrobial substances such as tree resin, thereby reducing the likelihood of infection among colony members (Gilliam et al. 1988; Christe et al. 2003; Chapuisat et al. 2007). In addition, they have the ability to produce their own antimicrobial substances (William L. Brown 1968; Turillazzi et al. 2006) and

cultivate bacteria on their bodies to aid in the production of antibiotics against parasites within the colonies (Currie et al. 1999). The evolution of social immunity is beneficial to the colony as a whole, even if it may not benefit certain individuals.

Ambrosia beetles (Scolytinae and Platypodinae) are among those animals that live in groups and exhibit social behaviours in various extends. Hence, pathogen pressure in these nests, where group members are closely related, is anticipated to be an important driver of sociality (Biedermann and Rohlfs 2017). As cooperative breeders, they provide a suitable model system for the study of the evolution of social life. Depending on various factors such as presence of dependent offspring, nutritional levels, and pathogen load, female ambrosia beetles may delay their dispersal and act as temporary workers, (Peer and Taborsky 2007; Biedermann and Taborsky 2011; Nuotclà et al. 2019). Furthermore, female ambrosia beetles are totipotent and can flexibly engage in dispersal, reproduction, and helping behaviours (Biedermann et al. 2011). This flexibility also makes them ideal for the experimental exploration of microbial communities and their functional importance for habitat properties (e.g. Costa 2006). Brood care, social hygiene, and dispersal delay are key pathogen-induced behaviours in these beetles, indicating their important role in driving social evolution in this group (Biedermann and Rohlfs 2017; Nuotclà et al. 2019).

Multiple processes are known to reduce the range of infection in eusocial species. Strategies such as building fragmented nests with multiple chambers (Pie et al. 2004), abandoning contaminated areas (Royce et al. 1991), boosting personal immunity by social interactions (Konrad et al. 2012) and removing infected group members (Rosengaus and Traniello 2001) may reduce the risk of parasite and pathogen infection. In addition, behavioural measures such as grooming, weeding or application of chemicals may be situationally applied (Currie and Stuart 2001; Pedrini et al. 2015; Grubbs et al. 2020). Many of these measures require neurophysiological abilities that enable the perception of such microbial threats (Yanagawa et al. 2012; Diehl et al. 2022). Similar to eusocial Hymenoptera, ambrosia beetles like the fruit-tree pinhole borer *Xyleborinus saxesenii* Ratzeburg (Nuotclà et al. 2019) exhibit comparable social immune

responses. The primary reaction in this case is expected to be hygienic behaviours, as suggested by Biedermann and Taborsky (2011) and Meunier (2015). Interestingly, beetle larvae in these nests also contribute to nest hygiene and waste management (Biedermann and Taborsky 2011). These collective behaviours can enhance the effectiveness of individual strategies aimed at suppressing harmful microbes, which, in turn, can lead to selection for individuals who are more inclined to remain and assist the community (Biedermann and Rohlfs 2017). In summary, social interactions play a vital role in the establishment of high-quality microbial environments for group-living insects, such as ambrosia beetles. These social behaviours can lead to positive evolutionary feedback processes that enhance the beetle's fitness and further strengthen the mutualism between the insects and microbes. As a result, the collective efforts of these insects can have a significant impact on the composition and quality of the microbial communities they rely on (Biedermann and Rohlfs 2017).

To protect themselves and their food source, ambrosia beetles must take precautions due to the inherent risks they face. Collaborative behaviours, such as cleaning, waste disposal, mutual grooming, weeding the fungus gardens, and even resorting to cannibalism or eliminating weak or diseased individuals, are observed in both larvae and adults (Biedermann and Taborsky 2011; Nuotclà et al. 2019). Moreover, chemical and microbial defence methods are available to them through oral secretions or associated bacteria (Cardoza et al. 2006; Scott et al. 2008; Grubbs et al. 2020). The bacterial defence mechanisms used by *X. saxesenii* and *Xyleborus affinis* are highly specific to fungal pathogens and do not harm the cultivar (Grubbs et al. 2020).

The strongest evidence for a positive feedback between sociality and fungus-farming, is that in some species, ambrosia fungus fruiting is only induced in the physical presence of the beetles (larvae, pupae or adults; Batra and Michie 1963; French and Roeper 1972; Biedermann 2014; Biedermann and Currie in prep.). Apparently, the resulting fruiting structures serve as the dominant food for the whole colony. Conversely, the absence of the insects leads to galleries that are quickly overgrown by contaminants (Batra 1966). The cultivated fungi, the mechanisms through which beetles establish monocultures, and other fungi

and bacteria that comprise the beetle's microbiome, all remain poorly understood (Hulcr and Stelinski 2017; Biedermann and Vega 2020). In *X. saxesenii*, recent studies could demonstrate that the presence of individuals and their hygienic behaviours are important to promote the nutritional ambrosia fungi, *Raffaelea sulphurea* and *R. canadensis*, remove fungal pathogens (e.g. *Aspergillus* sp.) (Biedermann and Taborsky 2011; Nuotclà et al. 2019; Diehl, Kowallik, et al. 2022). Laboratory breeding resulted in garden microbiomes composed of only a few highly abundant taxa, which are expected to build the core microbiome of essential microbes for the insect-fungus interactions (Diehl et al. under review; Diehl, Kowallik, et al. 2022). Modern methods such as next generation sequencing can provide information on the relative proportions of the core taxa in the communities and can help to identify key players in the mutualism. Knowledge on the microbial succession in field and laboratory fungus gardens is already gained and exposed a step-wise transition of fungal mutualists, very different from the ones of farming ants and termites (Diehl et al. under review). While gardens of ants and termites only hold a single dominating cultivar, more and more studies in facultative eusocial ambrosia beetles indicate a succession of different fungi (or yeasts) serving as food source (Diehl et al. under review; Ibarra-Juarez et al. 2020; Diehl, Kowallik, et al. 2022).

The primary threat to the beetles is likely to be from antagonistic pathogens such as *Aspergillus* sp., *Penicillium* sp. or *Chaetomium* sp. (Nuotclà et al. 2021), despite their otherwise well-protected environment. However, there is another type of microbial threat that targets the beetles directly. Entomopathogenic fungi, such as *Beauveria bassiana* Balsamo Vuillemin (Ascomycota: Hypocreales) and *Metarhizium brunneum* Petch (Ascomycota: Hypocreales), attack the insect directly and are known to be highly lethal. They can be found naturally in nests and can be isolated from beetles. Researchers explore their use as a potential biocontrol against economically harmful species. Experiments by Prazak (1991; 1997) have shown that *Trypodendron lineatum* Olivier is susceptible to *B. bassiana* and the number of broods in treated trees was reduced. Both strains of commercial *B. bassiana* and *M. brunneum*, were shown to be virulent against two other ambrosia beetles, *Xylesandrus germanus* Blandford and *X. crassiusculus* Motschulsky, and here, too, the pathogens have a significant impact on the development of the brood (Castrillo

et al. 2011; Castrillo et al. 2013). The mortality and brood success depend on the concentration applied (Castrillo et al. 2011; Castrillo et al. 2013).

Our research aimed to expand the current knowledge of ambrosia beetles and to identify the mechanisms that underly pathogen defence in the context of social evolution. To achieve this, we combined behavioural, developmental and microbial data. The focus of our experiment was on the behaviour and resistance of the ambrosia beetle *X. saxesenii* against two pathogens (*Aspergillus* sp. and *B. bassiana*), with a particular emphasis on social aspects such as nest hygiene and dispersal, as well as the symbiont community. We hypothesised that exposure to pathogens would lead to changes in beetle behaviour, with increased social activity and a stronger response to the more dangerous, *Beauveria*, compared to *Aspergillus*. As *Aspergillus* is a common garden pathogen, we expected that beetles would have some basic resistance to it, resulting in a lower mortality rate in nests exposed to this pathogen. Moreover, a positive correlation was anticipated between social behaviours and nest productivity. The aim was to investigate the microbiome in order to identify symbionts that play a critical role in pathogen defence. If the predictions were confirmed, it was expected that behavioural adaptations and beneficial symbionts would be passed on to future generations in response to permanent pathogen pressure. Additionally, genetic factors could influence pathogen resistance, with beneficial traits potentially inherited from the mother. By continuously exposing beetles to pathogens in every generation, we expected that offspring from parents previously exposed to the same pathogen treatment would have better adaptations, including a higher degree of sociality and lower susceptibility to pathogen-related mortality.

Material and Methods

Beetle collection and breeding

We collected two populations of *Xyleborinus saxesenii* for this experiment to avoid population specific traits and detect more general patterns. The first population from Steinbachtal near Wuerzburg, Germany (Decimal degrees

[DD]: 49.767500, 9.896770) originates from dispersing females by using ethanol baited traps (70% EtOH) in June of 2019. Another population from the Bavarian Forest (DD: 8.8816832, 13.5215362) was introduced into the laboratory breeding at the end of May 2019 from females collected in opened breeding systems from dead and wind-thrown beech tree logs (*Fagus sylvatica*) of the forest. All generations of these two populations were bred under standard breeding conditions in transparent plastic tubes using sterile artificial beech sawdust medium ('standard media' after Biedermann et al. 2009, using Millipore water to avoid Chloroplast 16S amplification from tap). Mated adult females were individually introduced into the tubes after rinsing them briefly with 70% EtOH followed by tap water and letting them dry on a cosmetic paper towel.

Naturally these beetles overwinter in diapause. To simulate this, we put the rearing tubes into the fridge for two to three months at 5°C after the observations concluded but before beetles started dispersing to found the F2 nests.

Spore solution preparation

Beauveria bassiana (GenBank Accession: MT159433.1) and *Aspergillus* sp. (strain F10006) were cultivated on Yeast-Extract-Malt agar medium (YEMA) for 11 days before spore solutions were made. To avoid clumping of the spores we transferred them into a 0.05% Tween 20/PBS (1x) buffer solution. Spores were collected from the agar plates by pouring sterile Tween 20/PBS solution on the air mycelium with spores and using a sterile glass Pasteur pipette to transfer the spores with the solution into a 50 ml falcon tube. Thereby the mycelium remained on the agar. Spore concentration was counted on an Improved Neubauer counting chamber and had a final concentration of 11.5 x 106 spores/ml for *B. bassiana* and 17.35 x 106 spores/ml for *Aspergillus* sp..

To secure pathogenicity of our used *Beauveria* strain towards *X. saxesenii*, we infected multiple individuals and let the fungus kill them and grow out, afterwards we collected the spores from the beetles and started new fungus cultures on YEMA agar. Dispersed females were surface sterilised by dipping into 70% Ethanol rinsing with tap water and letting them dry on cosmetic towel. Then we dipped the beetles under a gas cloud of the Bunsen burner into the

mycelium of *B. bassiana*, cultivated on YEMA agar medium, and kept them in a small petri dish with moist filter paper and sealed with parafilm till they showed fungal growth on the surface. These spores were rinsed of the individuals and used for the production of the final spore solution.

In a small side study, we tested the general lethality of the two used fungi with three different concentration (10^5, 10^6 and 10^7 spores/ml) to get a better estimate of the results (see SM, Tab. S11). Both active life stages in *X. saxesenii* (larvae and adult females) were included and thus gave us insightful information. Both stages died relatively quickly after infection with *B. bassiana* of every concentration (SM Tab. S11; Fig. S6 & S7), where the mortal danger for adults significantly increased the higher the spore concentration. There was a marginal higher risk for larvae with the 10^5 spores/ml concentration compared to the control (Log Rank Test: $p = 0.064$), which got significant for the higher concentrations (SM Tab. S11). Furthermore, we found a clear differentiation between larvae and adults with *Aspergillus* sp.. The highest concentration with 10^7 spores/ml showed a significant higher risk of death for adults (Log Rank Test: $p = 0.017$; SM Fig. S5), but not for the larvae (SM Tab. S11; Fig. S4). This result could be due to the artificial set-up in 96-well plates. We expect a longer survival time in nests, where larvae are not exposed to the additional risk of drying out.

Since in all treated nests Alphaproteobacteria were sequenced in a higher relative abundance, we wanted to exclude the possibility our Tween 20 solution might contain some unexpected bacteria. Therefore, we plated 200 µl of the stock solution on two Lysogeny broth (LB) agar plates and incubated them at 25°C for six days. No bacterial or fungal growth was detected.

Experimental set-up

For the natural variation of ambrosia beetle defensive behaviours and fungus garden communities we started with *X. saxesenii* nests (F0) from each of the two different field populations ($N = 66$, Tab. 1). This generation was treated as baseline for the following ones. Therefore, we recorded developmental times, total count of dispersed individuals and larval and female behaviours. From the first laboratory reared generation females of each population were selected as

foundresses for the next generation (starting day 39 ± 4 days after founding; min. 11 nests/population, Tab. 1), as well as one individual of each line for later amplicon sequencing of the microbiome (stored at -20°C until processing; Fig. 2 & 3). Following the last disperser, nests were dissected by knocking out the solid rearing medium and collecting additionally pieces of the nest walls from the nest centre under the sterile bench with a flame-sterilised sharp spoon. Fungus garden samples were aseptically stored in 1.5 ml Eppendorf tubes at -20°C until DNA extraction.

Same recording was done for nests in the F1 generation. When the first newly emerged females were detected (day 29 ± 3), nests were exposed to one of three treatments (Tab. 1): (i) 20 µl of an *Aspergillus*-buffer solution (1.7 x 10^7 spores/ml 0.05% Tween 20/PBS buffer; '*garden pathogen*'), (ii) 20 µl of a *Beauveria*-buffer solution (1.2 x 10^7 spores/ml 0.05% Tween 20/PBS buffer; '*entomopathogen*') or (iii) 20 µl of a 0.05% Tween 20/PBS buffer solution ('*control*') were injected with an insulin syringe with sterile interior (BD Micro-Fine™ + Demi, 0.3 ml U100, 0.3 x 8 mm) directly into the brood chamber and we sealed the hole with a hot glue gun (e.g. Nuotclà et al. 2019). After injection, larval and adult social behaviours were recorded (~d30). Like in the previous generation samples of fungus gardens and dispersing females were collected from the nests and stored at -20°C. At that point nest productivity (total offspring numbers) will be determined, when all offspring has emerged and the timing of first dispersal was noted.

Female offspring from the most productive nests per treatment of the two populations was selected as foundresses of the next generation (Tab. 1). We repeated the same treatment, observation and sampling for generations F2-F3. By breeding only with the most productive nests, we ensured that in each generation selected females which should be the ones with the best defence against the pathogens.

Table 1: Replicate number of the different datasets per population, generation and treatment that were analysed. Breeding data included total disperser count of nests and developmental times, observed data are the number of nests that were observed after first emerged female was detected (and treatment was applied for F1 generation onwards) and sequenced data includes the number of nests where DNA of gallery walls and dispersers (F0 and F3 generation) was extracted and amplicon sequenced.

Population + dataset		F0	F1			F2			F3		
			control	garden pathogen	entomo-pathogen	control	garden pathogen	entomo-pathogen	control	garden pathogen	entomo-pathogen
Steinbachtal	breeding	38	66	67	67	56	48	18	63	46	39
			16 lineages			13 lineages			13 lineages		
	observed	44	45	44	44	37	33	5	38	30	7
			15 lineages			9 lineages			7 lineages		
	sequenced	10	8	2	3	10	10	5	10	10	5
			8 lineages			9 lineages			9 lineages		
Bavarian Forest	breeding	15	65	63	65	74	40	7	84	58	14
			12 lineages			11 lineages			11 lineages		
	observed	22	39	33	37	55	33	10	40	41	27
			11 lineages			7 lineages			6 lineages		
	sequenced	7	11	8	4	11	11	9	10	11	8
			9 lineages			9 lineages			9 lineages		

Behavioural observations

After 30 days (± 3 days), when the first adult females emerged, larval and adult social behaviours were recorded with scan sampling for next five days. Data of the F0 generation was used to set the baseline behaviour for the different treatment lines in F1-3 generations. We recorded behaviour of a maximum of 10 individuals per life stage (larva or female) three times a day (Tab. 2). The recording of behaviour in treated nests, started one day after the injection of the treatment.

Table 2: Ethogram of observed behaviours.

Behaviour	Definition	Observed in
feeding	to feed (or weed) on the fungal layer of the gallery walls with the tasters and/or mandibles	L, F
allo-grooming	to groom on eggs, larvae, pupae or adult beetles with the mouth(L) or tasters (maxilla, labium)	L, F
shuffling/balling	to move frass and faeces with the mouth or whole body (L; legs and elytra (F))	L, F
cleaning	To groom or feed on the glass of the tube with tasters and mandibles	L, F
cannibalising	to eat an egg, larva, pupa or an adult beetle	L, F
resting	to be inactive without moving	L, F
walking	to creep (L) or walk (F)	L, F
copulating	to copulate with a male	F
hygienic behaviour	combination of allo-grooming, shuffling, cleaning, cannibalism	L, F

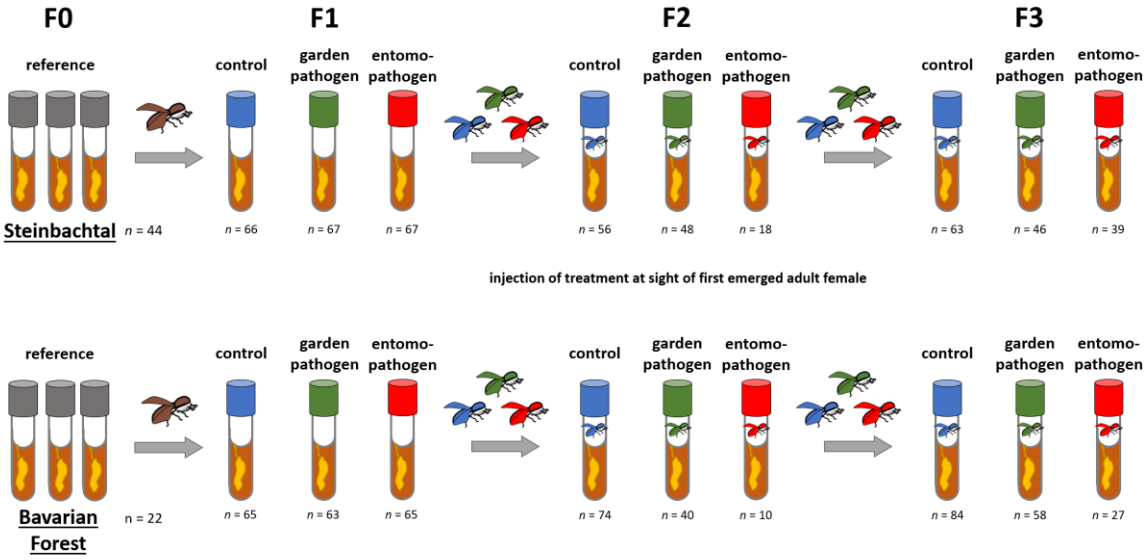

Figure 1: Experimental set-up of the selection line experiment. Two field populations ('*F0*') were bred for one generation in the laboratory. Dispersing females of at least eleven nests were used for three treatment groups: (i) '*control*' (blue), (ii) '*garden pathogen*' (green) and (iii) '*entomopathogen*' (red). At around day 30 after nest foundation treatment-injections were applied. Afterwards behaviour, dispersal, development and productivity were recorded. In the following two generations, females stayed in their treatment groups and the whole procedure was repeated. Additionally, samples of dispersing females ('*F0*' and '*F3*'), as well as fungus garden samples ('*F0-3*') were collected and stored at -20°C till DNA extraction.

Microbiome analysis

Since only behavioural adaptations, but not the participation of bacterial or fungal symbionts have been observed in this system, we wanted to make use of the fungus garden and beetle samples of the whole experiment to check for possible step-wise changes in the communities between the family lineages which could give a hint for presence of defensive symbionts. Therefore, we sequenced the bacterial and fungal communities across generations by Illumnia MiSeq amplicon sequencing (e.g. Nuotclà et al. 2021; Diehl, Kowallik, et al. 2022). We extracted DNA of all samples using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Germany) in accordance with the manufacturer's instructions and additional pre-processing steps using a ceramic bead and mixer mill (Retsch MM400), followed by vortexing with 0.1 mm & 0.5 mm glass beads on a Vortex Genie 2 (e.g. Supporting Material Nuotclà et al. 2021). Isolated DNA samples were stored at -20°C until final amplification and sequencing.

PCRs and library preparation were performed in the same way as described in Nuotclà et al. (2021) and Diehl et al. (2022). Therefore, we used the dual-indexing strategy with 515f and 806r primers (Caporaso et al. 2011; Kozich et al. 2013) to construct our bacterial 16S rRNA gene libraries (encompassing the full V4 region). Fungal LSU (28S) rRNA gene libraries were constructed with the dual-index primers of LIC15R and nu-LSU-355-3' (Nuotclà et al. 2021) amplifying the large subunit (LSU) region. Sequencing was performed on an Illumina MiSeq using 2 × 250 cycles v2 chemistry (Illumina Inc., San Diego, CA, USA). Each marker was processed on a separate chip.

Bioinformatics and statistics

All statistical analyses and visualisation of the data were performed in RStudio (Version 1.4.1106) with R version 4.0.5 (R Core Team 2021) using the 'phyloseq' package: McMurdie and Holmes 2013) for the microbial data, as well as 'ggplot2' (Wickham 2016) and 'ggpubr' (Kassambara 2020) to produce graphical output; see GitHub repository for information on the bioinformatic processing and R-script).

Since we analysed breeding, behavioural and microbiome data in this project, four sets with different replicate numbers were used (Tab. 1).

a) Breeding/Life-history dataset

During the breeding of *X. saxesenii* nests we were able to record the (i) total disperser count, the (ii) developmental time till first emerging female (days), the (iii) period of development between first detected larvae and first emerged female (days) and the (iv) period between first emerged female and first dispersing female (days). We ran linear mixed models (LMMs) using the 'lme' function ('nlme' package: Pinheiro et al. 2021) and used the 'transformTukey' function ('rcompanion' package: Mangiafico 2021) or the 'sqrt' function (base R) to find the power transformation that brings the response variable closest to a normal distribution. Each of the above-mentioned variables were applied as response variable to test for the influence of treatment ('*reference*', '*control*', '*garden pathogen*' and '*entomopathogen*') in the data of the 'F0' and 'F3' generation and in a second model including generation ('*F1', 'F2*' & '*F3*'; F0 excluded), as well. Due to previous analyses (Diehl, Kowallik, et al. 2022) we demonstrated a strong effect of the familiar lineage on the microbial community in *X. saxesenii*, therefore we wanted to consider this variance for other life-traits in our models, too, by including this information as random variable.

All LMMs were initially fitted with all interaction terms. Each model was then selected in a two-step procedure. First, we used the Akaike information criterion (AIC) to select an appropriate variance structure (using the weights-argument in the 'lme' function) where residual plots indicated a deviation from homogeneity (Zuur et al. 2009). Second, we simplified the fixed component by dropping non-significant interaction terms ($p > 0.05$). In a last step we used the AIC to choose, if necessary, the appropriate transformation method to produce a more normally distributed vector (using squared- or tukey-transformed response variable with the 'transformTukey' function of the 'rcompanion' package: Mangiafico 2021).

We obtained the p-values of effects in these models using the ANOVA function (which uses type II sums of squares by default; Fox and Weisberg 2019). Significant models were further analysed with a pairwise post-hoc test (tukey method; 'emmeans' package: Lenth 2021).

b) Behavioural observation data

Recorded behaviours of larvae and females over the five consecutive days were aggregated to a total for each life stage per observed nest. For the behavioural analysis we focused on three types of behaviours. As an expected response to pathogen exposure, we were especially interested in the '*hygienic behaviours*' (cleaning, allo-grooming, shuffling and cannibalism) and pooled them therefore. Further, '*resting*' and '*feeding*' were tested for effects of treatment and generation.

Here, we used a series of generalized linear mixed-effects models (GLMMs) with binomial error distribution. To examine the effects of repeated injection treatments on the different behaviours of larvae or females, the frequency of the respective behaviour was set as response variable for subsets of each treatment (including the data of none treated nests = '*reference*') and the '*generation*' as explanatory variable. To control for potential influences, we included '*population*', '*family lineage*' and '*nest ID*' as nested random variables and used the Akaike information criterion (AIC) and the 'performance' package (Lüdecke et al. 2021) to select the appropriate random variables for the model.

Again, we obtained the p-values of effects in these models using the ANOVA function (Fox and Weisberg 2019) and further analysed significant models with a pairwise post-hoc test (tukey method; 'emmeans' package: Lenth 2021).

Further, we combined data of hygienic behaviours of larvae and females and the total disperser count to test for a correlation. For that, we applied the Spearman's rank correlation test for the life stages on each the '*control*', '*garden pathogen*' and '*entomopathogen*' treatment between the two variables.

c) Bacterial and fungal community data

After filtering chloroplast genes and amplicon sequence variants that were only identified to domain level, we ran a contaminant removal method ('decontam' package: Davis et al. 2018) and visualised the taxonomic composition of the negative and Mock control samples, which were then excluded from the sample set, as well as samples with a read number less than 500 (see electronic supplementary material and GitHub repository). For the final analysis, 71 disperser samples with an average of 4 023.99 reads for 16S sequences (min. 501 reads; max. 28 577 reads) and 954 ASVs (amplicon

sequence variants, Edgar 2018) were included. Fungus gardens captured a total of 195 samples with an average of 21 334.72 reads (min. 879 reads; max. 56 311 reads) and 324 ASVs. For the LSU, 93 disperser samples with an average of 17 288.17 reads (min. 616 reads; max. 49 414 reads) and 448 ASVs were included in the analyses. The fungus gardens here included 176 samples with an average of 15 899.68 reads (min. 745 reads; max. 45 971 reads) and 574 ASVs. Microbial composition of bacteria and fungi was studied up to genus level.

Taxa composition bar plots (agglomerated to '*genus*' level) of fungal and bacterial communities for generations faceted by treatment were built of proportion transformed data for visualization of fungus garden communities. Since we sequenced only the dispersers of the F0 and F3 generation, bars were grouped into the treatments to detect changes.

Furthermore, we created tables of the mean and standard deviation of bacterial and fungal relative abundances of taxa per treatment and generation, each dispersers and fungus gardens.

Results

a) Breeding/Life-history

Treatment showed a significant effect on the number of dispersing females per nest (LMM: χ^2 = 263.39, *df* = 3, *p* < 0.001). In all treatments ('*control*', '*garden pathogen*' and '*entomopathogen*') disperser counts were lower than in the reference nests (EMM: all contrasts p < 0.001, SM Tab. S6). After excluding the 'F0' generation before the treatment, number of dispersers significantly increased from the first to the second/third generation in all treatments (Fig. 3; SM Tab. S7). In control nests the disperser number increased even further in the third generation (Fig. 3; SM Tab. S7).

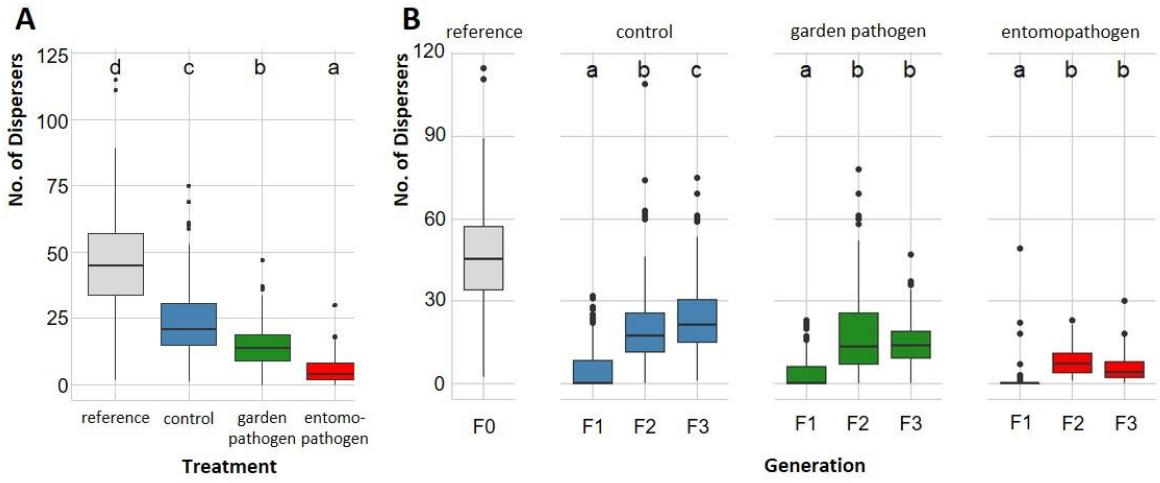

Figure 2: Number of adult female dispersers in relation to treatment and consecutive generations. (A) Comparison of treatments of disperser numbers over all generations. (B) Separate comparison of consecutive generations within the treatments. Boxplots with median, two hinges, two whiskers and all "outlying" points are visualised; different letters above boxplots denote significant differences (p < 0.05; Tukey's test).

The timing of the first female emergence got earlier every consecutive generation (LMM: χ^2 = 91.53, *df* = 2, *p* < 0.001; Fig. 4; SM Tab. S7), but this effect was not affected by treatment (SM Tab. S6). Further, we could not find a on philopatry (i.e. the period between the first hatched adult female until the first dispersal) (LMM: χ^2 = 6.05, *df* = 3, *p* = 0.109; SM Tab. S6), though the inclusion of generations exposed an interaction (LMM: χ^2 = 7.28, *df* = 2, *p* = 0.026; SM

Tab. S7) in nests treated with the entomopathogen. Females of the 'F3' generation in these nests stayed on average 1-2 days longer inside (EMM: *df* = 479, *t* = -2.9, *p* = 0.004).

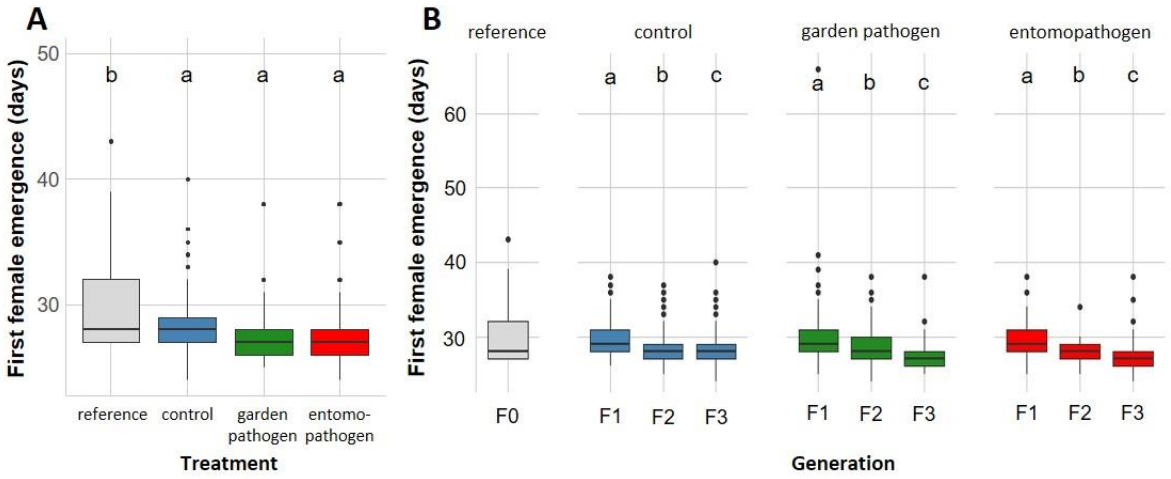

Figure 3: Timing of first adult female emergence in relation to treatment and consecutive generations. (A) Comparison of treatments for development time to first emerged female across all generations. (B) Separate comparison of consecutive generations within the treatments. Boxplots with median, two hinges, two whiskers and all "outlying" points are visualised; different letters above boxplots denote significant differences ($p < 0.05$; Tukey's test).

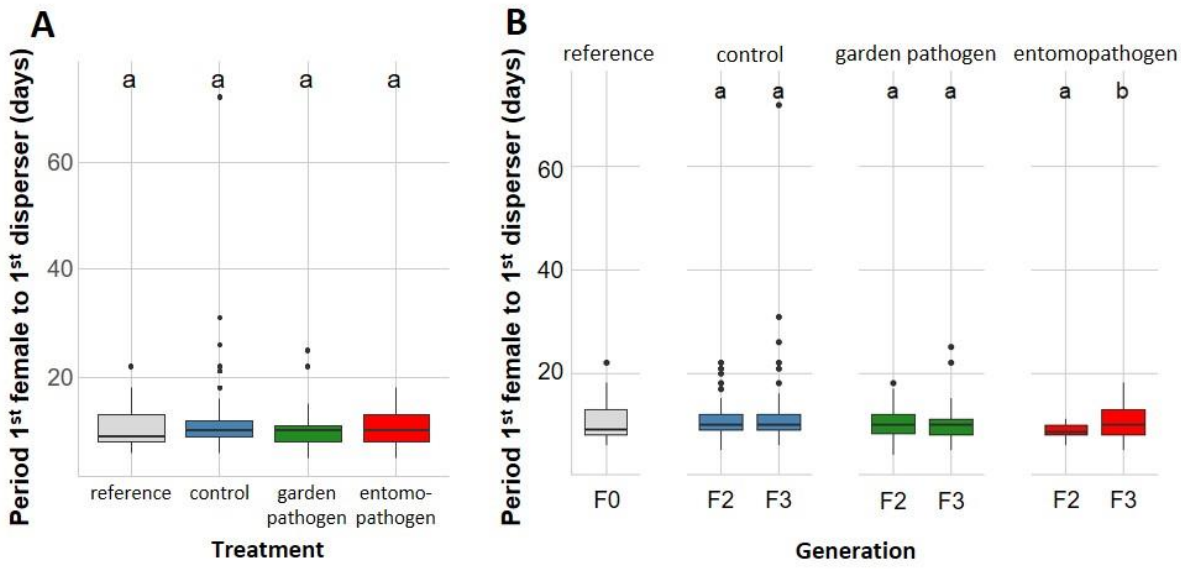

Figure 4: Philopatry of adult females in relation to treatment and consecutive generations. (A) Comparison of treatments for period from first emerged female to first dispersing female over all generations. (B) Separate comparison of consecutive generations within the treatments. Boxplots with median, two hinges, two whiskers and all "outlying" points are visualised; different letters above boxplots denote significant differences ($p < 0.05$; Tukey's test).

The development time from first larva to first emerged female in nests was marginal significant between treatments (LMM: χ^2 = 7.31, *df* = 3, *p* = 0.063; SM Tab. S6). The post-hoc test identified a shorter period for entomopathogen-treated nests in contrast to '*control*' nests (EMM: *df* = 296, *t* = 2.64, *p* = 0.044; Fig. 6; SM Tab. S6). Taking the generations into account, we found an interaction of treatment and generation (LMM: χ^2 = 9.77, *df* = 4, *p* = 0.045; SM Tab. S6 + S7). Periods increased in the third generation of '*control*' nests (EMM: '*F1 vs. F3*': *df* = 864, *t* = -2.34, *p* = 0.051; '*F2 vs. F3*': *df* = 864, *t* = -4.44, *p* = <0.001; Fig. 6; SM Tab. S7), whereas the development time was shorter in the entomopathogen-treated nests of the '*F2*' generation in contrast to the '*F1*' and '*F3*' generation (EMM: '*F1 vs. F2*': *df* = 864, *t* = 2.95, *p* = 0.009; '*F2 vs. F3*': *df* = 864, *t* = -4.52, *p* = 0.032; Fig.6; SM Tab. S7). In nests treated with the garden pathogen the development time was not affected (Fig. 6, SM Tab. S7).

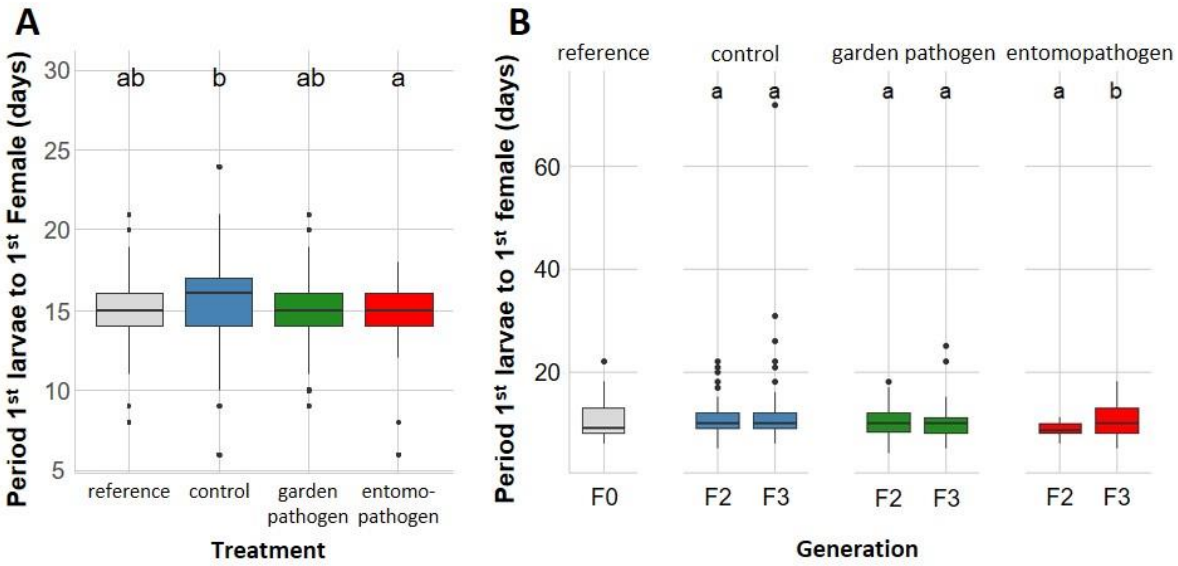

Figure 5: Development time from first larva till first emerging female in relation to treatment and consecutive generations. (A) Comparison of treatments for period from first larva to first emerged female over all generations. (B) Separate comparison of consecutive generations within the treatments. Boxplots with median, two hinges, two whiskers and all "outlying" points are visualised; different letters above boxplots denote significant differences ($p < 0.05$; Tukey's test).

b) Behavioural observations

For the analysis of the behaviours by larvae and females, we focused on the comparison of generations within each treatment. As a reference, we included the '*F0*'-data of untreated nests. Except for the larvae, who did not change their abundance of hygienic behaviours in nests injected with the entomopathogen (GLMM: χ^2 = 5.30, *df* = 3, *p* = 0.151; Fig. 7; SM Tab. S8), all behaviours differed significantly between the generations (SM Tab. S8).

Post-hoc contrasts of larval hygienic behaviours resulted in a significant reduction from a median of 40% in the reference to 30% in the '*F1*' and '*F2*' generation of the '*control*' and '*garden pathogen*' group. The hygienic actions increased again in the '*F3*' generation (Fig. 7A+B; Tab. 3). Larvae in the '*entomopathogen*' group showed a stable amount of hygienic behaviours throughout the generations (Fig. 7C; Tab. 3). Females behaved in a similar manner and showed a decrease of hygienic activity in the '*F1*' generation from originally a median of 35% to 25% in the '*control*' nests, which persisted in the following generation and increased a little again in the '*F3*' generation (Fig. 7D; Tab. 3). In '*garden pathogen*' and '*entomopathogen*' nests the hygienic behaviours decreased even up to a median of 20% (Fig. 7E+F; Tab. 3). While the behaviour of females in the entomopathogen-treated nests returned to the reference level in the third generation, those who were confronted with the garden pathogen already showed a slight increase in hygienic behaviour in the second generation. Still, they did not reach the level of the reference nests (Fig. 7E+F; Tab. 3).

Overall, larvae were quite active after the injection of the treatments. They only rested during the observed time with a median frequency of 10%, which decreased significantly in the third generation for all treatments (Fig. 8A-C; Tab. 3). Females, in turn, were already more resting in the untreated nests (median of 25%) than larvae and showed increased quiescence after injection of the treatments (around 50%). They got more active again in the third generation in '*control*' and '*entomopathogen*' nests, whereas '*garden pathogen*' nests already showed an adaptation in the second generation (Fig. 8D-F; Tab. 3).

Feeding behaviour was expressed differently in larvae (median 45%) and females (median 25%). While larvae were seen feeding more frequently after injection of

the treatments, this behaviour was reduced in adult females. Further, larvae kept feeding more, even though it decreased a bit in '*control*'- and '*entomopathogen*'-treated nests in the '*F2*' generation. However, the behaviour increased intensified again (60%) in the presence of the entomopathogen in the third generation (Fig. 7C; Tab. 3). Females reduced their feeding activity in the '*control*' and '*garden pathogen*' group during the first and second generation with treatment, but turned it up again in the third (Fig. 7D+E; Tab. 3). Confronted to the entomopathogen, they reduced their feeding activity, too, and raised it a little afterwards. Withal, there was no longer a significant difference, neither between the reference nor towards the first generation (Fig. 7F; Tab. 3).

The Spearman's rank correlation of total disperser count and hygienic behaviours performed by larvae or females revealed a positive correlation of larval hygienic behaviours with the disperser count for the '*control*' (R = 0.38, p < 0.001) and '*garden pathogen*' treatment (R = 0.34, p < 0.001). This correlation disappeared in the '*entomopathogen*' treatment (R = 0.002, p = 0.987).

Female' hygienic behaviour did not correlate in all treatments (SM Tab. S10).

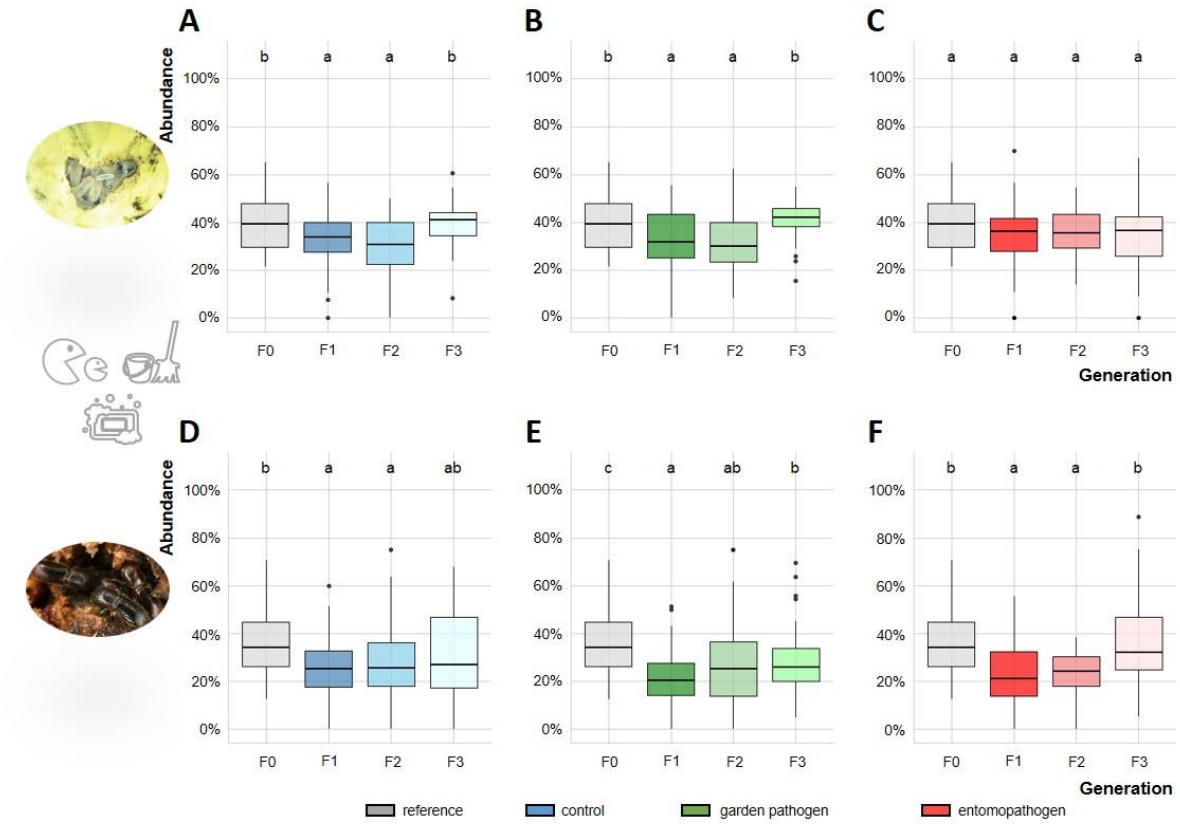

Figure 6: Distribution of the percentage of hygienic behaviours (cleaning, shuffling, grooming & cannibalism) in larvae (top) and females (bottom) divided by the three treatments. (A & D) Starting with the '*F1*' generation, nests were injected with the '*control*' (Tween 20/PBS) solution one day prior to the start of observations. (B & E) Starting with the '*F1*' generation, nests were injected with the '*garden pathogen*' (*Aspergillus*) solution one day prior to the start of observations. (C & F) Starting with the '*F1*' generation, nests were injected with the '*entomopathogen*' (*Beauveria*) solution one day before the start of observations. Boxplots with median, two hinges, two whiskers and all "outlying" points are visualised; different letters above boxplots denote significant differences ($p < 0.05$; Tukey's test).

Table 3: Statistical summary of post-hoc tests of the generalized linear mixed-effect models of observed nest behaviours. Each treatment ('*control*', '*garden pathogen*' and '*entomopathogen*') was tested for changes across generations. Data of the '*F0*' generation ('*reference*' group) served as reference. The summary of the ANOVAs of the models is in the Supplementary Material Tab. S8.

	control			**garden pathogen**			**entomopathogen**		
hygienic									
larvae									
contrast	***SE***	***z***	***p***	***SE***	***z***	***p***	***SE***	***z***	***p***
F0 – F1	0.070	3.892	0.001	0.071	3.058	0.012	0.081	2.259	0.108
F0 – F2	0.069	5.325	<0.001	0.076	3.959	<0.001	0.146	1.089	0.696
F0 – F3	0.068	-0.104	1.00	0.069	-1.032	0.731	0.111	1.350	0.531
F1 – F2	0.067	1.390	0.505	0.075	1.106	0.686	0.140	-0.166	0.998
F1 – F3	0.067	-4.190	<0.001	0.069	-4.182	<0.001	0.104	-0.305	0.990
F2 – F3	0.066	-5.706	<0.001	0.074	-5.024	<0.001	0.144	-0.058	1.00
females									
contrast	***SE***	***z***	***p***	***SE***	***z***	***p***	***SE***	***z***	***p***
F0 – F1	0.119	4.263	<0.001	0.110	6.542	<0.001	0.116	5.270	<0.001
F0 – F2	0.121	3.812	0.001	0.117	4.204	<0.001	0.202	2.834	0.024
F0 – F3	0.120	1.859	0.246	0.113	3.587	0.002	0.151	-0.171	0.998
F1 – F2	0.112	-0.416	0.976	0.114	-1.994	0.190	0.199	-0.192	0.998
F1 – F3	0.113	-2.518	0.057	0.108	-2.907	0.019	0.148	-4.302	<0.001
F2 – F3	0.112	-2.129	0.144	0.114	-0.775	0.866	0.222	-2.696	0.035
resting									
larvae									
contrast	***SE***	***z***	***p***	***SE***	***z***	***p***	***SE***	***z***	***p***
F0 – F1	0.247	0.469	0.966	0.258	0.974	0.764	0.204	1.791	0.278
F0 – F2	0.250	-0.638	0.920	0.278	0.335	0.987	0.370	0.310	0.990
F0 – F3	0.274	7.357	<0.001	0.280	5.822	<0.001	0.303	5.512	<0.001
F1 – F2	0.233	-1.179	0.640	0.257	-0.615	0.927	0.363	-0.689	0.901
F1 – F3	0.258	7.353	<0.001	0.257	5.361	<0.001	0.293	4.470	<0.001
F2 – F3	0.252	8.627	<0.001	0.263	5.825	<0.001	0.412	3.781	0.001
females									
contrast	***SE***	***z***	***p***	***SE***	***z***	***p***	***SE***	***z***	***p***
F0 – F1	0.246	0.487	0.962	0.094	-13.428	<0.001	0.102	-10.754	<0.001
F0 – F2	0.248	-0.653	0.915	0.101	-7.610	<0.001	0.175	-6.237	<0.001
F0 – F3	0.271	7.422	<0.001	0.097	-5.592	<0.001	0.137	-0.926	0.791
F1 – F2	0.233	-1.207	0.623	0.096	5.226	<0.001	0.171	0.018	1.00
F1 – F3	0.257	7.342	<0.001	0.091	7.984	<0.001	0.132	7.357	<0.001
F2 – F3	0.252	8.631	<0.001	0.096	2.347	0.088	0.194	4.978	<0.001
feeding									
larvae									
contrast	***SE***	***z***	***p***	***SE***	***z***	***p***	***SE***	***z***	***p***
F0 – F1	0.062	-6.556	<0.001	0.067	-5.659	<0.001	0.069	-5.765	<0.001
F0 – F2	0.062	-3.923	0.001	0.071	-3.512	0.003	0.123	-0.803	0.853
F0 – F3	0.061	-6.846	<0.001	0.067	-5.262	<0.001	0.090	-6.595	<0.001
F1 – F2	0.059	2.740	0.031	0.070	1.793	0.276	0.122	2.424	0.073
F1 – F3	0.058	-0.186	0.998	0.065	0.392	0.980	0.089	-2.233	0.114
F2 – F3	0.057	-3.036	0.013	0.069	-1.448	0.469	0.136	-3.653	0.002
females									
contrast	***SE***	***z***	***p***	***SE***	***z***	***p***	***SE***	***z***	***p***
F0 – F1	0.123	4.236	<0.001	0.121	5.375	<0.001	0.140	4.212	<0.001
F0 – F2	0.122	3.122	0.010	0.127	3.294	0.006	0.243	1.910	0.224
F0 – F3	0.122	0.535	0.951	0.119	-0.849	0.831	0.183	0.755	0.875
F1 – F2	0.116	-1.231	0.607	0.125	-1.829	0.260	0.240	-0.523	0.954
F1 – F3	0.117	-3.913	0.001	0.117	-6.432	<0.001	0.179	-2.520	0.057
F2 – F3	0.115	-2.733	0.032	0.124	-4.215	<0.001	0.268	-1.218	0.615

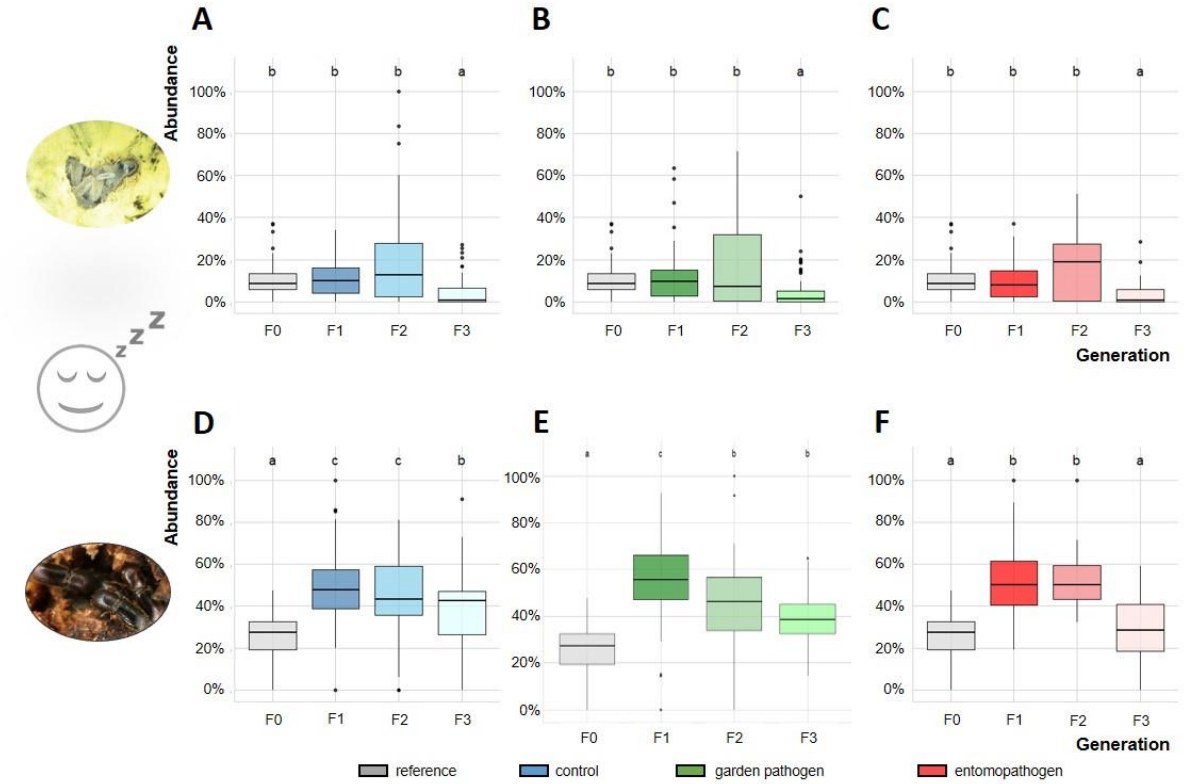

Figure 7: Distribution of the percentage of resting behaviour in larvae (top) and females (bottom) divided by the three treatments. (A & D) Starting with the '*F1*' generation, nests were injected with the '*control*' (Tween 20/PBS) solution one day prior to the start of observations. (B & E) Starting with the '*F1*' generation, nests were injected with the '*garden pathogen*' (*Aspergillus*) solution one day prior to the start of observations. (C & F) Starting with the '*F1*' generation, nests were injected with the '*entomopathogen*' (*Beauveria*) solution one day before the start of observations. Boxplots with median, two hinges, two whiskers and all "outlying" points are visualised; different letters above boxplots denote significant differences ($p < 0.05$; Tukey's test).

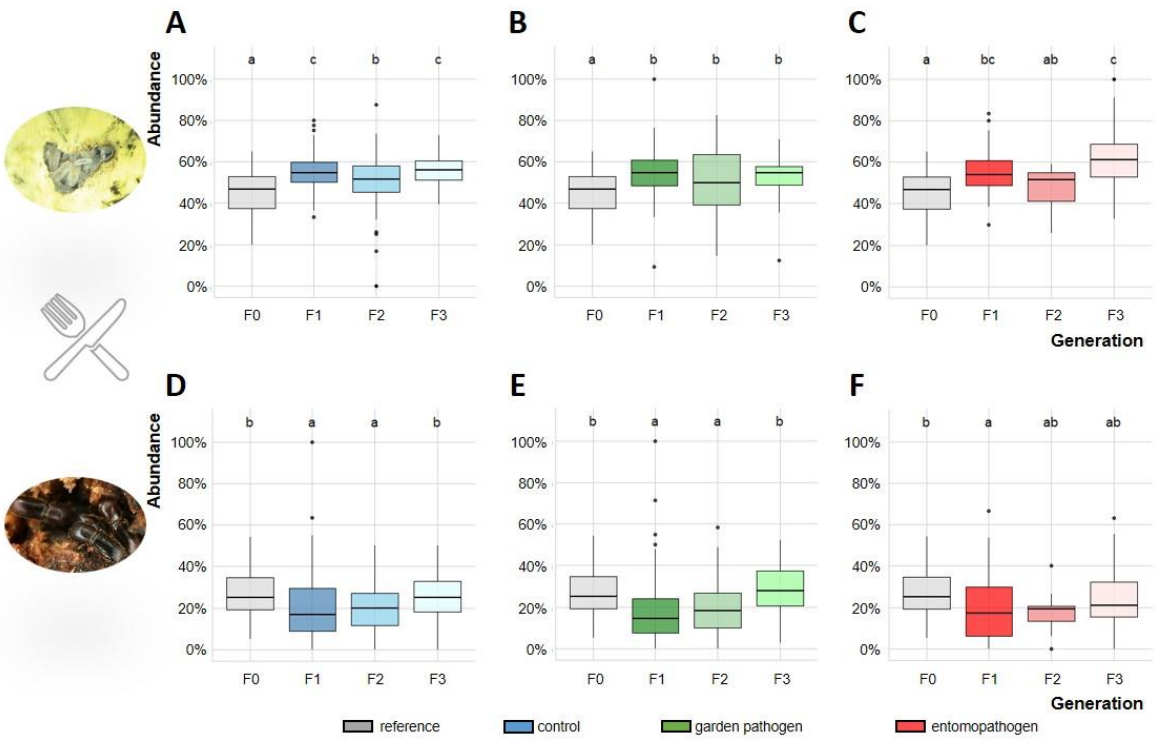

Figure 8: Distribution of the percentage of feeding behaviour in larvae (top) and females (bottom) divided by the three treatments. (A & D) Starting with the '*F1*' generation, nests were injected with the '*control*' (Tween 20/PBS) solution one day prior to the start of observations. (B & E) Starting with the '*F1*' generation, nests were injected with the '*garden pathogen*' (*Aspergillus*) solution one day prior to the start of observations. (C & F) Starting with the '*F1*' generation, nests were injected with the '*entomopathogen*' (*Beauveria*) solution one day before the start of observations. Boxplots with median, two hinges, two whiskers and all "outlying" points are visualised; different letters above boxplots denote significant differences ($p < 0.05$; Tukey's test).

a) Bacterial and fungal communities

Altogether, eight bacterial classes were detected in higher relative abundances across disperser samples. Among these were Actinobacteria, Bacilli, Clostridia, Deinococci, Flavobacteriia, Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria (>0.5% total mean RA; Fig. 10A). The most abundant class, Gammaproteobacteria, comprised ASVs of *Pseudoxanthomonas* (mean + s.d. = 25.24% ± 20.22 RA), *Erwinia* (23.64% ± 25.61), *Acinetobacter* (4.39% ± 10.21), *Pseudomonas* (3.29% ± 7.14), *Serratia* (1.44% ± 6.37), *Yersinia* (0.88% ± 3.5), *Gilliamella* (1.06% ± 2.41), *Enhydrobacter* (0.79% ± 1.0) and Azotobacter (2.45% ± 6.99). Betaproteobacteria were mostly represented by *Ralstonia* (4.44% ± 5.37), *Undibacterium* (4.85% ± 6.12), *Hydrogenophilus* (0.71% ± 1.11), *Comamonas* (1.0% ± 2.58) and *Massilia* (0.6% ± 0.87). Alphaproteobacteria were dominated by *Brucella* (4.22% ± 7.53). Actinobacteria were represented by the genus *Arthrobacter* (0.53% ± 3.61). Lastly, Bacilli were

found by *Staphylococcus* (2.71% ± 4.92) and *Anoxybacillus* (0.76% ± 1.37). Single ASVs in the classes of Clostridia (0.58% ± 1.3), Deinococci (0.64% ± 1.19) and Flavobacteriia (0.97% ± 1.15) did not reach the threshold of <0.5% total mean RA.

Fungus garden samples covered three classes which were recovered in higher abundances (Fig. 10B). The most dominant class here was again the Gammaproteobacteria with its high abundant *Pseudoxanthomonas* (47.85% ± 20.07), *Pseudomonas* (0.92% ± 4.48), Serratia (2.44% ± 9.69) and *Erwinia* (26.76% ± 23.92). Alphaproteobacteria were represented by *Brucella* (15.34% ± 21.38), *Bradyrhizobium* (1.76% ± 4.41) and *Methylobacterium* (2.51% ± 6.06). We also found Actinobacteria in an abundance of 0.55% (± 1.4) total mean RA.

The analyses of the dispersing females yielded seven high abundant fungal orders. Among these were Ophiostomatales, Sordariales, Eurotiales, Capnodiales, Pleosporales, Hypocreales and Togniniales (>0.5% mean RA; Fig. 10C). The highest relative abundance came from the *Chaetomium* (Sordariales; 62.87% ± 33.04). Followed by the order Ophiostomatales with the ambrosia fungus *Raffaelea* (15.53% ± 19.74). Eurotiales were represented by *Aspergillus* (2.59% ± 6.68), *Penicillium* (3.07% ± 7.10) and *Talaromyces* (0.89% ± 5.54). Other ASVs with a relative abundance greater than 0.5% total mean RA were *Phaeoacremonium* (Togniniales; 3.54% ± 10.82), *Epicoccum* (Pleosporales; 2.09% ± 7.75), *Cladosporium* (Capnodiales; 1.07% ± 5.04) and *Beauveria* (Hypocreales; 6.42 % ± 21.78).

Less diversity was found in the fungus garden nest samples. Here, we detected five higher abundant fungal orders (Fig. 10D). Again, the orders Eurotiales, Hypocreales, Ophiostomatales, Sordariales and Togniniales were found. Most abundant ASVs were *Chaetomium* (42.97% ± 37.02), *Phaeoacremonium* (31.94% ± 35.09), *Beauveria* (16.25% ± 28.52), *Raffaelea* (1.61% ± 9.47), *Aspergillus* (0.81% ± 2.56) and *Penicillium* (1.52% ± 5.84).

Detailed information on mean and s.d. of ASVs split into the treatments, as well as generations for fungus garden microbiomes, are listed in the Supplementary Material (Tab. S1 – S4).

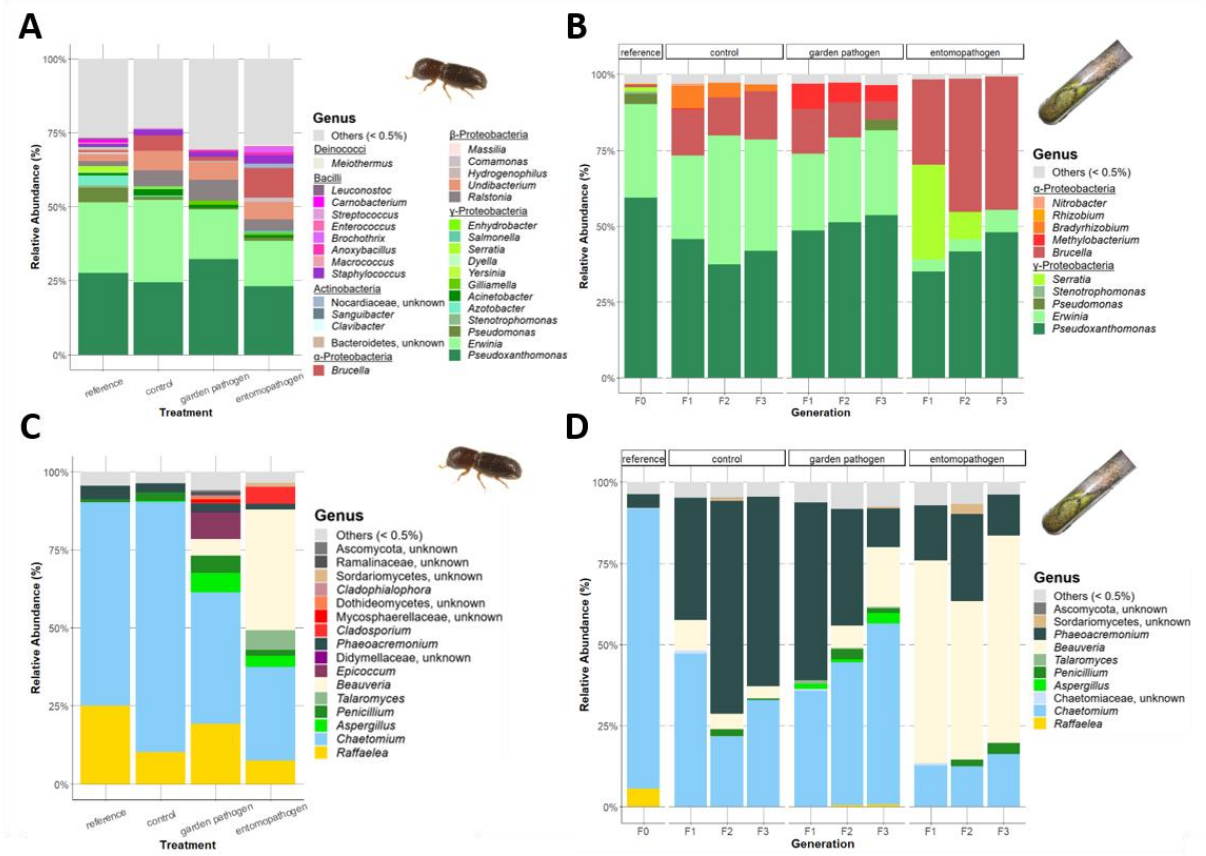

Figure 9: Relative abundance of symbiont taxa in disperser and fungus gardens in relation to the applied treatments and generations of *X. saxesenii* nests. Community of bacterial (A, B) and fungal genera (C, D) in the F0 and F3 generation of dispersing females (A, C) and fungus gardens across all generations (B, D) with a relative abundance of at least 0.5% (all else is combined in "others").

Discussion

Influence of pathogen pressure on the breeding and life-history of *X. saxesenii*

Overall, we observed a significant reduction in the numbers of adult female dispersers after injection compared to the reference group. Furthermore, the numbers varied depending on the treatment, with the greatest impact observed in the nests treated with the entomopathogen. The lineages appeared to recover from the disturbance within one generation, though differences among the treatments persisted. The strongest recovery over generations was observed in the control lines. The colonies also entered a diapause after the

first treatment. During this period, early developmental stages are more vulnerable, with mostly adults being able to survive (Weber and Mcpherson 1983). It is a natural occurrence, affecting every second or third generation in wild *X. saxesenii* populations (Hosking 1972). Therefore, the results from this scenario provide a valuable insight into the effect of these treatments under natural conditions. However, it is important to note that diapause might have further stressed the offspring and resulted in additional losses due to low temperatures.

The emergence of first females occurred earlier with each generation, regardless of the treatment applied. This suggests that this phenomenon is a general adaptation that occurs in connection with laboratory breeding. The availability of easily accessible nutrients allows the food fungi to grow quickly, which in turn can promote the development of the beetles. Interestingly, the residence time of mature females in their nests was extended by 1-2 days in the third generation of entomopathogen-treated nests. One possible explanation for this could be an adaptation to the required need for help as a result of repeated infection. This is another strategy for coping with the threat, as after the first injection with *Beauveria*, the development phase between larvae and adult females was shorter. Bark beetles are known to adapt their development speed and final size to adverse food conditions (e.g. Kajimura and Hijii 1994). However, it should be noted that the number of generations tested in this experiment is not conclusive in this regard and variations in life-history traits may manifest themselves with a higher number of generations. The beetle's reactions to pathogenic pressure on its survival may follow a process, where a temporary adjustment involves reducing the period of early life stages, and over the long term, social behaviours (primarily hygienic behaviours in this instance) may manifest as a general adaptation (Fig. 6F). Based on the results of our experimental period, life-history traits do not appear to be affected by the garden pathogens. Other studies have suggested that females have the ability to adjust their dispersal timing, especially under the influence of pathogens (Peer and Taborsky 2007; Nuotclà et al. 2019).

Behavioural adaptation of larvae and freshly emerged adult females

Behavioural observations did not reveal the expected extent of increased hygienic measures in response to pathogen exposure. Hygienic behaviours and feeding already constitute a significant proportion larvae's activity. Larval individuals were only inactive and resting in the brood chambers in 10% of the total observed time. This limits their ability to respond to unexpected situations, such as encountering a sudden increase of pathogens. In principle, larvae intensified their feeding behaviour slightly after the treatment, which could be due to a higher energy demand. However, we only observed a significant increase in this behaviour when the entomopathogen was introduced. In this context, we speculate that weeding played a role in reducing the spore load and limiting the spread of infection within the nest.

Females, on the other hand, showed an overall higher proportion of resting behaviour (~25%), which doubled after injection. Again, behavioural adaptation seems to take its time, since in the following generations females and larvae increased their overall activity and their relative amount of hygienic behaviours, in particular in galleries treated with the garden pathogen (Fig. 6). An increased proportion of allo-grooming and cannibalism behaviour has already been observed in females of *X. saxesenii* by Nuotclà et al. (2019). Here, the reaction occurred immediately after the treatment, but unlike our attempt, nests were observed before and after the injection, allowing a direct comparison of the nests. Interestingly, buffer solutions caused a reaction in females, as well (Nuotclà et al. 2019). In all treatment groups, females increased beyond hygienic behaviours, the proportion of feeding with advancing generations. Here, we would like to emphasize that the feeding behaviour of the beetles cannot be separated from weeding of the fungus garden by the observer. Therefore, it is possible that the adult's reaction to pathogens ('pathogen elimination') is also present in this context.

In our experiment, larvae appear to take over a greater proportion of social behaviours, especially in this specific period of nest development. The impact of larval hygienic activity for the success of the nest is supported by its positive correlation with the number of dispersing offspring. We expect a change in responsibilities in nests with older females who delayed their dispersal with the

intention to help. The study by Nuotclà et al. (2019) could detect an increase of allo-grooming frequencies of adult females with higher numbers of females within the brood chamber, which supports our theory. Here, females freshly emerged from their pupal stage and need to fully sclerotize before being able to take over all tasks. Furthermore, these young females are mostly involved in copulation and feeding in their first days, which later changes towards more philopatric behaviours (personal observation). We decided not to do the injection and observations at a later time, as we were interested in the behaviour of both larvae and adults and wanted to avoid a start of female dispersal during this five-day observation period (first dispersers were already seen 3-5 days later; personal observations).

Keeping the habitat and lifestyle of ambrosia beetles in mind, there is a clear difference between this taxon and fungus-farming ants and termites. While ants and termites are forced to leave their nests and forage for plant material to nourish the fungus gardens, ambrosia beetles live in a closed environment inside the wood, with the foundress blocking the entrance (Nuotclà et al. 2014). The chances of a sudden introduction of a severe threat are thereby relatively low, compared to the nests of social insects. Therefore, it is reasonable for us to assume that the response to pathogen pressure associated with this substrate-based lifestyle is delayed and occurs in the long term, recognizing the costs. Only under recurring pathogen pressure, adaptation is beneficial and worth being manifested. This could explain the reaction towards the entomopathogen. For the garden pathogen, *Aspergillus sp.*, one can argue that *X. saxesenii* is already well adapted and shows a high proportion of social and, therefore, also hygienic behaviours.

Impact of pathogens on the microbiome of dispersing females and the fungus garden community

Microbial community analysis of dispersers and fungus garden material showed that beetles are associated with more bacterial taxa than we can recover in their nests (Fig. 9 A+B). A comparison of both communities helps us disentangle which potential candidate bacteria is helpful in the context of fungus garden maintenance. The two dominant classes of fungus gardens are Alpha- and

Betaproteobacteria. Previous studies already identified these as important symbiont groups in association with ambrosia beetles. While Diehl et al. (2022; under review) found *Pseudoxanthomonas* and *Erwinia* to be the main symbionts, bacterial communities in treated nests had a higher relative abundance of the Alphaproteobacteria *Brucella, Rhizobium* and *Methylobacterium*. The role of these in relation to ambrosia beetles is still unknown. Brucellosis is a common disease in animals, but insects are not affected in the same manner, with studies mostly finding them to serve as a vector for their primary hosts (cf. Coelho et al. 2015; Spickler 2018). The chances the detected ASV could function as a secondary pathogen in relation to an artificial disturbance by an injection are rather unlikely. If and which function they may have, is a question for future studies.

Fungal communities, too, differed between dispersers and gardens. While the communities of '*reference*' and '*control*' females were very alike, pathogen-infected females were associated with multiple fungal taxa (Fig. 9C). Of course, the injected candidates were recovered, too. The relative abundance of the food fungus *Raffaelea* was low (15-20% mean RA), but still present, allowing beetles to successfully found a new nest. In fungus gardens, the biggest proportion of the community were antagonist fungi, such as *Chaetomium* and *Phaeoacremonium*. This is not unexpected, since the galleries were only sampled after the last females had left, which left the garden in a correspondingly poor condition. *Chaetomium* is a well-known contaminant in laboratory bred *X. saxesenii* nests, increasing in abundance in late nest development and beetle's absence (Diehl, Kowallik, et al. 2022; Diehl et al. under review). *Phaeoacremonium*, on the other hand, was detected in this high relative abundance in our laboratory breeding for the first time. This genus is associated with wood decay symptoms on trees like *Prunus* or stone fruit trees (Damm et al. 2008) and might be another antagonistic fungus succeeding the crop fungi. It can be frequently isolated from diseased grapevines, but Halleen et al. (2007) assumed that the fungus cannot be able to cause the disease on its own and rather needs the synergism with other fungi to harm the plant.

Our results presented evidence of successful infection of the brood chambers with the injected pathogen. Interestingly, the relative abundance of the

entomopathogen is higher than the garden pathogen (SM Tab. S4). We interpret this finding as proof of a successful response by the beetles to protect their crop. Some samples of galleries and dispersers in the '*control*' and '*garden pathogen*' groups contained DNA of *Beauveria*, as well. All treatment groups were bred together in racks over the whole experiment to guarantee blind testing. Therefore, we cannot exclude a potential for cross-contamination during the experimental period. Though *B. bassiana* can be easily cultivated on agar medium, it is naturally restricted to insect hosts. Here, it grows on cadavers and produces large numbers of spores that can only be vectored and start germinating in contact with insect bodies (Walstad et al. 1970; Doberski and Tribe 1980). Although the experiment was carried out with reasonable consideration to hygiene conditions, we cannot rule out the possibility that some of these spores could accidentally infect neighbouring nests.

Our side study on the fungal lethality revealed that a concentration of 10^7 spores/ml had the highest hazard ratios for both fungi in *X. saxesenii* (SM Fig. S4 + S5). Due to physical contact with other nestmates, a higher survival rate within the brood chambers can be expected. Infected individuals had the opportunity to receive grooming from others and were under a lower stress level compared to those in isolated situations (cf. Kohlmeier et al. 2016). As observed in the experiment with individuals, a distinct difference between the pathogens in the nests was also apparent, with a significantly greater number of successfully dispersed females present after exposure to the garden pathogen in comparison to the entomopathogen.

Conclusion

The study examined the effect of pathogen pressure on the breeding and life history of the ambrosia beetle *X. saxesenii*. The results showed a significant reduction in the number of adult female dispersers after injection, with the greatest impact observed in the nests treated with the entomopathogen. However, the lineages recovered from the disturbance within one generation, and the emergence of first females occurred earlier with each generation regardless of the treatment applied. Behavioural observations revealed that

larvae invest a high percentage of their activity into hygienic behaviours and feeding. On the other hand, females showed an overall higher proportion of resting behaviour and increased their relative amount of hygienic behaviours and feeding with advancing generations. Overall, larvae appear to take over the greater proportion of social behaviours, with the impact of larval hygienic activity supporting the success of the nest. The study provides valuable insights into the response of ambrosia beetles to pathogen pressure. This response is complex and involves multiple factors, including life-history traits, behavioural adaptations and microbial defences, indicating a process of adjustment that can lead to long-term behavioural adaptations. The results of our study open up new perspectives on previously existing theories, such as the involvement of pathogens in social evolution, or the adaptation of developmental stages and survival after confrontation with pathogens. Our interpretations require detailed verification in follow-up studies focusing on the individual trade-offs we observed. We found that this group of fungus-farmers is able to assess the danger they face and take action. Interestingly, these measures appear to differ from previous social insect responses. Understanding this multimodal framework of responses demands equal consideration to all aspects of individual and social immune responses, as well as changes in the environment.

References

Batra LR, Michie MD. 1963. Pleomorphism in some ambrosia and related fungi. Trans Kansas Acad Sci. 66(3):470–481. doi:10.2307/3626545. http://www.jstor.org/stable/3626545.

Batra LRR. 1966. Ambrosia fungi: extent of specificity to ambrosia beetles. Science. 153(3732):193–195. doi:10.1126/science.153.3732.193. http://www.sciencemag.org/content/153/3732/193.short.

Biedermann PHW. 2014. Evolution of cooperation in ambrosia beetles. Mitteilungen der Dtsch Gesellschaft für Allg und Angew Entomol. 19:191–201.

Biedermann PHW, Currie CR. Mechanisms of fungus gardening in ambrosia beetles. University of Bern.

Biedermann PHW, Klepzig KD, Taborsky M. 2011. Costs of delayed dispersal and alloparental care in the fungus-cultivating ambrosia beetle *Xyleborus affinis* Eichhoff (Scolytinae: Curculionidae). Behav Ecol Sociobiol. 65(9):1753–1761. doi:10.1007/s00265-011-1183-5. http://link.springer.com/10.1007/s00265-011-1183-5.

Biedermann PHW, Rohlfs M. 2017. Evolutionary feedbacks between insect sociality and microbial management. Curr Opin Insect Sci. 22:92–100. doi:10.1016/j.cois.2017.06.003. http://dx.doi.org/10.1016/j.cois.2017.06.003.

Biedermann PHW, Taborsky M. 2011. Larval helpers and age polyethism in ambrosia beetles. Proc Natl Acad Sci. 108(41):17064–17069. doi:10.1073/pnas.1107758108. http://www.pnas.org/cgi/doi/10.1073/pnas.1107758108.

Biedermann PHW, Vega FE. 2020. Ecology and evolution of insect-fungus mutualisms. Annu Rev Entomol. 65:431–455. doi:10.1146/annurev-ento-011019-024910.

Caporaso JG, Lauber CL, Costello EK, Berg-Lyons D, Gonzalez A, Stombaugh J, Knights D, Gajer P, Ravel J, Fierer N, et al. 2011. Moving pictures of the human microbiome. Genome Biol. 12(5):R50. doi:10.1186/gb-2011-12-5-r50. http://genomebiology.com/2011/12/5/R50.

Cardoza YJ, Klepzig KD, Raffa KF. 2006. Bacteria in oral secretions of an endophytic insect inhibit antagonistic fungi. Ecol Entomol. 31(6):636–645. doi:10.1111/j.1365-2311.2006.00829.x.

Castrillo LA, Griggs MH, Ranger CM, Reding ME, Vandenberg JD. 2011. Virulence of commercial strains of *Beauveria bassiana* and *Metarhizium brunneum* (Ascomycota: Hypocreales) against adult *Xylosandrus germanus* (Coleoptera: Curculionidae) and impact on brood. Biol Control. 58(2):121–126. doi:10.1016/j.biocontrol.2011.04.010. http://dx.doi.org/10.1016/j.biocontrol.2011.04.010.

Castrillo LA, Griggs MH, Vandenberg JD. 2013. Granulate ambrosia beetle, *Xylosandrus crassiusculus* (Coleoptera: Curculionidae), survival and brood production following exposure to entomopathogenic and mycoparasitic fungi. Biol Control. 67(2):220–226. doi:10.1016/j.biocontrol.2013.07.015. http://dx.doi.org/10.1016/j.biocontrol.2013.07.015.

Chapuisat M, Oppliger A, Magliano P, Christe P. 2007. Wood ants use resin to protect themselves against pathogens. Proc R Soc B Biol Sci. 274(1621):2013–2017. doi:10.1098/RSPB.2007.0531. https://royalsocietypublishing.org/doi/10.1098/rspb.2007.0531.

Christe P, Oppliger A, Bancalà F, Castella G, Chapuisat M. 2003. Evidence for collective medication in ants. Ecol Lett. 6(1):19–22. doi:10.1046/J.1461-0248.2003.00395.X. https://onlinelibrary.wiley.com/doi/full/10.1046/j.1461-0248.2003.00395.x.

Coelho AC, Díez JG, Coelho AM. 2015. Risk Factors for *Brucella* spp. in Domestic and Wild Animals. In: Updates on Brucellosis. IntechOpen. https://www.intechopen.com/state.item.id.

Costa JT. 2006. The other insect societies. Cambridge, Massachusetts and London, England: The Belknap Press of Havard University Press. www.hup.harvard.edu/catalog.php?isbn=9780674021631.

Cote IM, Poulin R. 1995. Parasitism and group size in social animals: a meta-analysis. Behav Ecol. 6(2):159–165. doi:10.1093/BEHECO/6.2.159. https://academic.oup.com/beheco/article/6/2/159/184622.

Cremer S, Armitage SAO, Schmid-Hempel P. 2007. Social immunity. Curr Biol CB. 17(16):R693-702. doi:10.1016/j.cub.2007.06.008. http://www.ncbi.nlm.nih.gov/pubmed/17714663.

Cremer S, Pull CD, Fürst MA. 2018. Social Immunity: Emergence and Evolution of Colony-Level Disease Protection. https://doi.org/101146/annurev-ento-020117-043110. 63:105–123. doi:10.1146/ANNUREV-ENTO-020117-043110. https://www.annualreviews.org/doi/abs/10.1146/annurev-ento-020117-043110.

Currie CR, Scott JA, Summerbell RC, Malloch D. 1999. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature. 398(6729):701–704. doi:10.1038/19519. https://www.nature.com/articles/19519.

Currie CR, Stuart AE. 2001. Weeding and grooming of pathogens in agriculture by ants. Proc R Soc London Ser B Biol Sci. 268(1471):1033–1039. doi:10.1098/rspb.2001.1605. https://royalsocietypublishing.org/doi/abs/10.1098/rspb.2001.1605.

Damm U, Mostert L, Crous PW, Fourie PH. 2008. Novel *Phaeoacremonium* species associated with necrotic wood of *Prunus* trees. Persoonia Mol Phylogeny Evol Fungi. 20:87–102. doi:10.3767/003158508X324227.

Davis NM, Proctor DiM, Holmes SP, Relman DA, Callahan BJ. 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome. 6(1). doi:10.1186/S40168-018-0605-2.

Diehl-Fleig E, Lucchese MDP. 1991. Reações comportamentais de operárias de *Acromyrmex striatus* (Hymenoptera, Formicidae) na presença de fungos entomopatogênicos. Rev Bras Entomol. 35(1):101–107.

Diehl JMC, Kassie D, Biedermann PHW. 2022. Friend or foe: Ambrosia beetle response to volatiles of common threats in their fungus gardens. bioRxiv.:2022.12.23.521835. doi:10.1101/2022.12.23.521835.. https://www.biorxiv.org/content/10.1101/2022.12.23.521835v1.

Diehl JMC, Keller A, Biedermann PHW. Succession of ambrosia beetle microbial community structure throughout development in field and laboratory galleries. Front Microbiol. under review:1–22.

Diehl JMC, Kowallik V, Keller A, Biedermann PHW. 2022. First experimental evidence for active farming in ambrosia beetles and strong heredity of garden microbiomes. Proc R Soc B Biol Sci. 289(1986). doi:10.1098/rspb.2022.1458. https://royalsocietypublishing.org/doi/10.1098/rspb.2022.1458.

Doberski JW, Tribe HT. 1980. Isolation of entomogenous fungi from elm bark and soil with reference to ecology of *Beauveria bassiana* and *Metarhizium anisopliae*. Trans Br Mycol Soc. 74(1):95–100. doi:10.1016/S0007-1536(80)80013-1.

Edgar RC. 2018. Updating the 97% identity threshold for 16S ribosomal RNA OTUs. Bioinformatics. 34(14):2371–2375. doi:10.1093/bioinformatics/bty113.

Fox J, Weisberg S. 2019. An R companion to applied regression. Thousand Oaks, CA: Sage Publications Inc.

French JRJ, Roeper RA. 1972. Interactions of the ambrosia beetle, *Xyleborus dispar* (Coleoptera: Scolytidae), with its symbiotic fungus *Ambrosiella hartigii* (Fungi Imperfecti). Can Entomol. 104(10):1635–1641. doi:10.4039/Ent1041635-10.

Gilliam M, Taber S, Lorenz BJ, Prest DB. 1988. Factors affecting development of chalkbrood disease in colonies of honey bees, *Apis mellifera*, fed pollen contaminated with *Ascosphaera apis*. J Invertebr Pathol. 52(2):314–325. doi:10.1016/0022-2011(88)90141-3.

Grubbs KJ, Surup F, Biedermann PHW, McDonald BR, Klassen JL, Carlson CM, Clardy J, Currie CR. 2020. Cycloheximide-producing *Streptomyces* associated with *Xyleborinus saxesenii* and *Xyleborus affinis* fungus-farming ambrosia beetles. Front Microbiol. 11(September):1–12. doi:10.3389/fmicb.2020.562140.

Halleen F, Mostert L, Crous PW. 2007. Pathogenicity testing of lesser-known vascular fungi of grapevines. Australas Plant Pathol. 36(3):277–285. doi:10.1071/AP07019/METRICS. https://link.springer.com/article/10.1071/AP07019.

Hosking GP. 1972. *Xyleborus saxeseni*, its life-history and flight behaviour in New Zealand. New Zeal J For Sci. 3(1):37–53.

Hulcr J, Stelinski LL. 2017. The ambrosia symbiosis: From evolutionary ecology to practical management. Annu Rev Entomol. 62(1):285–303. doi:10.1146/annurev-ento-031616-035105. http://www.annualreviews.org/doi/10.1146/annurev-ento-031616-035105.

Ibarra-Juarez LA, Burton MAJ, Biedermann PHW, Cruz L, Desgarennes D, Ibarra-Laclette E, Latorre A, Alonso-Sánchez A, Villafan E, Hanako-Rosas G, et al. 2020. Evidence for succession and putative metabolic roles of fungi and bacteria in the farming mutualism of the ambrosia beetle *Xyleborus affinis*. mSystems. 5(5):e00541-00520. doi:10.1128/MSYSTEMS.00541-20/ASSET/527FF6DB-D4E5-4920-8F5B-3208E1531817/ASSETS/GRAPHIC/MSYSTEMS.00541-20-F0010.JPEG.. https://journals.asm.org/doi/abs/10.1128/mSystems.00541-20.

Kajimura H, Hijii N. 1994. Reproduction and resource utilization of the ambrosia beetle, *Xylosandrus mutilatus*, in field and experimental populations. Entomol exp appl. 71:121–132.

Kassambara A. 2020. ggpubr: “ggplot2” based publication ready plots. https://cran.r-project.org/package=ggpubr.

Kohlmeier P, Holländer K, Meunier J. 2016. Survival after pathogen exposure in group-living insects: don’t forget the stress of social isolation! J Evol Biol. 29(9):1867–1872. doi:10.1111/jeb.12916.

Konrad M, Vyleta ML, Theis FJ, Stock M, Tragust S, Klatt M, Drescher V, Marr C, Ugelvig L V., Cremer S. 2012. Social transfer of pathogenic fungus promotes active immunisation in ant colonies. PLoS Biol. 10(4):e1001300. doi:10.1371/journal.pbio.1001300. http://dx.plos.org/10.1371/journal.pbio.1001300.

Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol. 79(17):5112–5120. doi:10.1128/AEM.01043-13.

Krause J, Ruxton GD. 2002. Living in groups. Oxford Series in Ecology and Evolution. https://global.oup.com/academic/product/living-in-groups-9780198508182?cc=de&lang=en&#.

Lenth R V. 2021. emmeans: Estimated marginal means, aka least-squares means. https://cran.r-project.org/package=emmeans.

Lüdecke D, Ben-Shachar MS, Patil I, Waggoner P, Makowski D. 2021. performance: An R package for assessment, comparison and testing of statistical models. J Open Source Softw. 6(60):3139. doi:10.21105/joss.03139. https://doi.org/10.21105/joss.03139.

Mangiafico S. 2021. rcompanion: Functions to support extension education program evaluation. https://cran.r-project.org/package=rcompanion.

Meunier J. 2015. Social immunity and the evolution of group living in insects. Philos Trans R Soc B Biol Sci. 370(1669):20140102–20140102.

doi:10.1098/rstb.2014.0102.
http://rstb.royalsocietypublishing.org/cgi/doi/10.1098/rstb.2014.0102.

Van Meyel S, Körner M, Meunier J. 2018. Social immunity: why we should study its nature, evolution and functions across all social systems. Curr Opin Insect Sci. 28:1–7. doi:10.1016/J.COIS.2018.03.004.

Myles TG. 2002. Alarm, aggregation, and defense by *Reticulitermes flavipes* in response to a naturally occurring isolate of *Metarhizium anisopliae*. Sociobiology. 40(2):243–255.

Nuotclà JA, Biedermann PHW, Taborsky M. 2019. Pathogen defence is a potential driver of social evolution in ambrosia beetles. Proc R Soc B Biol Sci. 286(20192332):1–9. doi:10.1098/rspb.2019.2332.
https://royalsocietypublishing.org/doi/abs/10.1098/rspb.2019.2332.

Nuotclà JA, Diehl JMC, Taborsky M. 2021. Habitat quality determines dispersal decisions and fitness in a beetle – fungus mutualism. Front Ecol Evol. 9:1–15. doi:10.3389/fevo.2021.602672.

Nuotclà JA, Taborsky M, Biedermann PHW. 2014. The importance of blocking the gallery entrance in the ambrosia beetle *Xyleborinus saxesenii* Ratzeburg (Coleoptera; Scolytinae). Mitteilungen der Dtsch Gesellschaft für Allg und Angew Entomol. 19:203–210.

Otterstatter MC, Thomson JD. 2007. Contact networks and transmission of an intestinal pathogen in bumble bee (*Bombus impatiens*) colonies. Oecologia. 154(2):411–421. doi:10.1007/S00442-007-0834-8.
https://pubmed.ncbi.nlm.nih.gov/17713789/.

Patterson JEH, Ruckstuhl KE. 2013. Parasite infection and host group size: a meta-analytical review. Parasitology. 140(7):803–813. doi:10.1017/S0031182012002259. https://pubmed.ncbi.nlm.nih.gov/23425516/.

Pedrini N, Ortiz-Urquiza A, Huarte-Bonnet C, Fan Y, Juárez MP, Keyhani NO. 2015. Tenebrionid secretions and a fungal benzoquinone oxidoreductase form competing components of an arms race between a host and pathogen. Proc Natl Acad Sci. 112(28):E3651–E3660. doi:10.1073/PNAS.1504552112.

Peer K, Taborsky M. 2007. Delayed dispersal as a potential route to cooperative breeding in ambrosia beetles. Behav Ecol Sociobiol. 61(5):729–739. doi:10.1007/s00265-006-0303-0.

Pie MR, Rosengaus RB, Traniello JFA. 2004. Nest architecture, activity pattern, worker density and the dynamics of disease transmission in social insects. J Theor Biol. 226(1):45–51. doi:10.1016/j.jtbi.2003.08.002.
https://pubmed.ncbi.nlm.nih.gov/14637053/.

Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team. 2021. _nlme: Linear and nonlinear mixed effects models_. https://cran.r-project.org/package=nlme.

Pražak R. 1991. Studies on indirect infection of *Trypodendron lineatum* Oliv. with *Beauveria bassiana* (Bals.) Vuill. J Appl Entomol. 111(1–5):431–441.

doi:10.1111/J.1439-0418.1991.TB00345.X.
https://onlinelibrary.wiley.com/doi/full/10.1111/j.1439-0418.1991.tb00345.x.

Pražak RA. 1997. Laboratory Evaluation of *Beauveria bassiana* (Bals.) Vuill. (Deu-teromycotina: Hyphomycetes) against *Trypodendron lineatum* Oliv. (Coleoptera: Scolytidae). J Plant Dis Prot. 104(5):459–465.
https://about.jstor.org/terms.

R Core Team (2021). R: A language and environment for statistical computing.

Rifkin JL, Nunn CL, Garamszegi LZ. 2012. Do animals living in larger groups experience greater parasitism? A meta-analysis. Am Nat. 180(1):70–82. doi:10.1086/666081. https://pubmed.ncbi.nlm.nih.gov/22673652/.

Rosengaus RB, Jordan C, Lefebvre ML, Traniello JFA. 1999. Pathogen alarm behavior in a termite: A new form of communication in social insects. Naturwissenschaften 1999 8611. 86(11):544–548. doi:10.1007/S001140050672.
https://link.springer.com/article/10.1007/s001140050672.

Rosengaus RB, Traniello JFA. 2001. Disease susceptibility and the adaptive nature of colony demography in the dampwood termite *Zootermopsis angusticollis*. Behav Ecol Sociobiol. 50(6):546–556. doi:10.1007/s002650100394. https://www.jstor.org/stable/4602004.

Royce LA, Rossignol PA, Burgett DM, Stringer BA. 1991. Reduction of tracheal mite parasitism of honey bees by swarming. Philos Trans R Soc London Ser B Biol Sci. 331(1260):123–129. doi:10.1098/RSTB.1991.0003.
https://royalsocietypublishing.org/doi/10.1098/rstb.1991.0003.

Schmid-Hempel P. 1998. Parasites in social insects. 60th ed. Princeton University Press.

Scott JJ, Oh D-C, Yuceer MC, Klepzig KD, Clardy J, Currie CR. 2008. Bacterial protection of beetle-fungus mutualism. Science. 322(5898):63. doi:10.1126/science.1160423.Bacterial.

Spickler AR. 2018. Brucellosis. Factsheet.:1–14.
http://www.cfsph.iastate.edu/DiseaseInfo/factsheets.php.

Stroeymeyt N, Casillas-Pérez B, Cremer S. 2014. Organisational immunity in social insects. Curr Opin insect Sci. 5(1):1–15. doi:10.1016/J.COIS.2014.09.001. https://pubmed.ncbi.nlm.nih.gov/32846736/.

Turillazzi S, Mastrobuoni G, Dani FR, Moneti G, Pieraccini G, La Marca G, Bartolucci G, Perito B, Lambardi D, Cavallini V, et al. 2006. Dominulin A and B: Two new antibacterial peptides identified on the cuticle and in the venom of the social paper wasp *Polistes dominulus* using MALDI-TOF, MALDI-TOF/TOF, and ESI-ion trap. J Am Soc Mass Spectrom. 17(3):376–383. doi:10.1016/J.JASMS.2005.11.017.
https://pubs.acs.org/doi/abs/10.1016/j.jasms.2005.11.017.

Walstad JD, Anderson RF, Stambaugh WJ. 1970. Effects of environmental conditions on two species of muscardine fungi (*Beauveria bassiana* and *Metarhizium anisopliae*). Journal of Invertebrate Pathology. 16:221-226.

Weber BC, Mcpherson JE. 1983. Life history of the ambrosia beetle *Xylosandrus germanus* (Coleoptera: Scolytidae). Annu Entomol Soc Am. 76:455–462.

Wickham H. 2016. ggplot2: Elegant graphics for data analysis. New York: Springer-Verlag New York.

William L. Brown J. 1968. An hypothesis concerning the function of the metapleural glands in ants. https://doi.org/101086/282536. 102(924):188–191. doi:10.1086/282536.. https://www.journals.uchicago.edu/doi/10.1086/282536.

Wilson EO. 1971. The Insect Societies. Cambridge: Belknap Press of Harvard University Press.

Wilson K, Knell R, Boots M, Koch-Osborne J. 2003. Group living and investment in immune defence: an interspecific analysis. J Anim Ecol. 72(1):133–143. doi:10.1046/J.1365-2656.2003.00680.X. https://onlinelibrary.wiley.com/doi/full/10.1046/j.1365-2656.2003.00680.x.

Yanagawa A, Fujiwara-Tsujii N, Akino T, Yoshimura T, Yanagawa T, Shimizu S. 2012. Odor aversion and pathogen-removal efficiency in grooming behavior of the termite *Coptotermes formosanus*. PLoS One. 7(10):e47412. doi:10.1371/journal.pone.0047412. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0047412.

ANNEX - CHAPTER 3

Supplementary Material

Supplementary Material

to manuscript “Ambrosia beetle response to pathogen pressure – A glance at life-history, behaviour and symbiont community over multiple generations” by JMC Diehl & PHW Biedermann

Table S1: Mean and standard deviation per ‘Treatment’ of bacterial genera (> 5% relative abundance) in disperser communities

Genus	reference	*N* nests	control	*N* nests	garden pathogen	*N* nests	entomo-pathogen	*N* nests
Acinetobacter	7.5 ± 1.5	2	9.3 ± 2.9	4	6.2 ± 0.8	2	5.2 ± 0.2	2
Anoxybacillus	/	/	/	/	6.3	1	6.1 ± 1.1	2
Azotobacter	31.6 ± 37.2	2	5.8	1	/	/	/	/
Brochothrix	/	/	/	/	/	/	20.9	1
Brucella	11.8	1	17.8 ± 12.7	5	6.9 ± 2.3	2	20.2 ± 5.8	6
Carnobacterium	22.1	1	/	/	/	/	/	/
Clavibacter	5.0	1	/	/	/	/	/	/
Comamonas	8.3	1	/	/	/	/	18.0	1
Dyella	5.6	1	/	/	/	/	/	/
Enhydrobacter	/	/	5.4	1	/	/	/	/
Enterococcus	/	/	/	/	/	/	5.1	1
Erwinia	32.3 ± 23.9	14	33.9 ± 23.9	14	30.7 ± 21.7	6	20.4 ± 29.3	9
Gilliamella	6.5	1	/	/	15.7	1	6.8	1
Hydrogenophilus	/	/	/	/	5.7	1	/	/
Leuconostoc	8.7	1	/	/	/	/	/	/
Macrococcus	6.4	1	/	/	/	/	/	/
Massilia	6.1	1	/	/	/	/	/	/
Meiothermus	/	/	/	/	/	/	5.5	1
Norcardiaceae, unknown	/	/	/	/	/	/	8.8 ± 4.5	2
Pseudomonas	23.5 ± 17.5	4	18.8	1	6.2	1	6.7 ± 2.0	2
Pseudoxanthomonas	29.2 ± 18.6	18	25.9 ± 19.6	16	35.5 ± 27.1	10	25.2 ± 18.3	11
Ralstonia	10.0 ± 2.7	3	9.0 ± 3.3	10	15.3 ± 10.1	5	7.8 ± 1.7	6
Salmonella	/	/	6.8	1	/	/	10.1	1
Sanguibacter	7.1	1	/	/	/	/	/	/
Serratia	35.5	1	/	/	/	/	/	/
Staphylococcus	8.9 ± 5.2	2	18.1 ± 6.2	3	20.3	1	16.6 ± 13.1	2
Stenotrophomonas	14.7	1	/	/	/	/	/	/
Streptococcus	/	/	5.8	1	/	/	/	/
Undibacterium	14.4 ± 6.4	3	11.2 ± 4.4	10	16.0 ± 5.8	4	10.0 ± 3.9	7
Yersinia	/	/	5.3	1	/	/	/	/
Total		19		17		11		12

Table S2: Mean and standard deviation per 'Treatment' of fungal genera (> 5% relative abundance) in disperser communities

Genus	reference	*N* nests	control	*N* nests	garden pathogen	*N* nests	entomo-pathogen	*N* nests
Aspergillus	/	/	5.6	1	19.5 ± 15.2	7	10.6 ± 3.8	4
Beauveria	/	/	/	/	39.3 ± 44.2	3	66.3 ± 36.8	7
Chaetomium	70.8 ± 28.2	23	80.2 ± 14.0	22	46.3 ± 24.7	20	51.5 ± 35.5	7
Cladophialophora	/	/	/	/	11.6	1	/	/
Cladosporium	/	/	/	/	7.6	1	32.4 ± 15.1	2
Didymellaceae, unknown	/	/	/	/	5.1	1	/	/
Dothideomycetes, unknown	/	/	/	/	16.0	1	/	/
Epicoccum	/	/	/	/	20.7 ± 16.0	9	/	/
Mycosphaerellaceae, unkown	/	/	/	/	19.9	1	/	/
Penicillium	8.1 ± 2.8	3	20.3 ± 11.8	3	20.1 ± 12.0	6	22.4	1
Phaeoacremonium	35.8 ± 20.1	3	21.4 ± 24.2	3	59.8	1	11.0 ± 7.8	2
Raffaelea	39.2 ± 25.5	15	16.0 ± 8.7	14	28.2 ± 18.7	15	17.9 ± 13.4	5
Ramalinaceae, unknown	/	/	/	/	23.6	1	/	/
Sordariomycetes, unknown	/	/	/	/	/	/	16.2	1
Talaromyces	/	/	/	/	/	/	37.6 ± 7.1	2
Total		25		22		22		12

Table S3: Mean and standard deviation per 'Treatment' and 'Generation' of bacterial genera (> 5% relative abundance) in gallery communities

Genus	reference	*N* nests	control	*N* nests	garden pathogen	*N* nests	entomo-pathogen	*N* nests
Bradyrhizobium	/	/						
F1			12.8 ± 9.6	11	/	/	/	/
F2			9.1 ± 4.1	11	/	/	/	/
F3			9.2 ± 2.5	5	/	/	/	/
Brucella	23.9	1						
F1			36.0 ± 10.5	8	29.6 ± 15.3	10	28.2 ± 13.2	8
F2			29.0 ± 14.0	9	40.7 ± 13.9	6	47.0 ± 21.6	13
F3			35.0 ± 19.4	9	43.0 ± 26.7	3	47.6 ± 12.3	12
Erwinia	40.0 ± 9.8	20						
F1			40.3 ± 15.3	13	39.0 ± 15.2	13	16.1 ± 2.2	2
F2			44.6 ± 11.7	20	45.1 ± 13.0	13	55.0	1
F3			49.0 ± 10.1	15	53.3 ± 8.3	11	32.0 ± 9.1	3
Methylobacterium	/	/						
F1			6.3	1	18.3 ± 10.2	9	/	/
F2			/	/	16.7 ± 7.1	8	/	/
F3			/	/	12.6 ± 5.6	9	/	/
Nitrobacter	/	/						
F1			7.1 ± 2.1	2	/	/	/	/
F2			5.2	1	/	/	/	/
F3			/	/	/	/	/	/
Pseudomonas	30.0 ± 10.9	3						
F1			/	/	/	/	/	/
F2			/	/	/	/	/	/
F3			/	/	12.1 ± 6.1	6	/	/
Pseudoxanthomonas	61.8 ± 21.7	25						
F1			45.7 ± 9.6	19	48.5 ± 22.2	20	35.0 ± 14.4	8
F2			37.3 ± 9.8	21	51.3 ± 16.6	21	44.8 ± 19.5	13
F3			41.8 ± 18.5	20	53.6 ± 17.4	21	47.9 ± 17.5	13
Rhizobium	8.4	1						
F1			/	/	/	/	/	/
F2			/	/	/	/	/	/
F3			/	/	/	/	/	/
Serratia	35.2	1						
F1			/	/	/	/	41.5 ± 10.4	6
F2			/	/	/	/	32.3 ± 17.0	4
F3			/	/	/	/	/	/
Stenotrophomonas	16.8	1						
F1			/	/	/	/	/	/
F2			/	/	/	/	/	/
F3			/	/	/	/	/	/
Total		26		60		62		35

Table S4: Mean and standard deviation per 'Treatment' and 'Generation' of fungal genera (> 5% relative abundance) in gallery communities

Genus	reference	*N* nests	control	*N* nests	garden pathogen	*N* nests	entomo-pathogen	*N* nests
Aspergillus	/	/						
F1			/	/	15.6	1	/	/
F2			/	/	9.4 ± 4.3	2	/	/
F3					10.0 ± 2.7	7	/	/
Beauveria	/	/						
F1			59.7 ± 41.9	3	/	/	87.4 ± 7.4	5
F2			33.2 ± 23.0	3	20.5 ± 9.8	7	62.2 ± 18.5	11
F3			24.8 ± 30.3	3	38.6 ± 20.0	10	64.0 ± 23.9	13
Chaetomiaceae, unknown	5.4	1						
F1			5.3 ± 0.5	3	5.1	1	5.0	1
F2			5.4	1	/	/	/	/
F3			/	/	/	/	/	/
Chaetomium	91.6 ± 12.9	16						
F1			49.8 ± 29.8	18	39.7 ± 24.2	9	29.7 ± 23.9	3
F2			32.2 ± 26.3	14	54.4 ± 26.5	17	24.9 ± 20.6	7
F3			50.4 ± 37.0	13	61.7 ± 29.3	19	30.1 ± 27.7	7
Penicillium	/	/						
F1			/	/	/	/	/	/
F2			14.4 ± 7.4	3	22.1 ± 19.5	3	27.4	1
F3			11.4	1	14.0 ± 7.2	2	44.5	1
Phaeoacremonium	18.2 ± 11.9	4						
F1			47.8 ± 27.0	15	68.6 ± 18.8	8	39.7 ± 24.7	3
F2			69.0 ± 29.0	20	44.6 ± 35.2	17	34.3 ± 26.4	11
F3			78.0 ± 25.6	15	31.4 ± 23.8	8	27.0 ± 16.4	6
Raffaelea	94.3	1						
F1			/	/	/	/	/	/
F2			/	/	9.4	1	/	/
F3			/	/	12.7	1	/	/
Sordariomycetes, unknown	/	/						
F1			/	/	/	/	/	/
F2			7.6 ± 2.6	2	/	/	7.1 ± 1.2	6
F3			/	/	6.9	1	/	/
Talaromyces	/	/						
F1			/	/	11.4	1	/	/
F2			/	/	6.1	1	/	/
F3			/	/	5.5 ± 0.5	2	/	/
Total		17		60		52		34

Table S5: Data summary of disperser count, days till first females emerged in nests and period from first female to first disperser in different treatments and generations

	Treatment	generation	mean	median	min	max	*N*
No. disperser	reference		46.5	45	2	115	53
	control	F1	4.95	0	0	32	131
		F2	20.5	17	0	109	130
		F3	24.1	21	1	75	147
	garden pathogen	F1	3.91	0	0	23	130
		F2	18.4	13	0	78	88
		F3	14.6	13.5	0	47	104
	entomopathogen	F1	0.89	0	0	49	132
		F2	8.12	7	1	23	25
		F3	5.66	4	0	30	53
Time till 1st female	reference		30.0	28	27	43	53
	control	F1	29.3	29	26	38	131
		F2	28.3	28	25	37	127
		F3	28.2	28	24	40	144
	garden pathogen	F1	29.7	29	25	66	129
		F2	28.8	28	24	38	88
		F3	27.6	27	25	38	104
	entomopathogen	F1	29.4	29	25	38	132
		F2	28.4	28	25	34	24
		F3	27.5	27	24	38	53
Period 1st female to 1st disperser	reference		10.8	9	6	22	53
	control	F2	10.9	10	5	22	125
		F3	11.4	10	6	72	126
	garden pathogen	F2	10.1	10	4	18	86
		F3	10.0	10	5	25	100
	entomopathogen	F2	8.8	8.5	6	11	24
		F3	10.7	10	5	18	47
Period 1st larvae to 1st female	reference		15.1	15	8	21	53
	control	F1	14.8	15	4	22	123
		F2	14.2	14	7	21	122
		F3	15.5	16	6	24	143
	garden pathogen	F1	15.0	16	2	42	120
		F2	14.5	14	9	23	86
		F3	15.0	15	9	21	103
	entomopathogen	F1	15.1	16	8	23	125
		F2	12.7	13	6	17	24
		F3	14.5	15	6	18	53

Table S6: Statistical summary of the linear mixed models and post-hoc test of the breeding and life-history dataset. For the general comparison of the treatment we used data of the '*F0*' and '*F3*' generation only.

Variable	Anova			Post-hoc			
	χ^2	*df*	*p*	contrast	*df*	*t*	*p*
Total number dispersers	263.39	3	<0.001	reference - control	301	6.71	<0.001
				reference – garden pathogen	301	10.54	<0.001
				reference – entomopathogen	301	14.57	<0.001
				control – garden pathogen	301	6.56	<0.001
				control - entomopathogen	301	12.52	<0.001
				garden pathogen - entomopathogen	301	7.73	<0.001
Time till 1st emerged female	36.96	3	<0.001	reference - control	298	4.62	<0.001
				reference – garden pathogen	298	4.96	<0.001
				reference – entomopathogen	298	5.73	<0.001
				control – garden pathogen	298	0.72	0.890
				control - entomopathogen	298	2.48	0.065
				garden pathogen - entomopathogen	298	1.92	0.221
Period 1st female to 1st disperser	6.05	3	0.109				
Period 1st larvae to 1st female	7.31	3	0.063	reference - control	296	-0.88	0.814
				reference – garden pathogen	296	0.08	1.0
				reference – entomopathogen	296	1.05	0.718
				control – garden pathogen	296	1.58	0.394
				control - entomopathogen	296	2.64	0.044
				garden pathogen - entomopathogen	296	1.42	0.491

Table S7: Statistical summary of the linear mixed models and post-hoc test of the breeding and life-history dataset. For the analysis of the treatment and generation we excluded the '*F0*' generation. (C = control, GP = garden pathogen, EP = entomopathogen)

Variable	Anova			Post-hoc			
	χ^2	*df*	*p*	contrast	*df*	*t*	*p*
Total number dispersers (treatment * generation)	45.38	4	<0.001	F1 – F2	904		C/GP/EP: <0.001
				F1 – F3	904		C/GP/EP: <0.001
				F2 – F3	904		C: 0.014, GP: 0.468; EP: 0.085
Time till 1st emerged female (generation)	91.53	2	<0.001	F1 – F2	902	4.64	<0.001
				F1 – F3	902	9.45	<0.001
				F2 – F3	902	3.66	< 0.001
Period 1st female to 1st disperser (treatment * generation)	7.28	2	0.026	control (F2-F3)	479	0.21	0.836
				GP (F2-F3)	479	0.09	0.926
				EP (F2-F3)	479	-2.90	0.004
Period 1st larvae to 1st female (treatment * generation)	9.77	4	0.045	F1 – F2	864		C: 0.391; GP: 0.858; EP: 0.009
				F1 – F3	864		C: 0.051; GP: 0.753; EP: 0.686
				F2 – F3	864		C: <0.001; GP: 0.183; EP: 0.032

Table S8: Statistical summary of Anovas of the generalized linear mixed-effect models of observed nest behaviors. Each treatment ('*control*', '*garden pathogen*' and '*entomopathogen*') was tested for changes over the generations. Data of the '*F0*' generation ('*reference*' group) served as reference.

	Larvae			**Females**		
hygienic						
	X^2	*df*	*p*	X^2	*df*	*p*
control	48.20	3	< 0.001	22.97	3	<0.001
garden pathogen	34.78	3	<0.001	44.02	3	<0.001
entomopathogen	5.30	3	0.151	36.95	3	<0.001
resting						
	X^2	*df*	*p*	X^2	*df*	*p*
control	87.65	3	< 0.001	87.97	3	<0.001
garden pathogen	48.24	3	<0.001	187.41	3	<0.001
entomopathogen	31.78	3	<0.001	142.6	3	<0.001
feeding						
	X^2	*df*	*p*	X^2	*df*	*p*
control	59.61	3	< 0.001	25.89	3	<0.001
garden pathogen	39.33	3	<0.001	52.73	3	<0.001
entomopathogen	57.43	3	<0.001	19.50	3	<0.001

Table S9: Statistical summary of post-hoc tests of the generalized linear mixed-effect models of observed nest behaviors. Each treatment ('*control*', '*garden pathogen*' and '*entomopathogen*') was tested for changes over the generations. Data of the '*F0*' generation ('*reference*' group) served as reference.

	control			garden pathogen			entomopathogen		
hygienic									
larvae									
contrast	***SE***	***z***	***p***	***SE***	***z***	***p***	***SE***	***z***	***p***
F0 – F1	0.070	3.892	0.001	0.071	3.058	0.012	0.081	2.259	0.108
F0 – F2	0.069	5.325	<0.001	0.076	3.959	<0.001	0.146	1.089	0.696
F0 – F3	0.068	-0.104	1.00	0.069	-1.032	0.731	0.111	1.350	0.531
F1 – F2	0.067	1.390	0.505	0.075	1.106	0.686	0.140	-0.166	0.998
F1 – F3	0.067	-4.190	<0.001	0.069	-4.182	<0.001	0.104	-0.305	0.990
F2 – F3	0.066	-5.706	<0.001	0.074	-5.024	<0.001	0.144	-0.058	1.00
females									
contrast	***SE***	***z***	***p***	***SE***	***z***	***p***	***SE***	***z***	***p***
F0 – F1	0.119	4.263	<0.001	0.110	6.542	<0.001	0.116	5.270	<0.001
F0 – F2	0.121	3.812	0.001	0.117	4.204	<0.001	0.202	2.834	0.024
F0 – F3	0.120	1.859	0.246	0.113	3.587	0.002	0.151	-0.171	0.998
F1 – F2	0.112	-0.416	0.976	0.114	-1.994	0.190	0.199	-0.192	0.998
F1 – F3	0.113	-2.518	0.057	0.108	-2.907	0.019	0.148	-4.302	<0.001
F2 – F3	0.112	-2.129	0.144	0.114	-0.775	0.866	0.222	-2.696	0.035
resting									
larvae									
contrast	***SE***	***z***	***p***	***SE***	***z***	***p***	***SE***	***z***	***p***
F0 – F1	0.247	0.469	0.966	0.258	0.974	0.764	0.204	1.791	0.278
F0 – F2	0.250	-0.638	0.920	0.278	0.335	0.987	0.370	0.310	0.990
F0 – F3	0.274	7.357	<0.001	0.280	5.822	<0.001	0.303	5.512	<0.001
F1 – F2	0.233	-1.179	0.640	0.257	-0.615	0.927	0.363	-0.689	0.901
F1 – F3	0.258	7.353	<0.001	0.257	5.361	<0.001	0.293	4.470	<0.001
F2 – F3	0.252	8.627	<0.001	0.263	5.825	<0.001	0.412	3.781	0.001
females									
contrast	***SE***	***z***	***p***	***SE***	***z***	***p***	***SE***	***z***	***p***
F0 – F1	0.246	0.487	0.962	0.094	-13.428	<0.001	0.102	-10.754	<0.001
F0 – F2	0.248	-0.653	0.915	0.101	-7.610	<0.001	0.175	-6.237	<0.001
F0 – F3	0.271	7.422	<0.001	0.097	-5.592	<0.001	0.137	-0.926	0.791
F1 – F2	0.233	-1.207	0.623	0.096	5.226	<0.001	0.171	0.018	1.00
F1 – F3	0.257	7.342	<0.001	0.091	7.984	<0.001	0.132	7.357	<0.001
F2 – F3	0.252	8.631	<0.001	0.096	2.347	0.088	0.194	4.978	<0.001
feeding									
larvae									
contrast	***SE***	***z***	***p***	***SE***	***z***	***p***	***SE***	***z***	***p***
F0 – F1	0.062	-6.556	<0.001	0.067	-5.659	<0.001	0.069	-5.765	<0.001
F0 – F2	0.062	-3.923	0.001	0.071	-3.512	0.003	0.123	-0.803	0.853
F0 – F3	0.061	-6.846	<0.001	0.067	-5.262	<0.001	0.090	-6.595	<0.001
F1 – F2	0.059	2.740	0.031	0.070	1.793	0.276	0.122	2.424	0.073
F1 – F3	0.058	-0.186	0.998	0.065	0.392	0.980	0.089	-2.233	0.114
F2 – F3	0.057	-3.036	0.013	0.069	-1.448	0.469	0.136	-3.653	0.002
females									
contrast	***SE***	***z***	***p***	***SE***	***z***	***p***	***SE***	***z***	***p***
F0 – F1	0.123	4.236	<0.001	0.121	5.375	<0.001	0.140	4.212	<0.001
F0 – F2	0.122	3.122	0.010	0.127	3.294	0.006	0.243	1.910	0.224
F0 – F3	0.122	0.535	0.951	0.119	-0.849	0.831	0.183	0.755	0.875
F1 – F2	0.116	-1.231	0.607	0.125	-1.829	0.260	0.240	-0.523	0.954
F1 – F3	0.117	-3.913	0.001	0.117	-6.432	<0.001	0.179	-2.520	0.057
F2 – F3	0.115	-2.733	0.032	0.124	-4.215	<0.001	0.268	-1.218	0.615

Table S10: Statistical summary of Spearman's rank correlation between disperser count and hygienic behaviours for each of the three treatments ('*control*', '*fungus pathogen*' and '*entomopathogen*').

Treatment	Larvae			Females		
	S	***rho***	***p***	***S***	***rho***	***p***
control	1353183	0.382	<0.001	2135011	0.062	0.343
garden pathogen	952956	0.336	<0.001	1243487	0.121	0.084
entomopathogen	266502	0.002	0.987	224114	0.160	0.084

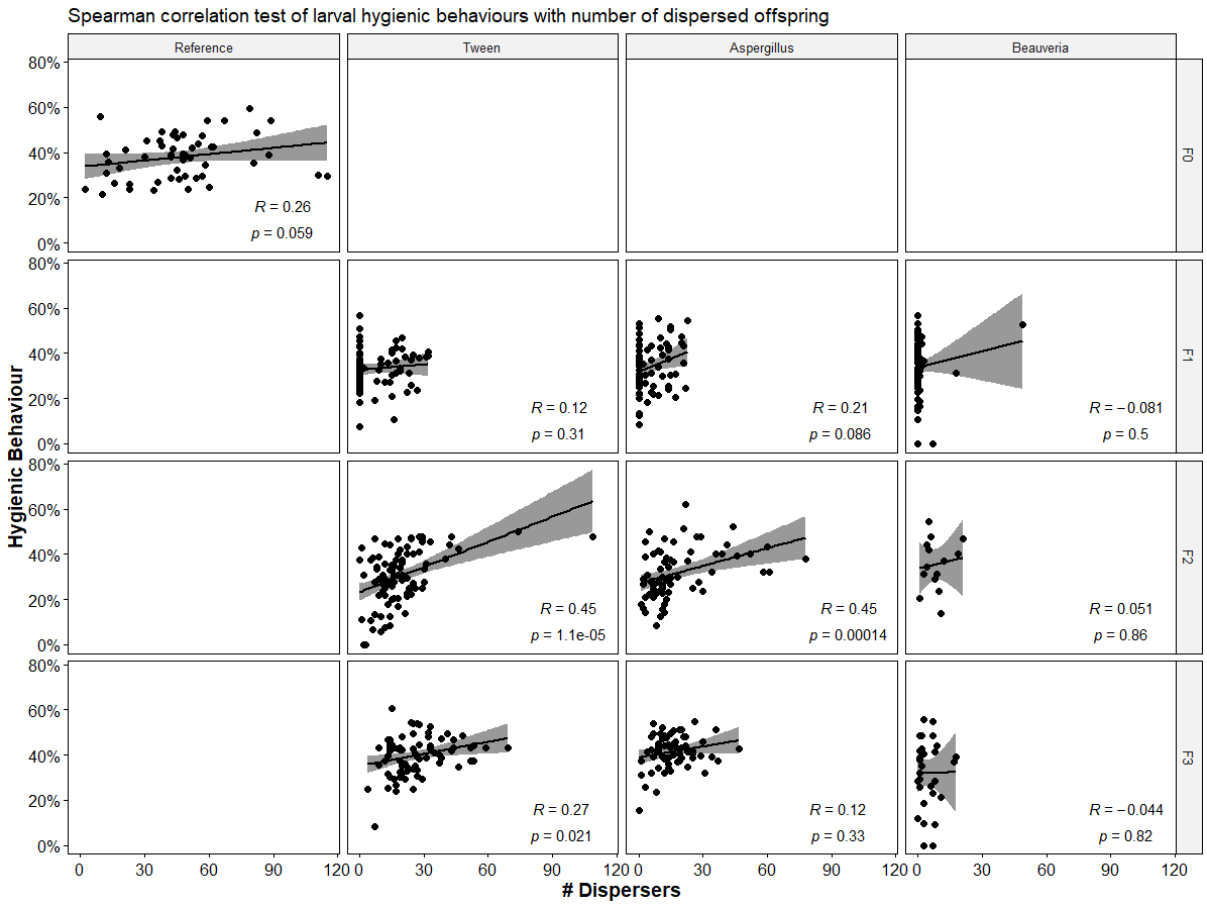

Figure S10: Scatterplots and summaries of the spearman's rank correlation tests for larval hygienic behaviours with the number of dispersed female offspring in the four treatments ('*reference*', '*control*', '*garden pathogen*' and '*entomopathogen*') over four generations ('*F0-3*').

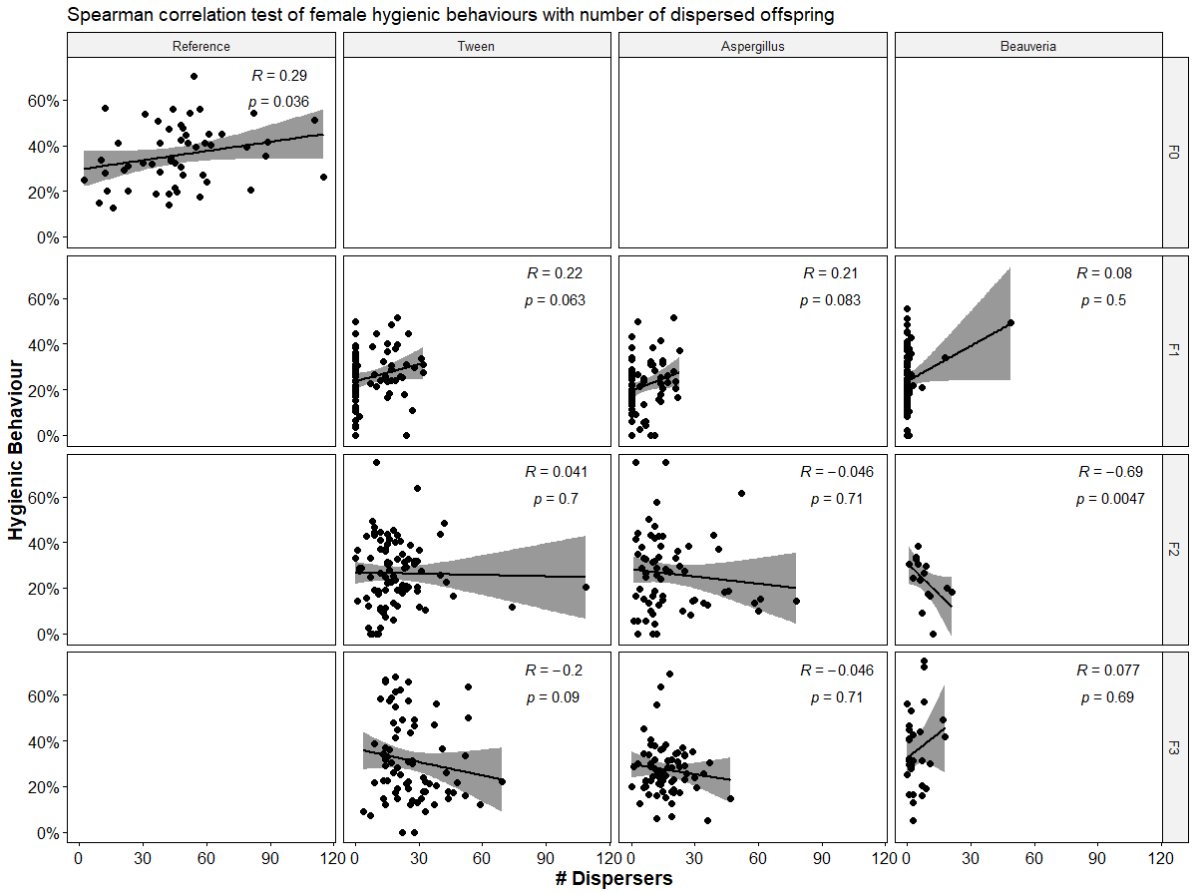

Figure S11: Scatterplots and summaries of the spearman's rank correlation tests for female hygienic behaviours with the number of dispersed female offspring in the four treatments ('*reference*', '*control*', '*garden pathogen*' and '*entomopathogen*') over four generations ('*F0-3*').

Lethality of used pathogens on larvae and mature females of *Xyleborinus saxesenii*

In a small side study, we tested in winter 2021 the general lethality of *Beauveria bassiana* and *Aspergillus* sp. with three different concentration (10^5, 10^6 and 10^7 spores/ml) to get a better estimate of our results. Both active life stages in *X. saxesenii* (larvae and adult/mature females) were included and thus gave us insightful information for the interpretation of our main results.

The production of spore solutions and beetle breeding were handled in the same way as described in the main methods. We used beetles of the same populations originally collected 2019 in the Steinbachtal near Wuerzburg, Germany (Decimal degree [DD]: 49.767500, 9.896770) and the Bavarian Forest (DD: 8.8816832, 13.5215362). The experiment was carried out in 96-well plates (BRANDplates® -pureGrade™-, BRAND) with 6 mm filter discs (Rotilabo®-test flakes, Carl Roth GmbH + Co. KG) in each well, where either a single female

beetle or larvae was placed in. Previously, individuals freshly extracted from nests were surface sterilized with 70% EtOH and washed with tap water. Per life stage, we applied 15 µl of three different solutions on the individuals into the well. (1) In the 'control' group, specimens were treated with a pure 0.05% Tween 20/PBS (1x) buffer solution (N_{Adults} = 132, N_{Larvae} = 45). The fungal spore solutions were applied in three concentrations a) 10^5 spores/ml (0.05% Tween 20/PBS (1x) buffer solution), b) 10^6 spores/ml and c) 10^7 spores/ml for each (2) *Aspergillus* sp. (N_{Adults} = 144, N_{Larvae} = 106) and (3) *B. bassiana* (N_{Adults} = 180, N_{Larvae} = 34) (Fig. S3). After the treatment, plates were closed with the lid and kept in a climate chamber at 25°C (70% humidity, permanent darkness). We checked vitality daily for the following month and categorized the individual dead, if they showed no movement even after poking with a sterile dissecting needle.

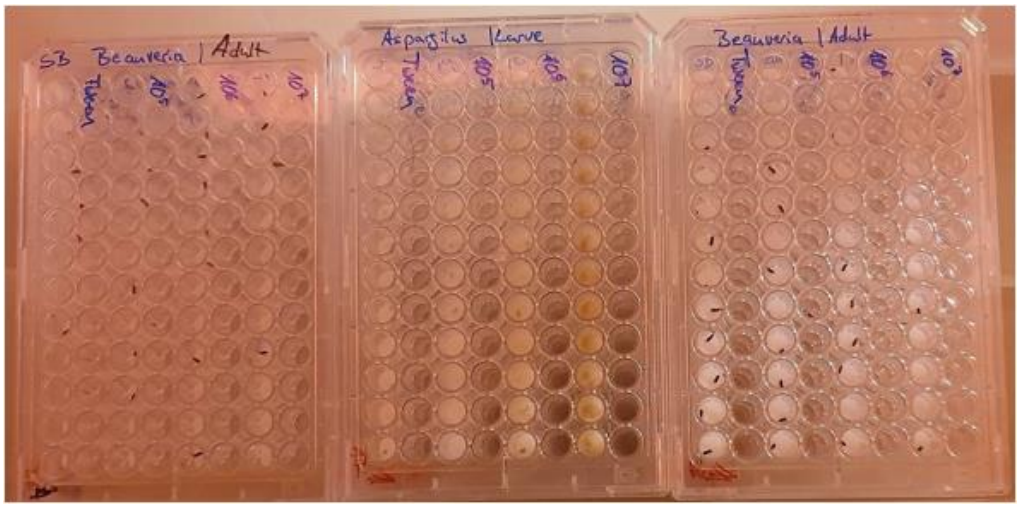

Figure S12: Experimental set-up for the survival study. Each pathogen (*Aspergillus* sp. and *Beauveria bassiana*) was separately tested in three concentrations (10^5, 10^6 or 10^7 spores/ml) on 96-well plates, including one row with the '*control*' solution. Every other row was left blank. We used separate plates for larvae and adult females.

All statistical analyses and visualisation of the survival data were performed in RStudio (Version 1.4.1106) with R version 4.0.5 (R Core Team 2021) using the 'survival' (Therneau 2021) and 'survminer' package (Kassambara et al. 2021), as well as 'ggplot2' (Wickham 2016) and 'ggpubr' (Kassambara 2020) to produce graphical output.

We ran Kaplan-Meier models for each pathogen with the control, including the life stage and spore concentration and plotted the survival curves. Afterwards we ran Log-Ranks test for a pairwise comparison of the survival of each life stage (larvae or adult female) in a treatment (*Aspergillus* or *Beauveria*) applying the 'BH' adjustment method. Here the 'control' was used as reference and compared against the three concentrations, as well as comparisons between concentrations. Finally, we calculated two Cox proportional hazards regression models with the and plotted the output in a Forest plot.

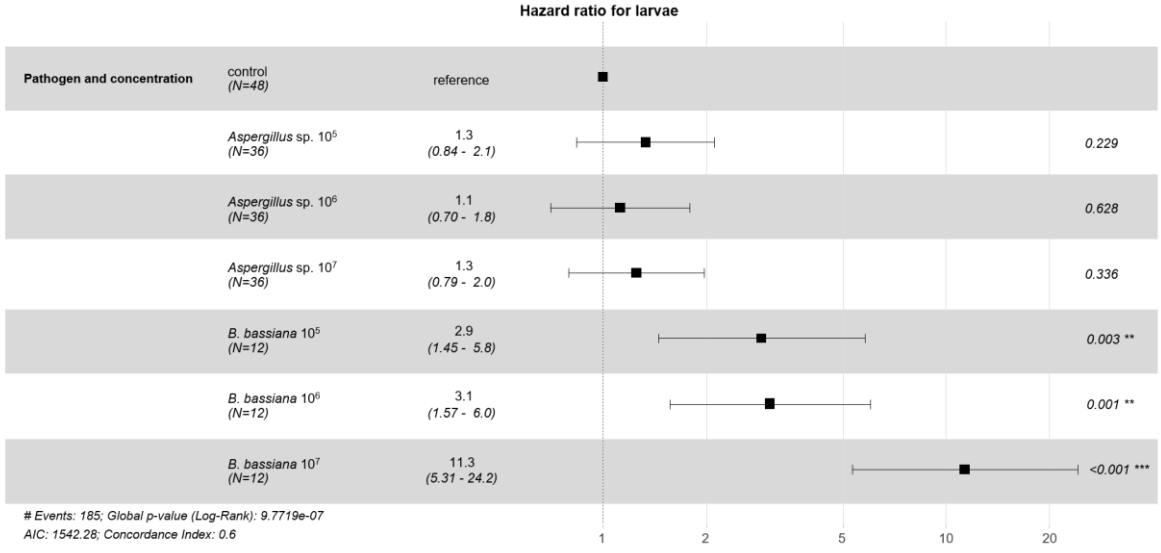

Figure S13: Forest plot for Cox proportional hazards model of tested *X. saxesenii* larvae.

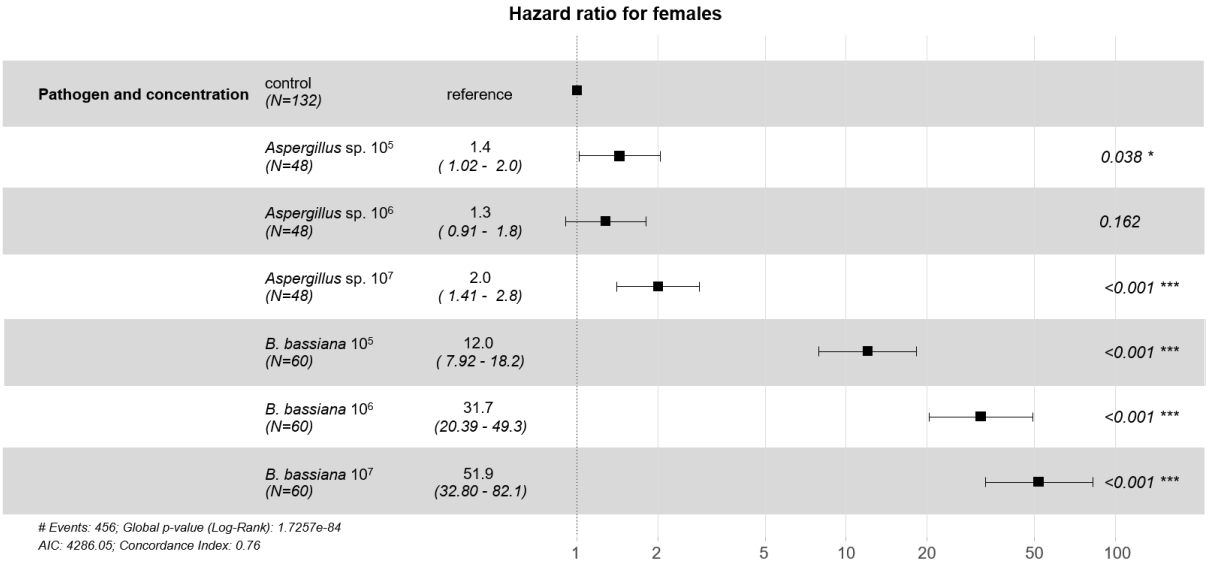

Figure S14: Forest plot for Cox proportional hazards model of tested *X. saxesenii* adult females.

Table S11: Median survival and number of events from Kaplan-Meier models and statistical output of Log Rank test comparing the survival of tested groups. Bold numbers signal significant differences in the pairwise comparison of tested groups with the "BH" p-value adjusting method (Benjamini and Hochberg, 1995).

Life stage	Pathogen/concentration	median survival (days)	events	Log Rank Test		
Adults	**control**	8	132	**control**	**10^5 spores/ml**	**10^6 spores/ml**
	Beauveria/10^5 spores/ml	5	60	**< 0.001**	-	-
	Beauveria/10^6 spores/ml	4	60	**< 0.001**	**< 0.001**	-
	Beauveria/10^7 spores/ml	4	60	**< 0.001**	**< 0.001**	**0.008**
	Aspergillus/10^5 spores/ml	9	48	0.129	-	-
	Aspergillus/10^6 spores/ml	9.5	48	0.361	0.510	-
	Aspergillus/10^7 spores/ml	8	48	**0.015**	0.072	**0.017**
Larvae	**control**	3	45	**control**	**10^5 spores/ml**	**10^6 spores/ml**
	Beauveria/10^5 spores/ml	3	11	0.064	-	-
	Beauveria/10^6 spores/ml	3	12	**0.042**	0.749	-
	Beauveria/10^7 spores/ml	2	11	**< 0.001**	**< 0.001**	**< 0.001**
	Aspergillus/10^5 spores/ml	3	36	0.82	-	-
	Aspergillus/10^6 spores/ml	4	34	0.82	0.82	-
	Aspergillus/10^7 spores/ml	4	36	0.82	0.82	0.82

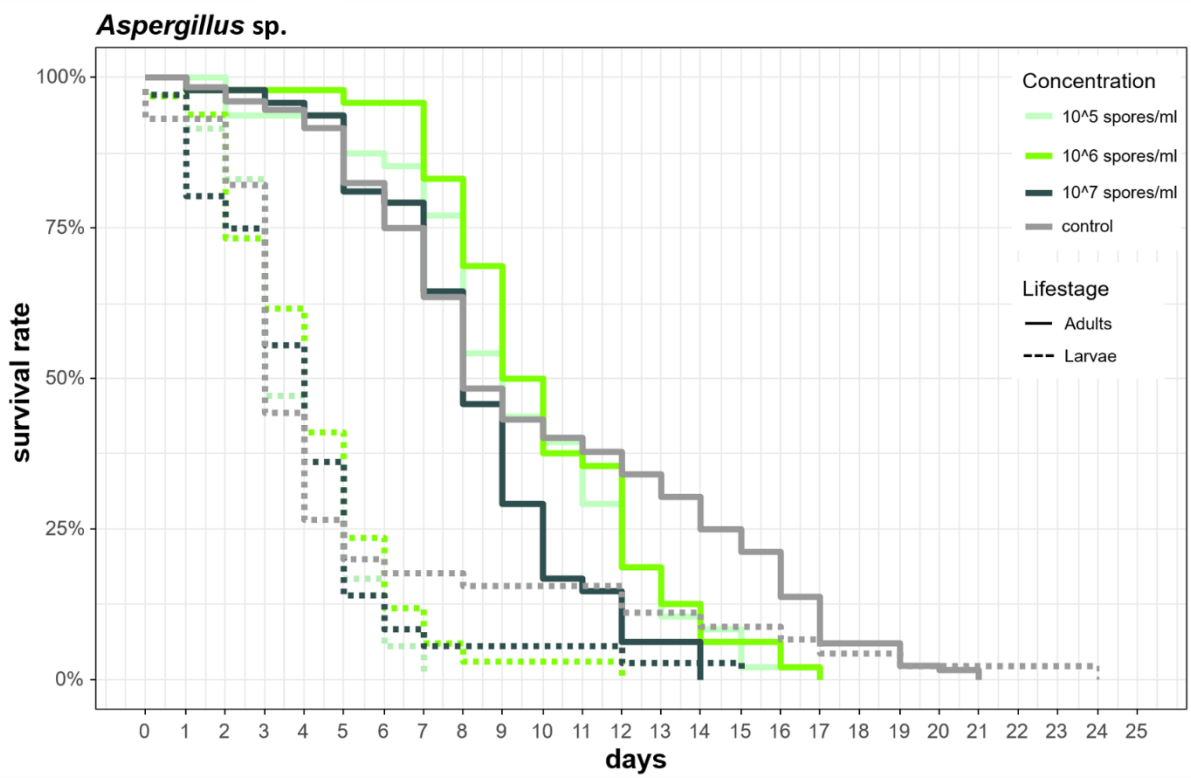

Figure S15: Survival curves of *X. saxesenii* larvae (dotted) and adult females (solid) infected with *Aspergillus* sp. in three concentrations. Control individuals were treated with buffer solution only.

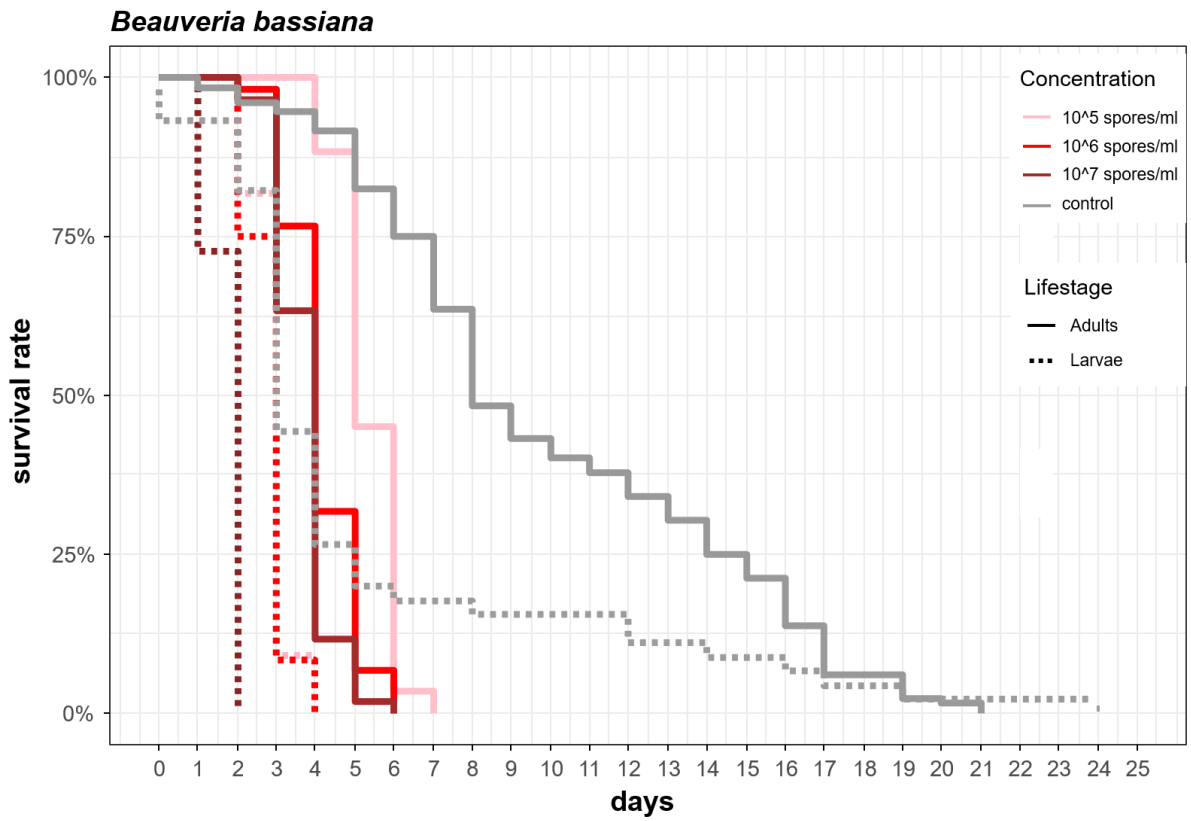

Figure S16: Survival curves of *X. saxesenii* larvae (dotted) and adult females (solid) infected with *Beauveria bassiana* in three concentrations. Control individuals were treated with buffer solution only.

References

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Stat Soc Ser B. 57(1):289–300. doi:10.1111/J.2517-6161.1995.TB02031.X. https://onlinelibrary.wiley.com/doi/full/10.1111/j.2517-6161.1995.tb02031.x.

Kassambara A. 2020. ggpubr: “ggplot2” based publication ready plots. https://cran.r-project.org/package=ggpubr.

Kassambara A, Kosinski M, Biecek P. 2021. survminer: Drawing survival curves using “ggplot2”. R package version 0.4.9. https://cran.r-project.org/package=survminer.

R Core Team. 2021. R: A language and environment for statistical computing. https://www.r-project.org/.

Therneau TM. 2021. A package for survival analysis in R. R package version 3.2-10. mran.revolutionanalytics.com. https://cran.r-project.org/package=survival.

Wickham H. 2016. ggplot2: Elegant graphics for data analysis. New York: Springer-Verlag New York.

CHAPTER 4

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Habitat quality determines dispersal decisions and fitness in a beetle – fungus mutualism.

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Habitat Quality Determines Dispersal Decisions and Fitness in a Beetle – Fungus Mutualism

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Delayed dispersal of sexually mature offspring is a fundamental component of cooperative breeding. In ambrosia beetles, female offspring temporarily remain in their natal nest and refrain from reproduction, instead investing in alloparental care. Previous work has demonstrated a link between helping behaviour and the increased need for pathogen defence, arising from their close association with fungal cultivars. In the ambrosia beetle *Xyleborinus saxesenii*, mature female offspring can effectively fight pathogen infections and manage the microbial composition within the nest by adjusting the frequency of different hygienic and nest maintenance behaviours. This suggests a potential to respond flexibly to the ecology of their nest, which calls for a better understanding of the connection between behaviour and the microbial community thriving within their nests. Here, we studied the significance of the mutualistic fungus garden composition for the beetles' nest ecology and fitness by experimentally varying substrate quality. We found that the vertically transmitted ambrosia fungus garden is composed of at least two fungus mutualist species and a wide variety of other microbes varying in their relative abundance. This is strongly affected by the moisture content of the substrate, which in nature depends on the age and type of wood. We found that the mutualist fungi complement each other in terms of dryness-resistance, allowing the beetles to utilise a broad range of substrates over prolonged time during which the wood gradually desiccates. Under suboptimal humidity conditions, the interaction between host and multiple fungus species has important ramifications for the behaviour of philopatric helpers, including their alloparental investment, sibling cannibalism and the timing of dispersal. Rearing five generations of beetles consecutively in dry substrate resulted in transgenerational effects on philopatry and alloparental care, probably mediated through the dominance of a particular fungus species that was driven by the experimental habitat condition. Interestingly, the nests of these selection lines produced much more offspring after five generations than any first-generation nest, which may have reflected increased egg laying by non-dispersing daughters. Our study highlights the importance of considering the interactions between the microbial community and their insect hosts for understanding social evolution in cooperatively breeding beetles.

Keywords: social evolution, habitat quality, cooperation, insect-fungus mutualism, ambrosia beetles, cooperative breeding, *Xyleborinus saxesenii*, dispersal

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INTRODUCTION

Cooperative breeding is characterized by alloparental care (i.e., brood care shown by non-parents), which evolved independently in many taxonomic groups including spiders, insects, fish, birds and mammals (Skutch, 1961; Taborsky, 1994; Choe and Crespi, 1997; Koenig and Dickinson, 2016; Rubenstein and Abbot, 2017). An alloparent's propensity to help caring for the offspring of others is often attributed to inclusive fitness benefits (Hamilton, 1964; Bourke, 2011) and correlated pay-offs (Taborsky et al., 2016). Adverse conditions like harsh environments, high predation risk and habitat saturation may cause dispersal delays, which sets the stage for the evolution of alloparental brood care by philopatric individuals (Stacey, 1979; Koenig et al., 1992; Heg et al., 2004; Mullon et al., 2018). In contrast to these environmental constraints, the potential significance of interspecific relations for the evolution of philopatry, alloparental care, task partitioning and social behaviour in cooperative breeders has received little attention. This is an important gap especially concerning invertebrates that are strongly affected by relationships with microbes such as fungi and bacteria (Hart et al., 2002; Mueller et al., 2005; Biedermann and Taborsky, 2011; Biedermann and Rohlfs, 2017).

Microbes can have various adverse effects on insects that may be mitigated by brood care. For instance, many social insects have evolved nesting behaviours where constant grooming of the offspring lowers the risk of infection and spread of pathogens (Ayasse and Paxton, 2002). Such nest-wide social pathogen defence behaviours have been conceptually framed as "social immunity traits" (Cremer et al., 2007). There is growing evidence that the evolution of social immunity traits goes hand in hand with the evolution of complex insect sociality, since they have been observed in a range of insects showing different degrees of parental and alloparental brood care, including eusocial taxa as well as burying beetles with biparental brood care and cooperatively breeding ambrosia beetles (Cotter et al., 2010; Meunier, 2015; Van Meyel et al., 2018; Nuotclà et al., 2019).

Social immunity in cooperatively breeding insects is apparently linked to their frequent exposure to microorganisms that compete for food or can have pathogenic effects, depending on the natural feeding ecology of the species. Burying beetles, for example, lay their eggs on buried carcasses of small vertebrates that are kept from rotting with the help of microorganisms (Shukla et al., 2018). Ambrosia beetles, on the other hand, dig their nests into the heartwood of trees, where they maintain a wood-colonizing garden of fungal mutualists as their sole food source. In these taxa, a central component of alloparental brood care consists of constant grooming of both the offspring and the food source. This keeps the offspring free from microbial growth (Biedermann and Taborsky, 2011) and it may be instrumental for the maintenance of a healthy mutualist community by selective removal of harmful microbes and spreading of beneficial species (Shukla et al., 2018; Nuotclà et al., 2019). Such intensive care for microbial mutualists is typically associated with highly social and fungiculturing attine ants and microtermitine termites, where the association with fungal crop "gardens" has led to extreme behavioural and physiological adaptations (Hölldobler and Wilson, 2009; Korb, 2010; Vesala et al., 2019; Biedermann and Vega, 2020). In some of these eusocial insects, part of the workers are even physically optimized for certain tasks associated with tending the fungal crop (Hart et al., 2002).

The importance of mutualistic microbes for the evolution of complex sociality is poorly understood. The association with mutualistic microbes may have facilitated the evolution of a social lifestyle in insects by providing them with a virtually endless food source, or under certain conditions sociality might be a prerequisite for maintaining a highly demanding fungiculture in the first place. As transitionary forms of sociality are rare in the eusocial insect taxa, the study of microbial influence on social evolution should focus on cooperatively breeding species relying on microbes for food or on other services. Recent studies suggest that under these circumstances, cooperation among siblings is necessary to compensate for increased pathogen pressure and a potential loss of parental care, if parents disappear (Nuotclà et al., 2019; Rebar et al., 2020).

Ambrosia beetles are a highly suitable model system to explore the relationship between insect hosts and their microbial mutualists since they independently evolved fungiculture at least 12 times (Kirkendall et al., 2015; Johnson et al., 2018), and different levels of social complexity can be found amongst the more than 3,500 known species. Brood care ranges from simple uniparental care to alloparental care and complex division of labour (Biedermann and Taborsky, 2011; Kirkendall et al., 2015). Compared to other wood-dwelling species that either consume nutrient rich phloem or wood tissue directly, fungus farming ambrosia beetle offspring might be much more dependent on brood care. In most species, the mutualistic fungus garden propagules are vertically transmitted by dispersing females and the garden needs to be first established before the female can begin to lay its eggs. Tunnels of wood boring insects offer ideal access points to a whole range of wood dwelling microbes, allowing them to penetrate trough the bark deeper into the heartwood of a tree (Ulyshen, 2016; Skelton et al., 2019). Some microbes may be in direct competition with the beetles' fungal mutualists and may even lead to a premature collapse of the nest (Nuotclà et al., 2019). Additionally, feeding on a fungus garden renders ambrosia beetle offspring less mobile compared to other wood dwelling species that constantly eat their way through fresh wood. This may render ambrosia beetles more vulnerable to pathogens entering the nest via the maternal entrance hole. Active microbial management may therefore be important not only in the establishment phase of a nest, but also throughout its entire lifetime. For instance, previous work on *Xyleborinus saxesenii* has demonstrated that these beetles show strong behavioural changes when the nest is challenged with a high pathogen load, leading to an effective social immune response and increased alloparental investment by daughters. This also caused delayed dispersal, apparently to fight the infection (Nuotclà et al., 2019). The present study expands on this work by shifting the focus from harmful to beneficial microbes in order to explore their potential role as drivers of dispersal decisions, parental care and social evolution. Newly developed methods for efficient lab rearing of the ambrosia beetles (Norris and Chu, 1985; Peer and Taborsky, 2004; Biedermann et al., 2009) and molecular tools for analysing

the microbial associates (Skelton et al., 2018, 2019) allow for an integrated approach to study microbe-driven social behaviour.

Our study system, *Xyleborinus saxesenii* dwells in relatively freshly dead wood of various tree species (Fischer, 1954). This breeding substrate may vary in humidity, depending on the degree of degradation as dead wood dries out constantly. The ephemerality of this dead wood habitat has been hypothesized to be a major hurdle for the evolution of complex sociality in ambrosia beetles, since it restricts the potential nest persistence and thus limits the overlap of multiple offspring generations (Kirkendall et al., 1997). In fact, the only candidate species where complex sociality beyond alloparental care has been assumed so far is also one of the very few species breeding exclusively in living trees, which allows for much longer lasting nests (Kent and Simpson, 1992; Smith et al., 2018). Besides the immediate limiting factor of diminishing nutrients in a dead tree (Ulyshen, 2016), the main limitation to nest longevity probably arises from the requirements of the ambrosia fungus garden, which was shown to be very sensitive to the humidity of the wood substrate. The chances that the beetles successfully establish a fungus garden is reduced when moisture levels are not within a certain range (cf. Hosking, 1973; Zimmermann and Butin, 1973; Biedermann et al., 2009; Ulyshen, 2016; personal observations). Since the beetles fully rely on these fungi for food, the initial quality of the substrate and the way it changes over time probably has a significant impact on fungus garden productivity, nest longevity and thus ultimately on the beetles' fitness. Behavioural strategies to slow down the drying of the wood, like plugging the entrance hole with a beetle's body or with faeces and frass, have been discussed but it is unclear to which degree the beetles or the fungus might be able to influence resource stability of the host tree (Kirkendall et al., 1997).

Under optimal humidity conditions, *X. saxesenii* shows a heterogeneous age-class structure where mature and fertilized daughters delay their dispersal and serve as alloparents for their mother's brood (Biedermann et al., 2012). This cooperative breeding strategy allows for increased offspring numbers as compared to ambrosia beetle species with less complex social organisation (cf. Fischer, 1954; Peer and Taborsky, 2005, 2007; Kirkendall et al., 2015). Since suboptimal conditions lower the fungus productivity, when shifting moisture levels away from the optimum we would expect either a decrease in the speed of offspring development or a reduction in the overall number of offspring produced. Consequently, this might change the propensity of adult offspring to invest in alloparental care. In the present study we thus tested how the beetles and their microbial community respond to experimentally varied moisture levels, which might reveal evolved strategies to counteract the natural ephemeral quality of dead wood. In this regard, especially a close observation of adult daughter dispersal timing is of importance, since it correlates directly with their alloparental investment (Peer and Taborsky, 2007; Biedermann et al., 2011). Substrate humidity may influence the whole microbial community in the nest, and special attention should be directed to the ambrosia fungus cultivars that constitute the main food source of both adults and larvae. *X saxesenii* gardens are comprised by the two species *Raffaelea sulphurea* and *R. canadensis*, which contrasts with most other ambrosia beetle species that are thought to be associated with only one species of mutualistic fungus. The reason for using more than one fungus species and how such associations can be maintained is hitherto unclear.

In a first step, we explored how the alloparenting daughters respond to adverse conditions, since their propensity to cooperate might depend on nest performance. We hypothesized that under harsh conditions, mature daughters would increase their propensity to cooperate for boosting their immature sisters' survival chances. Alternatively, daughters might divert more energy to their own future reproduction by refraining from cooperation and dispersing early. The second possibility might also be favourable if the incentives to inherit such poor-quality nests are low. To test whether the mature offspring adjust their dispersal strategies according to the maternal nest conditions, we reared nests under three humidity regimes, providing either (1) the "normal" (optimal) condition yielding the most offspring as determined in previous experiments (Biedermann et al., 2009; own pilot data), (2) a "dry" condition simulating older wood and thinner, fast drying branches, or (3) a "humid" condition resembling very recently dead wood that is usually not preferred by the beetles in the field. We regularly counted how many nest members of different age classes were present in the gallery and analysed their behaviours. Since timing of dispersal strongly relates to the extent of cooperative investment of daughters, we recorded all dispersal events and collected dispersing females. The microbial composition they carried on their body was analysed to test for potential treatment effects. We expected that the diverging treatment conditions apply differential pressures on the microbiome, favouring the growth of different species in dry and humid conditions. The fungus composition carried by the females largely represents the microbiome species composition of maternal nests at the time of dispersal, and it correlates with the composition of species that can be found in the newly founded nest, i.e., in the next generation. This first experiment thus allows assessing the influence of habitat quality on social decision making, and it should provide insight into potential transgenerational effects of environmental challenges via differential selection pressures on the transmitted microbiome.

Living under suboptimal humidity regimes might not only change the relative microbial species composition within the nest but even cause a complete loss of certain humidity-sensitive microbial species. Since inbreeding ambrosia beetle species like *X. saxesenii* transmit their mutualists vertically from parental to daughter nests, a loss of certain microbial species would influence the species composition of future fungus gardens. Therefore, we hypothesized that sub-optimal habitat conditions can influence the fitness and cooperative strategies of a matriline over multiple generations.

To test this hypothesis, we exposed the beetles in a second experiment to "dry" conditions for five subsequent generations and observed the nest development patterns and timing of adult daughter dispersal. This served to assess (1) whether selection for a certain microbial composition would influence the beetle's dispersal strategies over generations, and (2) whether such a potential "acclimatisation" to dry conditions might be

reversible when the beetles are subsequently exposed to optimal or "normal" nest growth conditions. If the microbial composition is changed during such multigenerational exposure to harsh conditions and cannot be easily reversed, we would expect a poorer performance of the fungal garden and a reduction in the beetles' fitness. This might change the incentives of mature daughters to either stay and cooperate or disperse and breed independently, which we determined by monitoring their dispersal timing.

MATERIALS AND METHODS

Study Species

Xyleborinus saxesenii is an inbreeding species of polyphagous ambrosia beetle native to Eurasia. The closely related species *Xylosandrus germanus* exhibits an extremely low degree of outbreeding (Keller et al., 2011) and a significant outbreeding depression (Peer and Taborsky, 2005), which may be characteristic for many cooperatively breeding Xyleborini that exhibit similarly high inbreeding rates (but see Storer et al., 2017, reporting regular outbreeding in a sib-mating species). *X. saxesenii* is haplodiploid and shows a highly female biased sex ratio of about 1/20 (Peer and Taborsky, 2007). Females are capable of flight and emerge from their natal nest already mated, whereas the smaller males disperse on foot after their sisters have been fertilized, as they are incapable of flight (Peer and Taborsky, 2004; Biedermann, 2010). Such males may later try to outbreed by entering conspecific nests on the same log. A new nest is initiated after dispersal of a single female that first bores a tunnel with a single egg niche into a relatively fresh dead tree. She then inoculates it with wood digesting mutualistic fungi brought from her natal nest within a mycetangium (fungus storing organ; Francke-Grosmann, 1975). Once the fungus garden is well established, she lays a clutch of eggs and regularly cleans the eggs, which prevents them from being overgrown by the ambrosia fungi covering the walls of the tunnel. The gallery is then extended by the wood-chewing larvae into one or multiple large nest chambers. After pupation, mature offspring delay their dispersal and invest heavily in alloparental care by taking over nursing duties of their mother, which then serves mainly as a gatekeeper blocking the entrance tunnel with her body (Peer and Taborsky, 2007; Biedermann and Taborsky, 2011; Nuotclà et al., 2014). Larvae are also workers like adults, but in contrast to them they mainly contribute trough enlarging the nest by consuming the fungus veined wood and helping the adults to dispose of waste by forming frass pellets that are then shifted trough the nest. This exclusive larval behaviour is called "balling" and constitutes, together with nest enlargement, a rare example for division of labour in holometabolous insects (Biedermann and Taborsky, 2011).

Laboratory Beetle Rearing

All beetles used in this study dispersed from seventh generation laboratory nests. This lab population was originally started using females caught with ethanol baited traps in the Bremgarten- and Könizer Berg Forests near Bern, Switzerland. Each new nest was initiated by a single female that originally mated with a brother inside her natal nest. The founding female was first roughly surface sterilised by rinsing it for a few seconds with bleach ("Javel Water" containing <5% potassium hypochloride), followed by 96% ethanol and finally with sterilised deionised water, before placing it in a laboratory rearing tube containing artificial wood substrate. Substrate preparation followed a standard protocol (Nuotclà et al., 2019), except that we completely dried the beech wood sawdust at 60°C before adding it to the mixture. The "normal" substrate contained 2.5 g sucrose, 2.5 g casein, 5 g starch, 2.5 g yeast extract, 0.63 g Wesson's salt mixture, 15 g agar, 100 g sawdust (beech), 5 ml peanut oil, 4 ml ethanol 97%, and 280 ml deionised water that were well mixed and then filled into clear polycarbonate tubes (Nalgene® centrifuge tubes, 16 ml, #3117-0160) before being capped and sterilized via autoclaving at 121°C for 20 min. We added different amounts of deionised water according to the treatments; 40% of the original formula for the "dry" condition, 100% for the "normal" condition, and 150% for the "humid" condition. The "normal" substrate had been demonstrated to yield the highest offspring numbers in previous experiments (Biedermann et al., 2009) and was thus chosen as a baseline for all comparisons in the experiments. The humidity content of the "dry" substrate was chosen based on an unpublished pilot experiment that showed this to be the lowest limit where beetles produce viable offspring regularly enough for experimental use. Nests with as little as 20% of the "normal" water content did yield viable offspring in the lab, but the nest establishing success rate was too low to be practical for our experiments. The water content of the "humid" condition represents the upper end at which we could follow all steps of our standard protocol. Higher humidity contents would result in separation of the sawdust and agarose into two separate phases during autoclaving. Our treatments corresponded to a gravimetric water content of 82% for the dry, 204% for the "normal" and 307% for the "humid" condition (gravimetric water content: water weight divided by all other components times 100, expressed in percent). The water content of artificial sawdust-agar substrate needs to be higher compared to natural wood, because it loses its moisture faster than wood, even at high relative air humidity. Beechwood infested by ambrosia beetles under natural conditions has a mean gravimetric water content of 87–90% at the beginning of the season (end of April), which steadily declines to 43–60% until the end of the beetles' dispersal phase end of August (Zimmermann, 1973). Nest establishment success in natural conditions is expected to be around 20%, similarly to the closely related species *X. germanus* (Peer and Taborsky, 2007). Under laboratory conditions, the nest establishment success can vary between 5 and 50% (Biedermann et al., 2009; personal observations). Only nests with good visibility of the nest chambers were used in this study. Therefore, only nests where the tunnels and nest chambers were built near the tube wall were utilised while all others were discarded. All nests were stored in complete darkness in a ventilated climate chamber at controlled ambient conditions with 23°C and 75% relative humidity.

Humidity Experiment

In the first experiment we manipulated the humidity content of our standard lab rearing substrate to change the conditions for microbial growth. We created three humidity regimes to approximate different wood substrates reflecting the total range of natural conditions. The "dry" substrate was meant to resemble older stages of dead wood or thin branches that have lost most of their humidity. The "normal" substrate represented the optimal conditions that were found to yield the highest nest establishment success (Biedermann et al., 2009; own pilot data). The "humid" substrate was meant to reflect freshly dead trees that still contain considerable sap flow. We established a high number of nests because of an expected low nest success rate and our confinement to tubes with good nest visibility. A pilot experiment showed that suboptimal humidity results in even lower nesting success compared to the "normal" condition ("dry"∼1/2 as successful and "humid"∼1/3 less successful than "normal"). We thus adjusted the number of initial tubes for each humidity category accordingly (start "dry": 150 tubes; "normal": 70 tubes; "humid":100 tubes). After successful establishment of a fungus garden and start of egg laying, the nests were monitored every 2–3 days. We noted the number of eggs, larvae, pupae, adult females and males and recorded their behaviours by scan sampling (cf. Nuotclà et al., 2019). From the moment a nest contained more than one adult individual, its tube cap was exchanged for a cap that allowed dispersing beetles to be captured, and the nest was turned so that the entrance tunnel pointed downward. This prevents dispersed beetles from crawling back into the nest, which helps obtaining precise information about dispersal timing. Dispersed beetles where either collected and snap-frozen on the dispersal day in a minus 80°C freezer where they were stored until molecular analysis of the microbial community they carried, or they were used for subsequent laboratory rearing (see section "Selection Experiment," below). After all beetles had dispersed from a nest, the substrate was removed from the tube and the maximal depth to which the beetles had dug their nest chamber was measured.

Some nests that successfully produced offspring collapsed. Thus, only nests that had at least two dispersing females were used for the final analysis to exclude unsuccessful nests. The success rate was 15% for dry, 19% for normal and 13% for humid nests. The final sample size for the *humidity experiment* was 23 nests with a total of 115 dispersing females for the "dry" treatment, 13 nests with a total of 136 dispersing females for the "normal" treatment, and 13 nests with a total of 84 dispersing females for the "humid" treatment.

Selection Experiment

In our second experiment we repeatedly reared dispersing females from the "dry" nest condition for five consecutive generations in "dry" substrate. In each generation we let 20 dispersing beetles initiate nests in 20 fresh tubes filled with "dry" substrate. After five generations exposed to this suboptimal substrate, we randomly assigned 100 dispersing beetles to start a new nest, half of them in "normal" and half of them in "dry" substrate. We monitored them similarly to the nests used for the *humidity experiment*, but without behavioural record and the microbial community assay. Again, we only analysed data for nests producing at least two dispersing females to exclude unsuccessful nests. The success rate here was 38% for dry and 14% for normal nests. For the final test of the *selection experiment* we obtained a sample size of 19 nests with a total of 974 dispersing females for the "dry" condition and 7 nests with a total of 255 dispersing females for the "normal" condition.

Collection of Samples for Microbial Analyses and DNA Extraction

We randomly selected five nests from each of the three treatments in the *humidity experiment*. From each of these nests we chose a beetle that dispersed on the very first day ("early disperser") and one beetle that dispersed on the very last day at which dispersal occurred ("late disperser"). The DNA extraction of these selected snap frozen beetles was conducted using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Germany) in accordance with the manufacturer's instructions. Additionally, we included a treatment of the whole snap frozen beetles with ceramic beads in a bead beater, followed by another step with glass beads (0.1 and 0.5 mm) and swirling on a Vortex Genie 2 to break up cells at the beginning of the extraction. The isolated DNA samples were stored at −20°C until further molecular analysis. Partial sequences for the 28S large subunit (LSU) ribosomal DNA (rDNA) were obtained from all samples for fungal identification using the newly designed dual-index primers LIC15R (originally from Miadlikowska et al., 2002) and nu-LSU-355-3' (originally from Döring et al., 2000), whereas sequences for the 16S rDNA for the identification of bacteria were obtained using the dual-index primers for the V4 region (Kozich et al., 2013). Our paired-end sequencing approach was performed on the Illumina MiSeq platform (see **Supplementary Material** for full protocol, primer design and details on bioinformatics processing).

Estimation of Day With Highest Individual Density

Based on previous lab studies with detailed individual counts over time (Mizuno and Kajimura, 2002, 2009; Biedermann et al., 2012), we expected that the growth of larval and adult numbers (individual density) in a nest will follow a cubic regression in the form $y = f(ax + bx\text{^}2 + cx\text{^}3 + d)$, with x representing the number of days since nest foundation, y representing the number of individuals, and the coefficients a, b, c and d describing the shape of the regression. Individual density is expected to initially increase as more individuals develop into that particular age class, reaching a maximum (point of highest individual density) before decreasing steadily while larvae develop into adults and adults disperse. The parameters a, b, c and d where determined for each nest and age class (larvae + pupae, adult females) using the function lme() in R (weighted least squares estimate). The derivative of f at the point $y = 0$ provided an estimate for the time point with the highest individual density of a certain age class for every nest (i.e., the two solutions for x in $0 = a + 2bx + cx\text{^}2 + d$ provide the two local extrema of the function, where x at the local maximum represents the day of highest individual

density). This method was preferred over the use of the point in time at which the highest count of individuals was obtained, since it helps mitigating uncertainties caused by non-detected individuals over multiple observation days. This is necessary since perfect visibility into nest chambers rarely exists, even after selecting only the nests with good visibility for the experiment as described above. Post-hoc graphical evaluation for all individual nests confirmed that the individual counts for every age-class fitted well with the described regression, and that the calculated maxima of the function represented plausible estimates for the time point of a nest's highest individual density.

Analyses of Density, Dispersal, and Nest Depth

Significant deviation from homogeneity of variances (Levene test; for subadult offspring: Df = 2, $F = 3.239$ $P = 0.048$; for adult females: Df = 2, $F = 8.459$ $P < 0.001$) revealed the need for a test without assumption of homogenous variances. We thus performed pairwise *t*-tests with non-pooled standard deviations to analyse whether the treatments differ in the point in time at which the highest individual densities of a certain age class were observed. The same method was used to determine differences between the treatments regarding the total number of dispersing adult females and nest depth. All p-values resulting from these pairwise *t*-tests were corrected for multiple testing using the Benjamini and Hochberg (1995) method. Linear models were used to test whether (a) the time points at which the highest densities of the different age classes were determined, (b) the total number of dispersing adult females, and (c) the nest digging depth differed significantly between the two humidities tested in both experiments (interaction of "dry" vs "normal" substrate, and of the *humidity experiment* vs the *selection experiment*). To assess whether the dispersal timing of adult females differed between the treatments of both experiments, we analysed the dispersal day data using a Cox proportional hazards model likelihood ratio test (Therneau and Grambsch, 2000).

Behavioural Data Analyses

Behavioural data were analysed using generalised linear mixed models (GLMMs) with binomial error distribution and logit-link function. For this analysis we focused on the general activity, grooming, cannibalism, entrance blocking and balling behaviours of the beetles (see (Nuotclà et al., 2019) for a full ethogram of *X. saxesenii*). To examine the effects of the humidity treatment ("dry," "normal" or "humid") and time since nest initiation ("nest age") on the different behaviours of the nest members, the frequency of the respective behaviour was set as the response variable, and the humidity treatment, nest age and their interaction served as explanatory variables. As nests were measured repeatedly over multiple days, we included the nest ID as a random variable. We performed log-likelihood tests to examine the significance of the explanatory variables. Stepwise backward elimination of non-significant terms was used to simplify the maximal model containing the interaction of treatment and nest age. Overdispersion was corrected for by incorporating an additional observation-level random variable in the model (Browne et al., 2005; Engqvist, 2005; Bolker et al., 2009).

Analysis of Molecular Data

Data files containing the attained zOTU (zero-radius Operational Taxonomic Unit; strictly speaking the amplicon sequence variant found by sequencing, in a broader sense the clearly distinguishable taxa) table, taxonomic table and sample data were analysed using the software R through merging into a phyloseq object (see **Supplementary Material** for information about the bioinformatics processing). The amplicon sequences of the fungi *Chaetomium globosum* and *Penicillium sp.* were excluded from the analysis since they were overabundant in all samples relative to other fungal species. DNA of these fungi is probably overabundant because they produce high amounts of spores that are passively transmitted on the surface of the beetles. Also, fast growing fungi such as *Chaetomium globosum* and *Penicillium sp.* may have higher rRNA copy numbers than slow-growing taxa (e.g., the ambrosia fungus *R. sulphurea*), as has been shown for prokaryotes (Maleszka and Clark-Walker, 1993; Weider et al., 2005). Similarly, the parasitic bacteria *Wolbachia sp.* where overrepresented in the bacterial 16S ribosomal RNA dataset due to their high numbers contained in the beetles' cells. We decided to exclude *Wolbachia* amplicon sequence variants to get a better resolution of the remaining species, since our focus was mainly on the microbial community that lives within the nest. We ran a GLMM with nest ID as random variable assuming a Poisson error distribution (Bolker et al., 2009) to test the influence of the humidity treatment ("dry" vs "normal" vs "humid") and time of dispersal ("early disperser" vs "late disperser") on the microbial community (number of observed zOTU's). Next, we performed a mixed non-metric multidimensional scaling (NMDS) and calculated the Bray-Curtis dissimilarity of taxa abundances between samples (Clarke et al., 2006). A permutational ANOVA test with 999 permutations was conducted using the R package vegan (Oksanen et al., 2016) to compare microbial communities between the treatments and time of dispersal, including "nest ID" as random variable. We ran another set of GLMM's to test whether there were differences depending on humidity treatment and dispersal timing between the relative abundance of carried ambrosia fungi and all other fungi. This enabled us also to determine whether the relative abundance of the two ambrosia fungi, *R. sulphurea* and *R. canadensis*, varied with these factors. Here, the relative read abundances of the fungi where set as the response variable, and the humidity treatment, dispersal timing and their interaction served as explanatory variables. The analysis followed the method described earlier for behavioural data. Post-hoc Tukey HSD tests with correction for multiple testing following Benjamini and Hochberg (1995) were used to describe differences between the treatments and between dispersal timings.

We should like to point out that the results of whole community analyses need to be interpreted with caution since the relative read abundance determined by analysis of whole beetles may not adequately represent the community that a beetle transmits to a new nest due to over- or underrepresentation of certain species. In addition, the ecological importance of

most non-ambrosia mutualists is unknown. Therefore, to enable conclusions about the influence of habitat conditions on the whole microbial community, future studies should rather focus on the analysis of samples dissected from the mycangia alone, as the beetles actively spread material contained in them onto the walls of their newly founded nests. The present study reports the community composition found when crushing whole beetles, hence for the mentioned issues we only draw conclusions about the read abundance of the two known garden mutualists relative to each other; their relative abundance is likely determined mainly by the material contained within the mycangia and the guts (thus either purposely transmitted or previously ingested for food from the garden), and to a much lesser degree by accidental surface contamination. Besides this, our analysis of species richness for whole beetles may provide important clues about the influence of habitat on the microbial community, as these results are not affected by over- or underrepresentation of certain species; apart from the ambrosia fungi, we only determined the diversity of microbial species contained in each nest when a beetle disperses (see **Supplementary Material** for more details on the analysis of the sequencing output).

All statistical analyses were performed with R version 3.6.1 with additional packages "lme4" (Bates et al., 2015), "survival" (Therneau and Grambsch, 2000), "multcomp" (Hothorn et al., 2008), "car" (Fox and Weisberg, 2019), "phyloseq" (McMurdie and Holmes, 2013), "nlme" (Pinheiro et al., 2018), "mgcv" (Wood, 2017), "permute" (Simpson, 2019), "lattice" (Sarkar, 2008), "ggplot2" (Wickham, 2016), "plyr" (Wickham, 2011), "dplyr" (Wickham et al., 2019), "scales" (Wickham and Seidel, 2019), and "emmeans" (Lenth et al., 2019).

RESULTS

Nest Development

In the *humidity experiment*, nests reared in "normal" substrate reached their highest individual density earlier than those reared in "dry" (larvae $P = 0.028$; adult females $P = 0.004$) or "humid" substrate (larvae $P = 0.057$; adult females $P = 0.027$). The day at which individual density peaked did not differ significantly between the "humid" and "dry" nests in this experiment (larvae $P > 0.1$; adult females $P = 0.069$; **Figures 1A–D**).

The *selection experiment* showed that after five generations reared in "dry" substrate, the beetles showed a significantly delayed peak nest density, both in nests reared for the final test in "dry" substrate (larvae $P < 0.001$; adult females $P = 0.004$) and in those reared in "normal" substrate (significant for larvae only: larvae $P = 0.041$; adult females $P = 0.1$). For these analyses, the intervals between nest founding and peak density were compared to the corresponding intervals in "normal" substrate in the *humidity experiment*, which served as the baseline. There was no difference in this parameter between nests from the selection line compared between the "dry" and "normal" test substrate ($P > 0.1$). A linear model checking for an interaction between treatment ("dry" vs "normal") and experiment (*humidity experiment* vs *selection experiment*) regarding the time point of peak nest density showed significant effects of rearing the beetles over multiple generations under dry conditions (larvae $DF_{residuals} = 58$, $F = 5.591$, $P = 0.021$; adult females $DF_{residuals} = 58$, $F = 5.652$, $P = 0.021$).

Visual inspection of eggs over time did not reveal any second egg batches after the first adult daughters were visible in the 13 "normal" and 13 "humid" nests during the *humidity experiment*, but 1 of 23 nests reared in "dry" substrate contained eggs at this late nest stage. In the *selection experiment,* 15 of 19 "dry" substrate nests and 4 of 7 "normal" substrate nests contained late egg batches in the final test.

Nests reared in "dry" substrate during the *humidity experiment* and nests reared in both "dry" and "normal" substrate in the final test of the *selection experiment* were dug significantly deeper into the substrate than those reared in "normal" substrate in the *humidity experiment*, which served as baseline (all $P < 0.001$; **Figure 1E**), whereas there was no difference in nest depth between "dry" and "normal" substrate conditions in the final test of the *selection experiment* test ($P = 0.129$). Nest depth did not differ between "normal" and "humid" substrate conditions in the *humidity experiment* ($P = 402$). A linear model used to evaluate the effect of five generations of "dry" substrate rearing on nest depth revealed a significant interaction between treatment ("normal" vs "dry") and experiment (*humidity experiment* vs *selection experiment*; DF residuals = 56, $F = 13.252$, $P < 0.001$).

Fitness and Timing of Dispersal

In the *humidity experiment*, significantly more dispersing females were produced in nests reared in "normal" substrate than in those reared in "dry" ($P < 0.001$) or "humid" ($P = 0.017$) substrates, whereas the latter two did not differ from each other ($P = 0.208$; **Figure 2A**). Nests reared in both "dry" and "normal" substrate in the final test of the *selection experiment* produced significantly more dispersing females than the nests of all three treatments in the *humidity experiment* (all $P < 0.05$), whereas they did not differ from each other ($P = 0.248$). There was no significant interaction between treatment ("normal" *vs.* "dry") and experiment (*humidity experiment* vs. *selection experiment*) on the total number of dispersing females (DF residuals = 58, $F = 2.8177$, $P = 0.099$; **Figure 2A**).

A Cox proportional hazards model ($n = 1564$ dispersing beetles; robust score test = 23.46; $P < 0.001$) revealed that in the *humidity experiment*, female dispersal was delayed in nests reared in "dry" ($P < 0.001$) compared to "normal" substrate, which was not true for the comparison between "humid" and "normal" substrate ($P = 0.496$). The same test also showed that female dispersal in nests reared in both "dry" ($P < 0.001$) and "normal" ($P < 0.001$) substrate in the final test of the *selection experiment* was delayed when compared to the nests reared in "normal" substrate in the *humidity experiment,* which served as a baseline (**Figure 2B**).

Behaviour

GLMMs of total behavioural activity (all behaviours combined) indicated that the adult females were generally more active under "dry" than under "normal" conditions ($P = 0.006$), whereas there was no difference in the quantity of activity between the

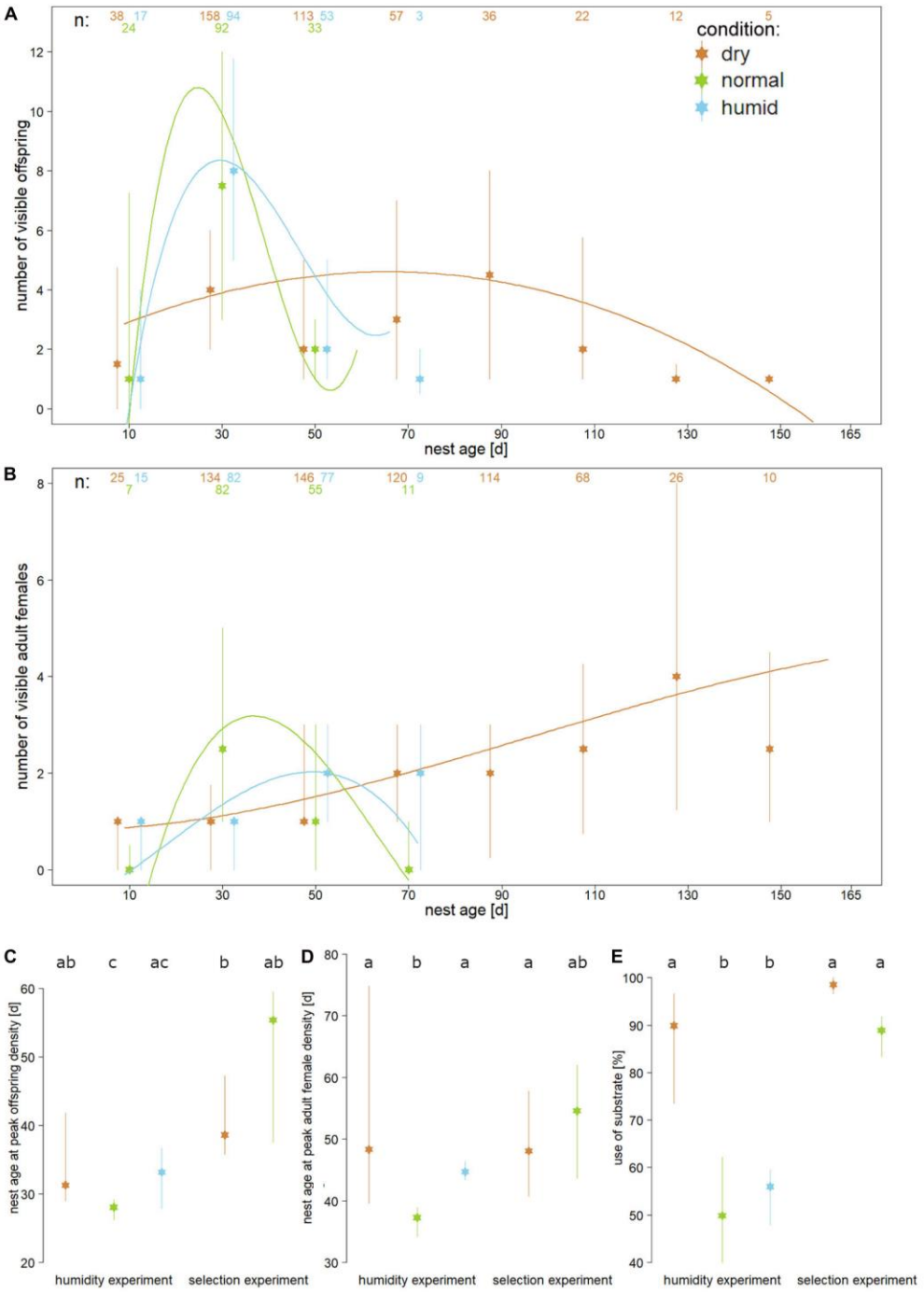

FIGURE 1 | Medians and interquartile ranges of relevant nest development parameters are displayed for three substrate humidities ("dry" = brown, "normal" = green, and "humid" = blue) measured during either the *humidity experiment* **(A–E)** or during the *selection experiment* **(C–E)**. The *humidity experiment* tested the influence of different substrates on first generation nests, whereas the *selection experiment* tested whether the beetles develop differently in "normal" or "dry" substrate after they were reared over five generations in "dry" substrate. Panel **(A)** shows sub-adult (pupae and larvae) and panel **(B)** shows adult female peak densities for 20 day intervals. Numbers on top indicate the number of cumulative observations that were made for each treatment during each interval. Curves indicate regressions modelled as y = f(ax+bx^2+cx^3+d) for all data of each treatment, to illustrate the overall development of individual density across the treatments. For the statistical analysis we calculated the points in time of the highest individual density for every individual nest [shown in panels **(C,D)**]. Panel **(E)** shows how deep the beetles dug into the substrate in both experiments. Different lower-case letters in the top line of panels **(C–E)** indicate statistically significant differences within each panel (*t*- tests, corrected for multiple testing; $P < 0.05$).

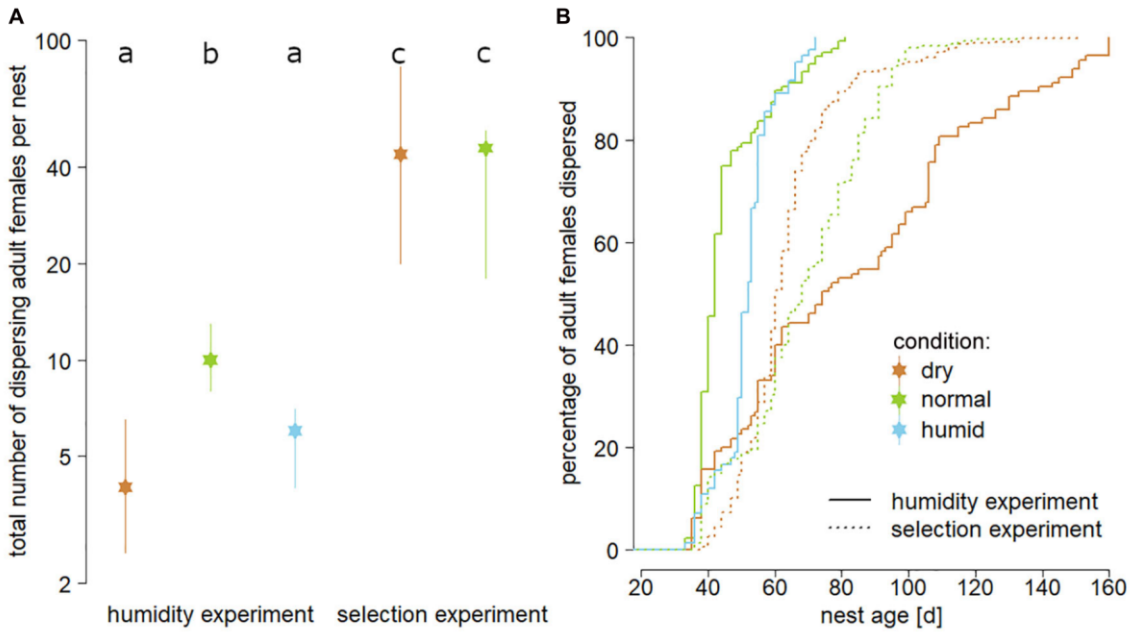

FIGURE 2 | Panel **(A)** illustrates the overall fitness impact of the three tested substrate humidities, indicated by medians and interquartile ranges of the total number of dispersing females per nest (logarithmic scale; "dry" = brown, "normal" = green, "humid" = blue). Different lower-case letters in the top line indicate statistically significant differences (*t*-tests corrected for multiple testing; $P < 0.05$). Panel **(B)** shows differences in the length of dispersal delay of adult females for both experiments and all treatments. Solid lines represent first generation nests of the *humidity experiment*, whereas dotted lines represent nests that had been reared for a final test in either "dry" (brown) or "normal" (green) substrate after the beetles had been reared in dry substrate for five generations (*selection experiment*).

"normal" and "humid" treatments ($P = 0.478$). Larvae reared in "dry" conditions were generally more active than those reared in "normal" medium ($P = 0.004$). Adult females tended to get more active with increasing nest age under "normal" and "humid" conditions of the *humidity experiment* ($P = 0.06$), whereas this trend was reversed in females under "dry" conditions; this was revealed by a significant interaction between nest age and humidity treatment ($P = 0.01$). Larvae became less active over time in all treatments ($P = 0.012$; see **Supplementary Figure 1** for frequencies of relevant behaviours, and **Supplementary Tables 4, 5** for model outputs).

Female grooming did not differ in frequency between the humidity treatments and this factor was removed from the final model. A separate GLMM for larval grooming showed that they groomed less in "dry" ($P = 0.018$) and "humid" ($P = 0.038$) than in "normal" conditions. Both, adult females ($P < 0.001$) and larvae ($P = 0.002$) generally groomed less the older the nests where. However, a significant positive interaction between nest age and humidity treatment indicates that this decrease over time was less strong in larvae reared under "dry" ($P = 0.008$) and "humid" ($P = 0.028$) conditions than in those reared in "normal" substrate.

Female cannibalism on nestmates occurred more under "humid" than under "normal" conditions ($P = 0.009$), whereas "normal" and "dry" conditions did not differ from each other ($P = 0.565$). Cannibalism by larvae did not differ between the treatments and did not change with nest age. Adult females cannibalised less with increasing nest age ($P = 0.001$). The frequency in which the entrance tunnel was blocked by an adult female did not differ between treatments and did not change with the course of time. Balling behaviour was generally shown more often by larvae under "dry" ($P = 0.040$) and "humid" conditions ($P = 0.011$) than in nests reared in "normal" substrate and its frequency decreased over time ($P = 0.005$).

Microbial Species Composition

Microbial species richness was approximated by analysing the zOTU richness (see Methods). The number of fungus 28S ribosomal RNA zOTUs in all samples ranged from 5 to 20, whereas the number of bacterial 16S ribosomal RNA zOTUs ranged from 48 to 257. GLMMs revealed a non-significant interaction between the humidity treatment ("normal" (reference) vs "dry" vs "humid") and the time of dispersal ("early" (reference) vs "late") for the fungal richness (GLMM: $P = 0.062$), and a significant interaction for the bacterial richness ($P = 0.004$). Post-hoc tests revealed that nests reared in "humid" substrate contained significantly more fungus species ("early" and "late" dispersers combined) than the ones reared in "dry" (TukeyHSD: $P = 0.010$) or "normal" ($P = 0.048$) substrate (no statistical difference between "early" and "late"). Fungal species richness did not differ between "normal" and "dry" substrate nests, and there was no difference in bacterial species richness between any of the three humidity treatments (all $P > 0.1$; see **Figures 3, 4** and **Supplementary Table 1**).

After the exclusion of the overrepresented *Penicillium sp.* and *Chaetomium globosum,* ten dominant fungus taxa with a mean relative abundance (MRA) of over 0.5% could be assigned. The

family Ophiostomatacea was represented with the two important fungus garden mutualists *Raffaelea sulphurea* and *R. canadensis,* and with a member of the genus *Sporothrix* that is potentially associated with ambrosia beetles (Harrington et al., 2010; Oranen, 2013). We found two fungus garden pathogens of the family Trichocomaceae, *Talaromyces rugulosus* and *Aspergillus flavus*. All other taxa where common saprobionts: *Petriella sp.* (Microascaceae), *Aureobasidium leucospermi, Alternaria sp., Cladosporium sp.*, and a member of the Nectriaceae that could not be determined more specifically (see **Figure 3A** and **Supplementary Table 2**).

Bacteria were dominated by Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria, which accounted for about 50% of total sequences (**Figure 3B** and **Supplementary Table 3**). Taxa from the phyla Acidobacteria, Planctomycetes, Verrucomicrobia, Armatimonadetes, Chlamydiae, Chloroflexi and Deionococcus-Thermus were detected in very low abundance (MRA of under 5%).

The overall microbial community composition carried by dispersing adult females neither significantly differed between the humidity treatments ("normal" (reference), "dry" and "humid"), nor for the time of dispersal ("early" vs "late"; PERMANOVA: all $P > 0.1$). Plotting the Bray-Curtis dissimilarity in NMDS plots also illustrated no obvious separation of the samples in "treatment" or "time of dispersal" (**Supplementary Figures 2A,C**). However, beetles dispersing from "normal" substrate nests had significantly higher read numbers for the two ambrosia fungi *R. sulphurea* and *R. canadensis* than those dispersing from "dry" (GLMM with TukeyHSD; $P = 0.005$) or "humid" ($P = 0.043$) substrate nests, whereas there was no difference between "humid" and "dry" substrate treatments in this respect ($P = 0.761$). Late dispersers carried more ambrosia fungi than those leaving early ($P < 0.001$), and there was a significant interaction effect of humidity treatment ("dry" vs "normal" vs "humid") and the time of dispersal ("early" vs. "late") on the ratio of ambrosia fungi to all other fungi ($P < 0.001$).

The ratio of the two ambrosia fungi *R. sulphurea* and *R. canadensis* tended to be lower in beetles that dispersed from "humid" nests ($P = 0.055$), and it was significantly lower in those dispersed from "dry" substrate ($P < 0.001$), than the corresponding ratio of beetles dispersing from "normal" substrate. There was no significant difference in the ratio of these two fungi between beetles dispersing from the "humid" and "dry" treatments ($P = 0.146$), and no significant influence of dispersal time on the ambrosia fungus ratio ("early" vs "late": $P = 0.3$; factor removed from final model; **Figure 4B**).

DISCUSSION

The results of the *humidity experiment* indicate that under "humid" and "dry" nest substrate conditions, which correspond to either very freshly dead or desiccated parts of long-dead trees, foundresses may suffer considerable fitness loss compared to "normal" humidity conditions. This is due to decreased offspring numbers and a delayed maturation time of the offspring (**Figures 1A,C**, **2A**). Delayed maturation time also caused later dispersal of adult daughters in "dry" substrate (**Figure 2B**) and larval density in these conditions was permanently lower and never reached a peak comparable to those reached under "normal" or "humid" conditions (**Figures 1A,C**). Besides the considerably slowed development, we also observed a more steady dispersal pattern in "dry" substrate compared to nests reared in "normal" or "humid" substrate, where the timing of dispersal seems to be more clustered (**Figure 2B**).

The altered nest development and dispersal patterns under suboptimal "humid" and, especially, "dry" conditions (**Figures 1**, **2**) might be explained by changes in the microbial community found in these nests. The relative abundance of ambrosia fungi reads compared to reads of other fungus species carried by dispersers in the "dry" and "humid" treatment nests was lower than for those in the "normal" treatment (**Figure 4B**). This may indicate that the fungus garden of these suboptimal substrates yielded less food for the beetles than the "normal" condition. We found that beetles from nests reared on "humid" substrate carried a significantly more variable fungus community than those dispersing from "normal" or "dry" substrate nests. Humid substrate seems to allow more fungus species to thrive, possibly leading to increased competition between them, which might put the mutualistic ambrosia fungi at a disadvantage (see **Figure 4A**). In contrast, dry initial conditions might cause lower growth of the mutualistic ambrosia fungi, which thrive better at higher humidity (Zimmermann and Butin, 1973). Thus, both suboptimal conditions probably yielded less food for the beetles, but for different reasons.

Importantly, the ambrosia fungus species *Raffaelea canadensis* represented a much greater proportion of fungi carried by dispersing beetles from "dry" nests than from those dispersing from "normal" nests, whereas the latter carried *Raffaelea sulphurea* as the dominating mutualistic fungus species when dispersing from their natal nest (**Figures 3A**, **4B**). Experimental data indicate that *R. canadensis* is a slower growing fungus than *R. sulphurea*, but it grows much better under dry than normal conditions (Nuotclà and Taborsky, unpubl. data). We therefore hypothesize that carrying multiple species of mutualists that are adapted to different humidity regimes may help the beetles to thrive in variable conditions. Such mutualist complementarity was reported also for the fungus-associated bark beetle *Dendroctonus ponderosae*, which carries at least two associated ambrosia fungus species that vary in abundance depending on the temperature regime (Six and Bentz, 2007).

The ephemerality of dead wood has been suggested to be a crucial factor impeding social evolution in ambrosia beetles. It limits the potential nest lifetime and thus may lower the chances for generational overlap and reduce the incentive for offspring to remain philopatric, cooperate and reproduce in their natal nest at a later stage (Alexander et al., 1991; Kirkendall et al., 1997). Only few examples are known in ambrosia beetles where this limitation does not apply. Amongst those we find some of the most remarkable examples of social complexity for ambrosia beetles, as for instance in the platypodine species *Trachyostus ghanensis* or *Austroplatypus incompertus*, the nests of which can survive many years inside living trees and can harbour multiple overlapping offspring generations (Roberts, 1960; Kent and Simpson, 1992).

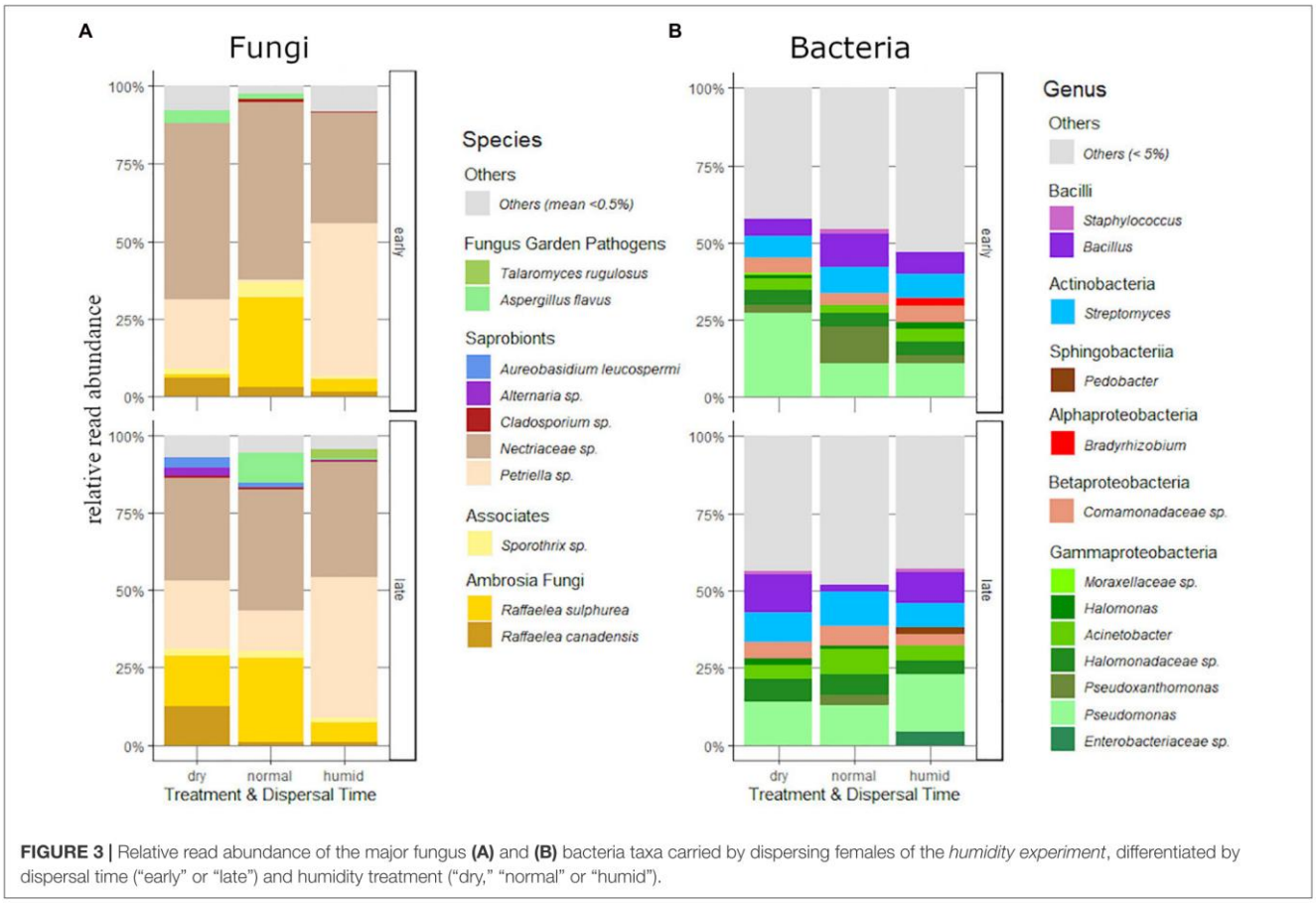

FIGURE 3 | Relative read abundance of the major fungus **(A)** and **(B)** bacteria taxa carried by dispersing females of the *humidity experiment*, differentiated by dispersal time ("early" or "late") and humidity treatment ("dry," "normal" or "humid").

The species of the genera *Ambrosiophilus* and *Ambrosiodmus* have overcome the problem of dwindling resources in aging dead trees by associating with a highly competitive wood-decaying fungus. This has led to long lived nests that harbour multiple generations of offspring (Kasson et al., 2016). Similarly, carrying a variety of complementary mutualistic fungi may buffer environmental conditions and enhance the viability of the alloparenting strategy of *X. saxesenii* by increasing the timespan in which the wood can be used for fungiculture. Initially, the beetles can rely on the fast growing and highly productive *R. sulphurea*, but over time the primary abundance of ambrosia fungi in the gallery may change to the more dry-tolerant *R. canadensis*, depending on humidity. Prolonged nest viability should increase the incentive for adult daughters to stay in the natal gallery and help raising sisters, since it increases the survival chances of the latter. Thus, even if our "dry" treatment initially led to less and more slowly growing offspring, the future prospects for the daughters might have been better due to a more favourable fungus composition than in the "humid" treatment, where the ambrosia fungi may be outcompeted by other microorganisms.

Prolonged nest viability might also allow daughters to eventually take over the nest from their mother. In the "dry" treatment of the *humidity experiment* we found one case where second egg batches occurred, which might indicate that daughters have fostered offspring in their maternal nest. In the *selection experiment*, after we had reared the beetles over five generations in "dry" conditions, 73% of nests exhibited late egg batches. Nevertheless, we are currently unable to determine whether these eggs were indeed produced by daughters, or whether they reflected second egg batches produced by their mother. In any case, these additional clutches should raise the total fitness across a nest, which is corroborated by the considerable increase of the total number of dispersing adult females in these nests.

Enhanced long term offspring production in dry conditions was confirmed by the *selection experiment*, where after keeping the beetles in the "dry" substrate treatment for five generations, the final test yielded much higher numbers of dispersing beetles in both test conditions, "dry" and "normal," than any of the treatments in the *humidity experiment* (**Figure 2A**). The enhanced productivity was probably linked to the fungus garden composition of the nests, with the dryness-resistant *R. canadensis* becoming the dominating mutualistic fungus species. Visual inspection of galleries in the *humidity experiment* indicated a yellowish colour of the fungus garden especially in the "normal" and "humid" treatment conditions, which is typically attributed to metabolic compounds produced by the mutualist *R. sulphurea* (hence its name). In contrast, the fungus garden of nests at the late stages of the "dry" condition in the *humidity experiment*, and at both conditions of the final test in the *selection experiment*, had mostly a whitish

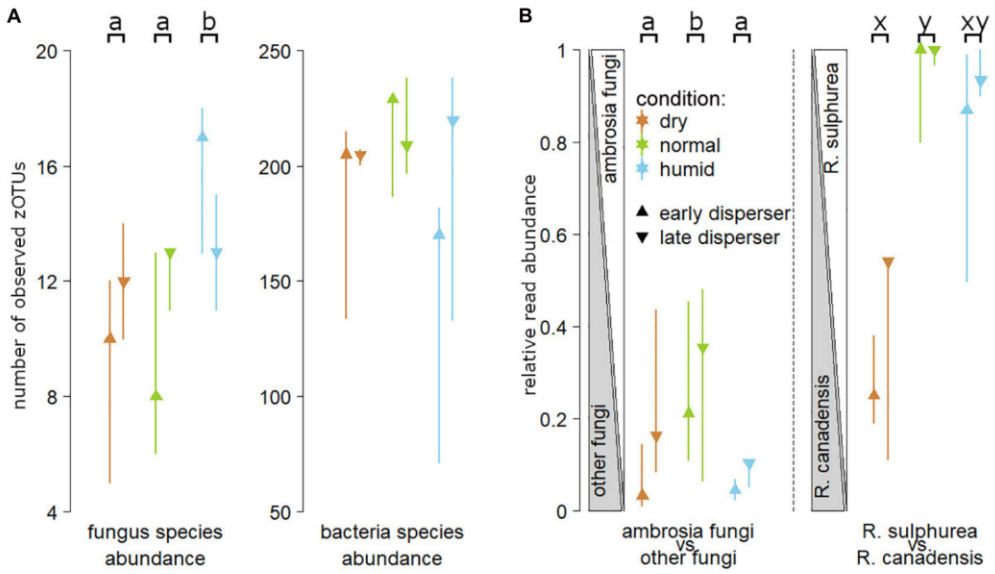

FIGURE 4 | Panel **(A)** illustrates the numbers of zero radius Operational Taxonomic Units (zOTU, ≈ number of clearly distinguishable species) for fungus and bacteria species that are carried by first ("early") or last ("late") dispersing females from nests reared in "normal," "dry" and "humid" substrate during the *humidity experiment*. Panel **(B)** illustrates the relative read abundance of ambrosia fungi over all fungi, and the relative read abundance of the two main ambrosia fungus species carried by *Xyleborinus saxesenii* females, *Raffaelea sulphurea* and *Raffaelea canadensis*. Medians and interquartile ranges are shown. Different lower case letters in the top line indicate statistically significant differences within each panel (TukeyHSD; $P < 0.05$).

colour, presumably indicating the dryness-condition specialist *R. canadensis*. Since this fungus grows only slowly, the beetles may reach adulthood comparatively late due to nutritional limitation. But this drawback is compensated by longer nest maintenance and consequently higher productivity, as under dry conditions these ambrosia fungi may be less challenged by competing microorganisms.

The higher productivity of nests after selection in "dry" substrate, relates also to the enhanced utilisation of the offered substrate (**Figure 1E**). Already first generation foundresses in the humidity experiment dug deeper into dry substrate and the chambers excavated by the larvae were thus nearer to the bottom of the experimental tubes in "dry" than "normal" and "humid" conditions. This might reflect a strategy to reach deeper into the humid core of the wood. It could be that a nest foundress digs as long as it takes for the fungus garden to grow enough biomass to cover the beetles' nitrogen requirements (wood being a nitrogen-poor substrate) before laying the eggs. Ophiostomatoid fungi are known to concentrate nitrogen, phosphorus and other trace elements from the surrounding wood and to provide it to the beetles trough fungal tissues in the beetles' tunnels (Six and Elser, 2019). In accordance with this idea, when fungus is experimentally removed, *Dendroctonus* bark beetles are known to dig longer tunnels to cover their nitrogen requirements (Ayres et al., 2000). Reversing the humidity back from "dry" to "normal" conditions in the final test of the *selection experiment* did not reverse the pattern of digging depth, which might indicate that this experimental selection resulted in permanent changes of the microbial community.

Not only nest foundress digging behaviour changed according to the humidity conditions, but also the offspring seem to adjust their behavioural patterns. Larvae were more active and showed more balling behaviour in both sub optimal treatment conditions. Balling is a crucial nest keeping behaviour only shown by larvae, which facilitates the removal of debris from the nest by adult females. Increased nest depth in "dry" and accelerated grow of competing fungi in "humid" nests thus seem to necessitate more work by the larvae. Besides, adult females notably increased their cannibalisation of larvae in "humid" nests when compared to such reared in "normal" substrate. Cannibalisation was described to be a form of destructive sanitation that allows removal of nestmates that are infected by pathogenic fungi (Nuotclà et al., 2019). Increased cannibalisation rates might be thus further evidence for increased microbial competition in humid wood. However, we found no increased adult female grooming frequency which would also be predicted in the presence of pathogens.

In conclusion, our data show that when the substrate is very dry, the ambrosia fungus garden is mainly composed of less productive, drought resistant fungi, which leads to slower offspring development but may also limit the invasion of antagonistic fungus species. This obviously enables long-lasting nests and increases total offspring numbers, perhaps at least partly due to some daughters refraining from dispersal and instead producing own offspring in the natal nest. The success of this strategy may depend on the availability of alternative nesting possibilities, dispersal conditions and the progression of the season. Hence, the dry conditions that finally render higher offspring numbers but retard the offspring development, may

work out well early in the season but rather reflect a "best-of-a-bad-job" response when the season has further progressed.

We further demonstrate that the substrate choice of a foundress not only has direct consequences for the cooperative investment of her daughters but can have long-lasting effects for future generations, since primary fungal mutualists can be selected depending on substrate humidity. It seems prudent for dispersing offspring to seek wood conditions matching those in their natal nest in order to provide optimal conditions for the microbial mutualist community they bring along. Since nest longevity and productivity appear to depend heavily on the mutualist community composition, which is also linked to philopatry and cooperative investment, the incentive for habitat matching may have selected for cooperative traits over evolutionary time. Testing this "habitat matching hypothesis" in future experiments could help to answer whether primary fungal mutualists can act as drivers of sociality in ambrosia beetles.

DATA AVAILABILITY STATEMENT

Raw data on the nest member density, behavior frequency, digging depth, and dispersal as well as the molecular reference files and shell scripts for bioinformatics processing can be found in the **Supplementary Material**. The nucleotide data associated with this study are accessible at the European Nucleotide Archive (accession number PRJEB44223; https://www.ebi.ac.uk/ena/browser/view/PRJEB44223).

AUTHOR CONTRIBUTIONS

JN and MT conceived and designed the experiments. JN carried out the experiments and analysed the data. JD developed and carried out the molecular analysis and analysed the molecular data. JN, JD, and MT wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.602672/full#supplementary-material

REFERENCES

Alexander, R. D., Noonan, K. M., and Crespi, B. J. (1991). "The evolution of eusociality," in *The Biology of the Naked Mole-Rat*, eds P. W. Sherman, J. U. M. Jarvis, and R. D. Alexander (Princeton, NJ: Princeton University Press), 3–44. doi: 10.1515/9781400887132

Ayasse, M., and Paxton, R. J. (2002). "Brood protection in social insects," in *Chemoecology of Insect Eggs and Egg Deposition*, eds M. Hilker and T. Meiners (Berlin: Blackwell Verlag GmbH), 117–148.

Ayres, M. P., Wilkens, R. T., Ruel, J. J., Lombardero, M. J., and Vallery, E. (2000). Nitrogen budgets of phloem-feeding bark beetles with and without symbiotic fungi. *Ecology* 81, 2198–2210.

Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67, 1–48. doi: 10.18637/jss.v067.i01

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

Biedermann, P. H. W. (2010). Observations on sex ratio and behavior of males in *Xyleborinus saxesenii* Ratzeburg (Scolytinae. Coleoptera). *Zookeys* 56, 253–267. doi: 10.3897/zookeys.56.530

Biedermann, P. H. W., Klepzig, K. D., and Taborsky, M. (2009). Fungus cultivation by ambrosia beetles: behavior and laboratory breeding success in three xyleborine species. *Environ. Entomol.* 38, 1096–1105. doi: 10.1603/022.038.0417

Biedermann, P. H. W., Klepzig, K. D., and Taborsky, M. (2011). Costs of delayed dispersal and alloparental care in the fungus-cultivating ambrosia beetle *Xyleborus affinis* Eichhoff (Scolytinae: curculionidae). *Behav. Ecol. Sociobiol.* 65, 1753–1761. doi: 10.1007/s00265-011-1183-5

Biedermann, P. H. W., Peer, K., and Taborsky, M. (2012). Female dispersal and reproduction in the ambrosia beetle *Xyleborinus saxesenii* Ratzeburg (Coleoptera; Scolytinae). *Mitteilungen der Dtsch. Gesellschaft für Allg. und Angew. Entomol.* 18, 231–236.

Biedermann, P. H. W., and Rohlfs, M. (2017). Evolutionary feedbacks between insect sociality and microbial management. *Curr. Opin. Insect Sci.* 22, 92–100. doi: 10.1016/j.cois.2017.06.003

Biedermann, P. H. W., and Taborsky, M. (2011). Larval helpers and age polyethism in ambrosia beetles. *Proc. Natl. Acad. Sci. U.S.A.* 108, 17064–17069. doi: 10.1073/pnas.1107758108

Biedermann, P. H. W., and Vega, F. E. (2020). Ecology and evolution of insect–fungus mutualisms. *Annu. Rev. Entomol.* 65, 431–455. doi: 10.1146/annurev-ento-011019-024910

Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H., et al. (2009). Generalized linear mixed models: a practical guide for ecology and evolution. *Trends Ecol. Evol.* 24, 127–135. doi: 10.1016/j.tree.2008.10.008

Bourke, A. F. G. (2011). *Principles of Social Evolution*. Oxford: Oxford University Press.

Browne, W. J., Subramanian, S. V., Jones, K., and Goldstein, H. (2005). Variance partitioning in multilevel logistic models that exhibit overdispersion. *J. R. Stat. Soc. Ser. A Stat. Soc.* 168, 599–613. doi: 10.1111/j.1467-985X.2004.00365.x

Choe, J. C., and Crespi, B. J. (1997). *The Evolution of Social Behaviour in Insects and Arachnids*. Cambridge: Cambridge University Press.

Clarke, K. R., Somerfield, P. J., and Chapman, M. G. (2006). On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray-Curtis coefficient for denuded assemblages. *J. Exp. Mar. Biol. Ecol.* 330, 55–80. doi: 10.1016/j.jembe.2005.12.017

Cotter, S. C., Topham, E., Price, A. J. P., and Kilner, R. M. (2010). Fitness costs associated with mounting a social immune response. *Ecol. Lett.* 13, 1114–1123. doi: 10.1111/j.1461-0248.2010.01500.x

Cremer, S., Armitage, S. A. O., and Schmid-Hempel, P. (2007). Social immunity. *Curr. Biol.* 17, R693–R702. doi: 10.1016/j.cub.2007.06.008

Döring, H., Clerc, P., Grube, M., and Wedin, M. (2000). Mycobiont-Specific PCR primers for the amplification of nuclear its and LSU rDNA from lichenized ascomycetes. *Lichenologist* 32, 200–204. doi: 10.1006/lich.1999.0250

Engqvist, L. (2005). The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. *Anim. Behav.* 70, 967–971.

Fischer, M. (1954). Untersuchungen über den Kleinen Holzbohrer (Xyleborinus Saxeseni Ratz.). *Pflanzenschutzberichte* 12, 137–180.

Fox, J., and Weisberg, S. (2019). *An {R} Companion to Applied Regression*, 3rd Edn. Thousand Oaks, CA: Sage.

Francke-Grosmann, H. (1975). The epizoic and endozoic transmission of the symbiotic fungus of the ambrosia beetle *Xyleborus saxeseni* (Coleoptera: scolytidae). *Entomol. Ger.* 1, 279–292.

Hamilton, W. D. (1964). The genetical evolution of social behaviour. I&II. *J. Theor. Biol.* 7, 1–52. doi: 10.1016/0022-5193(64)90039-6

Harrington, T. C., Aghayeva, D. N., and Fraedrich, S. W. (2010). New combinations in Raffaelea, Ambrosiella, and Hyalorhinocladiella, and four new species from the redbay ambrosia beetle, *Xyleborus glabratus*. *Mycotaxon* 111, 337–361.

Hart, A. G., Anderson, C., and Ratnieks, F. L. W. (2002). Task partitioning in leafcutting ants. *Acta Ethol.* 5, 1–11. doi: 10.1007/s10211-002-0062-5

Heg, D., Bachar, Z., Brouwer, L., and Taborsky, M. (2004). Predation risk is an ecological constraint for helper dispersal in a cooperatively breeding cichlid. *Proc. R. Soc. B Biol. Sci.* 271, 2367–2374. doi: 10.1098/rspb.2004.2855

Hölldobler, B., and Wilson, E. O. (2009). *The Superorganism: The Beauty, Elegance, and Strangeness of Insect Societies*. New York, NY: WW Norton & Company.

Hosking, G. P. (1973). Xyleborus saxeseni, its life-history and flight behaviour in New Zealand. *N. Zeal. J. For. Sci.* 3, 37–53.

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biom. J.* 50, 346–363. doi: 10.1002/bimj.200810425

Johnson, A. J., McKenna, D. D., Jordal, B. H., Cognato, A. I., Smith, S. M., Lemmon, A. R., et al. (2018). Phylogenomics clarifies repeated evolutionary origins of inbreeding and fungus farming in bark beetles (Curculionidae, Scolytinae). *Mol. Phylogenet. Evol.* 127, 229–238. doi: 10.1016/j.ympev.2018.05.028

Kasson, M. T., Wickert, K. L., Stauder, C. M., Macias, A. M., Berger, M. C., Simmons, D. R., et al. (2016). Mutualism with aggressive wood-degrading *Flavodon* ambrosius (Polyporales) facilitates niche expansion and communal social structure in Ambrosiophilus ambrosia beetles. *Fungal Ecol.* 23, 86–96. doi: 10.1016/j.funeco.2016.07.002

Keller, L., Peer, K., Bernasconi, C., Taborsky, M., and Shuker, D. M. (2011). Inbreeding and selection on sex ratio in the bark beetle *Xylosandrus germanus*. *BMC Evol. Biol.* 11:359. doi: 10.1186/1471-2148-11-359

Kent, D. S., and Simpson, J. A. (1992). Eusociality in the beetle Austroplatypus incompertus (Coleoptera: Curculionidae). *Naturwissenschaften* 79, 86–87.

Kirkendall, L. R., Biedermann, P. H. W., and Jordal, B. H. (2015). "Evolution and diversity of bark and ambrosia beetles," in *Bark Beetles*, eds F. E. Vega and R. W. Hofstetter (San Diego, CA: Elsevier), 85–156. doi: 10.1016/B978-0-12-417156-5.00003-4

Kirkendall, L. R., Kent, D. S., and Raffa, K. F. (1997). "Interactions among males, females and offspring in bark and ambrosia beetles: the significance of living in tunnels for the evolution of social behavior," in *The Evolution of Social Behaviour in Insects and Arachnids*, eds J. C. Choe and B. J. Crespi (Cambridge: Cambridge University Press), 181–214.

Koenig, W. D., and Dickinson, J. L. (2016). *Cooperative Breeding in Vertebrates: Studies of Ecology, Evolution, and Behavior*. Cambridge: Cambridge University Press.

Koenig, W. D., Pitelka, F. A., Carmen, W. J., Mumme, R. L., and Stanback, M. T. (1992). The evolution of delayed dispersal in cooperative breeders. *Q. Rev. Biol.* 67, 111–150. doi: 10.1086/417552

Korb, J. (2010). "Termite mound architecture, from function to construction," in *Biology of Termites: A Modern Synthesis*, eds D. E. Bignell, Y. Roisin, and N. Lo (Dordrecht: Springer Netherlands), 349–373. doi: 10.1007/978-90-481-3977-4_13

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., and Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* 79, 5112–5120. doi: 10.1128/AEM.01043-13

Lenth, R., Singman, H., Love, J., Buerkner, P., and Herve, M. (2019). *Estimated Marginal Means, aka Least-Squares Means.* R Packag. version 1.15-15. doi: 10.1080/00031305.1980.10483031<.License

Maleszka, R., and Clark-Walker, G. D. (1993). Yeasts have a four-fold variation in ribosomal DNA copy number. *Yeast* 9, 53–58. doi: 10.32388/ry98ex

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Meunier, J. (2015). Social immunity and the evolution of group living in insects. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370:20140102. doi: 10.1098/rstb.2014.0102

Miadlikowska, J., McCune, B., and Lutzoni, F. (2002). Pseudocyphellaria perpetua, a New Lichen from Western North America. *Bryologist* 105, 1–10.

Mizuno, T., and Kajimura, H. (2002). Reproduction of the ambrosia beetle, *Xyleborus* pfeili (Ratzeburg) (Col., Scolytidae), on semi-artificial diet. *J. Appl. Entomol.* 126, 455–462. doi: 10.1046/j.1439-0418.2002.00691.x

Mizuno, T., and Kajimura, H. (2009). Effects of ingredients and structure of semi-artificial diet on the reproduction of an ambrosia beetle, *Xyleborus* pfeili (Ratzeburg) (Coleoptera: Curculionidae: Scolytinae). *Appl. Entomol. Zool.* 44, 363–370. doi: 10.1303/aez.2009.363

Mueller, U. G., Gerardo, N. M., Aanen, D. K., Six, D. L., and Schultz, T. R. (2005). The evolution of agriculture in insects. *Annu. Rev. Ecol. Evol. Syst.* 36, 563–595. doi: 10.1146/annurev.ecolsys.36.102003.152626

Mullon, C., Keller, L., and Lehmann, L. (2018). Social polymorphism is favoured by the co-evolution of dispersal with social behaviour. *Nat. Ecol. Evol.* 2, 132–140. doi: 10.1038/s41559-017-0397-y

Norris, D. M., and Chu, H.-M. (1985). "Xyleborus ferrugineus," in *Handbook of Insect Rearing*, Vol. I, eds P. Singh and R. F. Moore (Amsterdam: Elsevier), 303–315.

Nuotclà, J. A., Biedermann, P. H. W., and Taborsky, M. (2019). Pathogen defence is a potential driver of social evolution in ambrosia beetles. *Proc. R. Soc. B Biol. Sci.* 286:20192332. doi: 10.1098/rspb.2019.2332

Nuotclà, J. A., Taborsky, M., and Biedermann, P. H. W. (2014). The importance of blocking the gallery entrance in the ambrosia beetle *Xyleborinus* saxesenii Ratzeburg (Coleoptera; Scolytinae). *Mitteilungen der Dtsch. Gesellschaft für Allg. und Angew. Entomol.* 19, 203–207.

Oksanen, A. J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., et al. (2016). *Community Ecology Package.* 0–291.

Oranen, H. (2013). *The Striped Ambrosia Beetle, Trypodendron lineatum (Olivier), and its Fungal Associates.* Available online at: https://helda.helsinki.fi/handle/10138/40117 (accessed April 16, 2021).

Peer, K., and Taborsky, M. (2004). Female ambrosia beetles adjust their offspring sex ratio according to outbreeding opportunities for their sons. *J. Evol. Biol.* 17, 257–264. doi: 10.1111/j.1420-9101.2003.00687.x

Peer, K., and Taborsky, M. (2005). Outbreeding depression, but no inbreeding depression in haplodiploid Ambrosia beetles with regular sibling mating. *Evolution (N. Y)* 59:317. doi: 10.1554/04-128

Peer, K., and Taborsky, M. (2007). Delayed dispersal as a potential route to cooperative breeding in ambrosia beetles. *Behav. Ecol. Sociobiol.* 61, 729–739. doi: 10.1007/s00265-006-0303-0

Pinheiro, J. C., Bates, D., DebRoy, S., Sarkar, D., and Team, R. C. (2018). *_nlme: Linear and Nonlinear Mixed Effects Models_*. Available online at: https://cran.r-project.org/package=nlme (accessed April 16, 2021).

Rebar, D., Bailey, N. W., Jarrett, B. J. M., and Kilner, R. M. (2020). An evolutionary switch from sibling rivalry to sibling cooperation, caused by a sustained loss of parental care. *Proc. Natl. Acad. Sci. U.S.A.* 117, 2544–2550. doi: 10.1073/pnas.1911677117

Roberts, H. (1960). *Trachyostus ghanaensis Schedl (Col., Platypodidae) an Ambrosia Beetle Attacking Wawa, Triplochiton scleroxylon*. London: West African Timber

Borer Research Unit by the Crown Agents for Oversea Governments and Administrations, 1–17. doi: 10.1017/CBO9781107415324.004

Rubenstein, D. R., and Abbot, P. (eds). (2017). *Comparative Social Evolution*, Cambridge: Cambridge University Press. doi: 10.1017/9781107338319

Sarkar, D. (2008). *Lattice: Multivariate Data Visualization with R*. New York, NY: Springer US.

Shukla, S. P., Plata, C., Reichelt, M., Steiger, S., Heckel, D. G., Kaltenpoth, M., et al. (2018). Microbiome-assisted carrion preservation aids larval development in a burying beetle. *Proc. Natl. Acad. Sci. U.S.A.* 115, 11274–11279. doi: 10.1073/pnas.1812808115

Simpson, G. L. (2019). *permute: Functions for Generating Restricted Permutations of Data*. Available online at: https://cran.r-project.org/package=permute (accessed April 16, 2021).

Six, D. L., and Bentz, B. J. (2007). Temperature determines symbiont abundance in a multipartite bark beetle-fungus ectosymbiosis. *Microb. Ecol.* 54, 112–118. doi: 10.1007/s00248-006-9178-x

Six, D. L., and Elser, J. J. (2019). Extreme ecological stoichiometry of a bark beetle–fungus mutualism. *Ecol. Entomol.* 44, 543–551. doi: 10.1111/een.12731

Skelton, J., Jusino, M. A., Carlson, P. S., Smith, K., Banik, M. T., Lindner, D. L., et al. (2019). Relationships among wood-boring beetles, fungi, and the decomposition of forest biomass. *Mol. Ecol.* 28, 4971–4986. doi: 10.1111/mec.15263

Skelton, J., Jusino, M. A., Li, Y., Bateman, C., Thai, P. H., Wu, C., et al. (2018). Detecting symbioses in complex communities: the fungal symbionts of bark and ambrosia beetles within asian pines. *Microb. Ecol.* 76, 839–850. doi: 10.1007/s00248-018-1154-8

Skutch, A. F. (1961). Helpers among Birds. *Condor* 63, 198–226. doi: 10.2307/1365683

Smith, S. M., Kent, D. S., Boomsma, J. J., and Stow, A. J. (2018). Monogamous sperm storage and permanent worker sterility in a long-lived ambrosia beetle. *Nat. Ecol. Evol* 2, 1009–1018. doi: 10.1038/s41559-018-0533-3

Stacey, P. B. (1979). Habitat saturation and communal breeding in the acorn woodpecker. *Anim. Behav.* 27, 1153–1166. doi: 10.1016/0003-3472(79)90063-0

Storer, C., Payton, A., McDaniel, S., Jordal, B., and Hulcr, J. (2017). Cryptic genetic variation in an inbreeding and cosmopolitan pest, *Xylosandrus crassiusculus*, revealed using ddRADseq. *Ecol. Evol.* 7, 10974–10986. doi: 10.1002/ece3.3625

Taborsky, M. (1994). "Sneakers, satellites, and helpers: parasitic and cooperative behavior in fish reproduction," in *Advances in the Study of Behavior*, eds P. J. B. Slater, J. S. Rosenblatt, C. T. Snowdon, and M. Milinski (New York, NY: Academic Press), 1–100. doi: 10.1016/S0065-3454(08)60351-4

Taborsky, M., Frommen, J. G., and Riehl, C. (2016). Correlated pay-offs are key to cooperation. *Philos. Trans. R. Soc. B Biol. Sci.* 371:20150084. doi: 10.1098/rstb.2015.0084

Therneau, T. M., and Grambsch, P. M. (2000). *Modeling Survival Data: Extending the Cox Model*. New York, NY: Springer New York. doi: 10.1007/978-1-4757-3294-8

Ulyshen, M. D. (2016). Wood decomposition as influenced by invertebrates. *Biol. Rev.* 91, 70–85. doi: 10.1111/brv.12158

Van Meyel, S., Körner, M., and Meunier, J. (2018). Social immunity: why we should study its nature, evolution and functions across all social systems. *Curr. Opin. Insect Sci.* 28, 1–7. doi: 10.1016/j.cois.2018.03.004

Vesala, R., Harjuntausta, A., Hakkarainen, A., Rönnholm, P., Pellikka, P., and Rikkinen, J. (2019). Termite mound architecture regulates nest temperature and correlates with species identities of symbiotic fungi. *PeerJ* 6:e6237. doi: 10.7717/peerj.6237

Weider, L. J., Elser, J. J., Crease, T. J., Mateos, M., Cotner, J. B., and Markow, T. A. (2005). The functional significance of ribosomal (r)DNA variation: impacts on the evolutionary ecology of organisms. *Annu. Rev. Ecol. Evol. Syst.* 36, 219–242. doi: 10.1146/annurev.ecolsys.36.102003.152620

Wickham, H. (2011). The split-apply-combine strategy for data analysis. *J. Stat. Softw.* 40, 1–29.

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer US.

Wickham, H., François, R., Henry, L., and Müller, K. (2019). *A Grammar of Data Manipulation*. doi: 10.18637/jss.v080.i01<. (accessed April 16, 2021).

Wickham, H., and Seidel, D. (2019). *scales: Scale Functions for Visualization*. Available online at: https://cran.r-project.org/package=scales.

Wood, S. N. (2017). *Generalized Additive Models: An Introduction with R, Second Edition*, 2nd editio Edn. Boca Raton, FL: Chapman and Hall/CRC. doi: 10.1201/9781315370279

Zimmermann, G. (1973). *Vergleichende Ökologisch-Physiologische Untersuchungen an Ambrosiapilzen, Assoziierten Bläuepilzen und Luftbläuepilzen.* Doctoral thesis. Germany: Georg-August University Göttingen.

Zimmermann, G., and Butin, H. (1973). Untersuchungen über die Hitze- und Trockenresistenz holzbewohnender Pilze. *Flora* 162, 393–419. doi: 10.1016/S0367-2530(17)31722-X

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ANNEX - CHAPTER 4

Supplementary Material

Supplementary Material

To publication **Habitat Quality Determines Dispersal Decisions and Fitness in a Beetle – Fungus Mutualism** by **JA Nuotclà, JMC Diehl & M Taborsky** in **Front. Ecol. Evol.**

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Construction of 16S and 28S ribosomal RNA gene libraries and amplicon sequencing
PCRs were performed in three separate 10µL reactions in order to avoid PCR bias. Each reaction contained 5µL 2 × Phusion Master Mix (New England Biolabs, Ipswich, MA, USA), 0.25µl each of the forward and reverse primers, 3.5µL DNase/RNase Free Water (ZymoBiomics Kit) and 1µL DNA template. One sample (150.5L; see **Supplementary Table 1** for explanation about sample naming) showed low bacterial presence, so we repeated the 16S ribosomal RNA PCR for this sample with 3µl of DNA template.

Bacterial 16S ribosomal RNA gene libraries were constructed using a dual-indexing strategy (Kozich et al., 2013) to amplify the V4 region. Here, adapters and dual indices were incorporated directly into the PCR primers, which allows multiplexing of the samples. Conditions for this PCR were as follows: initial denaturation at 95°C for 4 min, 30 cycles of denaturation at 95°C for 40 sec, annealing at 55°C for 30 sec and elongation at 72°C for 1 min, followed by a final extension step at 72°C for 5 min. Each sample was assigned a different forward/reverse index combination for sample-specific labelling. Triplicate reactions of each sample were combined after the PCR and further processed as described by Kozich et al. (2013), including between-sample normalization using the SequalPrep™ Normalization Plate Kit (Invitrogen GmbH, Darmstadt, Germany) and pooling of all samples. These pools were cleaned-up with the AMPure Beads Purification (Agilent Technologies, Inc. Santa Clara, CA, USA) and quality controlled using a Bioanalyzer High Sensitivity DNA Chip (Agilent Technologies, Santa Clara, CA, USA), quantified with the dsDNA High Sensitivity Assay (Life Technologies GmbH, Darmstadt, Germany), and afterwards combined to a single pool containing all samples. This was diluted to 8 pM, denatured and spiked with 5% Phix Control Kit v3 (Illumina Inc., San Diego, CA, USA) according to the Sample Preparation Guide (llumina Inc. 2013). Sequencing was performed on an Illumina MiSeq using 2 × 250 cycles v2 chemistry (Illumina Inc., San Diego, CA, USA).

For fungal analysis, we used the combination of amplifying barcoding primers LIC15R (Miadlikowska et al., 2002) and nu-LSU-355-3' (Döring et al., 2000). This combination was already used for fungus species identification based on Sanger sequencing in our lab and proved to be suitable for a meta-barcoding attempt. We chose this LSU primer pair to allow species identification of *X. saxesenii*'s known fungal symbionts, *Raffaelea sulphurea* and *R. canadensis*, of which reliable identification has been a common problem in previous meta-barcoding studies using ITS markers (Kostovcik et al., 2015; Malacrinò et al., 2017). Dreaden et al. (2014) already argued that an amplification of this region is difficult and inconsistent, especially for many of the fungal taxa in association with bark beetles. The newly chosen LSU primers deliver a fragment of suitable size for MiSeq v2 sequencing using 500 cycles and add up to other primer pairs used in bark beetle studies in recent works (e.g. Skelton et al., 2019).

Fungal 28S ribosomal RNA gene libraries were constructed the same way as the 16S gene libraries to amplify the large subunit (LSU) region. Conditions for this PCR were as follows: initial denaturation at 98°C for 30 sec, 35 cycles of denaturation at 98°C for 30 sec, annealing

at 55°C for 30 sec and elongation at 72°C for 15 sec; followed by a final extension step at 72°C for 10 min. Each sample was assigned a different forward/reverse index combination for sample-specific labelling. Like the 16S ribosomal RNA gene libraries, triplicate reactions of each sample were combined after PCR and further processed as described above.

Dual-indexing design for 28S ribosomal RNA gene libraries

For MiSeq-conformity, we expanded each of the primers (LIC15R and nu-LSU-355-3') according to the overall oligo scaffold described in Kozich et al., (2013). This scaffold consists of MiSeq-specific adapters, an 8nt index sequence, a 10nt pad as well as a 2nt linker sequence and lastly the amplifying primers. To successfully transfer the scaffold design to LSU sequencing, we ensured by minor modifications that the melting temperature (Tm) of the combined pad, linker and amplifying primer was ~65°C (see Additional file of Kozich et al., 2013) enabling the read primers to bind during the later sequencing procedure. In the forward scaffold, we adapted the pad sequence from 5′-TATGGTAATT -3′ to 5′-TATGGT**GC**T**G**-3′ (adapted nucleotides in bold). The pad of the reverse scaffold remained unchanged. Complete sequences of the final oligos were forward (fw):
5′AATGATACGGCGACCACCGAGATCTACAC-NNNNNNNN-TATGGT**GC**T**G**-GT-**GGAGGAAAAGAAACCAACAG**-3′
and reverse (rev):
5′-CAAGCAGAAGACGGCATACGAGAT-NNNNNNNN-AGTCAGTCAG-CC-**GCTTTTCATCTTTCGATCACTC**-3′
where adapted nucleotides are denoted in bold and NNNNNNNN indicates the index sequences used for multiplexing.

Primer sequences were thus 32nt (fw) and 34nt (rev) long, had a Tm of ~ 65°C, a ~ 47% GC content and exhibited low self-complementarity (longest dimer complement: 4 bp). They amplify a total fragment of approximately 330–340 bp. The actual sequenced part of this fragment covers 280–320 bp (target only) and is thus within the range of 2 × 250 cycles sequencing, leaving some buffer for joining the paired end reads. We used 16 forward index sequences SA501– SB508 and 24 reverse indices SA701–SB712, allowing a total of 384 unique combinations for sample indexing (Additional file of Kozich et al., 2013). With LSU-specific modifications, it was also necessary to modify the sequencing primers that are added to the MiSeq flow cell. We thus changed read and index primers as follows (adapted nucleotides in bold):
Read1: 5'-TATGGT**GC**T**G**-GT- **GGAGGAAAAGAAACCAACAG**-3‘
Read2: 5`-AGTCAGTCAG-CC- **GCTTTTCATCTTTCGATCACTC**-3‘
Index: 5`- **GAGTGATCGAAAGATGAAAAGC** – GG – CTGACTGACT – 3`

Bioinformatics processing

Raw sequence reads were obtained from the Illumina MiSeq output directly, which includes sample reads already demultiplexed by the MiSeq Reporter v. 2.5.1.3 with perfect index matches only. Forward and reverse reads were merged using the -fastq_mergepairs command in USEARCH v11.0-2.667 (Edgar, 2010), by applying additional parameters, a first quality filtering (sequence length >200 and maximum differences in the alignment =30) was performed and three output files, including either merged, not merged forward and reverse reads, were created. Overall quality was assessed using the command –fastq_filter specifying a high expected error threshold of 1 (fastq_maxee). Unique sequences in our FASTq file were identified with the command –fastx_uniques and sorted by decreasing size annotation with a minimum size of 4 (-sortbysize). Before clustering of operational taxonomic units (OTUs), we denoised the amplicon reads with –unoise3 of the UNOISE algorithm (Edgar, 2016b). Instead

of commonly used OTUs, our attempt identified all correct biological sequences in the reads, which are called zOTUs (zero radius operational taxonomic units or amplicon sequence variants; Callahan et al., 2017) after denoising. With the –usearch_global command we set the identity threshold to 99% identity and created an uc-map (USEARCH cluster format) of the input sequences that are now clustered and matched in a database search. The resulting .uc file was converted to a zOTU table by the command –uc2otutab.py of the Python algorithm (https://www.python.org/). ZOTUs were taxonomically classified with the –sintax command and the rdp_16s_v16_sp.fa reference database (Edgar, 2016a) for the 16S ribosomal RNA sequences using a cutoff of 0.8, which gives predictions of similar accuracy to RDP at 80% bootstrap cutoff. Two new reference databases found their application for the 28S ribosomal RNA sequences. The first database applied, is a collection of 28S ribosomal RNA sequences of fungal stocks at the lab of Peter Biedermann (University of Wuerzburg, Germany). Since this library is gathered from known symbionts and pathogens of our model organism, *X. saxesenii*, we lowered the identity threshold to 97% identity. zOTUs with no hit were classified a second time with a custom reference database of fungi from NCBI, created with BCdataBaser v.1.1.1. (Keller et al., 2019), using the cutoff of 0.8 and the usual identity threshold of 99% identity. Finally, both tables were combined into the syntax table. For details on parameter settings for all steps above, see the supplemented Shell-scripts.
Laboratory control samples suggested 243 of the bacterial 16S ribosomal RNA and 56 of the fungal 28S ribosomal RNA zOTUs to originate from kits, plasticware and/or laboratory contamination, or they could not be assigned further than to Kingdom/Domain level, which were removed prior to follow-up analyses.

Sequencing output and data analysis
In total we obtained 36,625,164 raw 16S ribosomal RNA reads and 24,009,500 28S ribosomal RNA reads, which accounted for an average of 95,378 reads for 16S ribosomal RNA and 62,525 reads for 28S ribosomal RNA per sample in our two sequencing runs. The total read size for this project was 850,392 raw 16S ribosomal RNA reads and 756,564 raw 28S ribosomal RNA reads, which accounted for an average of either 25,769 or 22,252 reads per sample. After data processing (merging, low quality <Q20, short reads <200 bp, ambiguous base-pairs) and further removal of Chloroplast genes and amplicon sequence variants that were only identified to domain level, left a mean of 13,084 reads per 16S ribosomal RNA sample and 17,481 per 28S ribosomal RNA sample for downstream analysis (16S: SD 8,068; median 11,338 / 28S: SD 9,841; median 16,079). Species accumulation curves (**Supplementary Figure 2 B, D**) show that almost all samples were sequenced to saturation after approximately 5,000-6,000 high quality reads for both. There were in total 804 amplicon sequence variants included in the 16S ribosomal RNA analysis after deletion of control samples. For the 28S ribosomal RNA in total 103 amplicon sequence variants were included in the analysis. After checking for the most abundant amplicon sequence variants in our controls and samples, *Chaetomium globosum* and *Penicillium sp.* appeared to be quite overrepresented in terms of abundance and seemed to cloud all other species, since it already appeared in the negative controls. Consequently, we expected these species to show cross-contamination, too, and removed zOTUs 1, 2, 20, 22, 25, 29, 32, 34, 36, 40, 38, 41, 48, 51, 52, 64, 65, 68, 73 and 77 from further analysis for a final look on changes of the more interesting ambrosia fungi. A similar situation was found for the 16S dataset, where Wolbachia sp. (zOTU 1) was overrepresented in the treatments by over 60% relative read abundance. It was thus removed as well to allow focussing on the rest of the bacterial community. *Wolbachia* is a known endosymbiont of ambrosia beetles (Kawasaki et al., 2010, 2016) and as such not relevant for the present study, which has focused on the microbial community within the nest. Nevertheless, the occurrence of Wolbachia in our samples may be interesting for future research, since Wolbachia infections have not yet been

found in *X. saxesenii* (Kawasaki et al., 2016).

A closer look at the controls showed a sufficient sequencing result of the microbial community standard from ZymboBiomics. All bacterial species contained in the standard are represented in the bar graph of relative bacterial taxa abundance (**Supplementary Figure 3**). Negative controls (autoclaved rearing medium for beetle breeding and PCR water control) showed some bacterial species which can be neglected, since the first control of these samples with gel electrophoresis ahead to sequencing revealed no visible bands and rarefaction curves and richness estimates suggest a low input of single sequences due to possible cross-contamination (**Supplementary Figures 3, 4**). Control samples extracted from the nesting chambers of nests separately reared under 'normal' standard conditions and their emerging dispersing females indicated that dispersing females have a more diverse bacterial community compared to their nest (**Supplementary Figure 3**). Similarly, a closer look on the fungal controls yielded important information on the quality of our newly developed 28S MiSeq primers. Our own mock community of known fungi associated with *X. saxesenni* (*R. sulphurea, R. canadensis, C. globosum, Ophiostoma stenosterans* and *Pichia sp.*) revealed that the two symbionts *R. canadensis* and *R sulphurea* can be distinguished, as well as other fungi of the orders Eurotiales, Sordariales, Hypocreales, Capnodiales, Onygenales and Dothideales, but yeasts including Saccharomycetales (e.g. *Pichia sp.*, *Candida sp.*) were not differentiated (**Supplementary Figure 5**). The negative controls showed some fungal taxa too, which can also be neglected since the first control of these samples with gel electrophoresis ahead to sequencing revealed no visible bands and rarefaction curves as well as richness estimates again suggest a low input of single sequences due to potential cross-contamination (**Supplementary Figure 5, 6**). Interestingly, the comparison of nest chamber material and dispersing females demonstrated, that by removing the overrepresented *Chaetomium globosum*, ambrosia fungi were revealed that had not been visible before (**Supplementary Figure 5**). Illustrating the output of the bacterial and fungal dataset after removal of overrepresented taxa thus allows for a better overview of the relevant ambrosia fungi within our samples (**Supplementary Figures 3-6**).

Reference database

Our own reference database from known fungal species of ambrosia beetles included eighteen reference sequences of twelve unique fungal species. The new reference database created with BCdatabaser included 85,250 fungal sequences.

Details of bacterial species composition

The regular nest community consisted of mainly *Pseudomonas*, *Halomonas* (or Halomonadaceae sp.), *Streptomyces*, *Acinetobacter* and Comamonadaceae sp. Some samples were lacking some of these genera but had all *Bacillus* in common (40.8E, 40.8L, 100.8L, 100.9E, 150.5E, 150.5L & 150.6E; see **Supplementary Table 1** for explanation about sample naming). In three samples (100.9E, 150.5L & 40.8L) *Staphylococcus* was present. All nests had *Pseudomonas* present, except in sample 150.5E, where a *Bradyrhizobium*, *Bacillus* and Comamonadaceae sp. built the main community, and sample 40.8E with a community of *Pseudoxanthomas, Bacillus*, Comamonadaceae sp. and Moraxellaceae sp. (>5%). Finally, the sample 150.6L showed a bacterial community with the highly abundant genera *Pseudomonas, Pedobacter* and Enterbacteriaceae sp. and in sample 40.10E, *Pseudomonas* (85.2% RA) was the only high abundant genus >5% relative abundance.

The class of Gammproteobacteria was the most abundant with its representatives

Pseudoxanthomonas, Pseudomonas, Acinetobacter and *Moraxellaceae sp.* with a mean abundance of 4.74%, 16.0%, 5.77% and 1.42% (mean RA). The last three Gammaproteobacteria could only be assigned up to family level and were Halomonadaceae sp. (mean RA = 5.53%), *Halomonas* (mean RA = 3.71%) and Enterobacteriaceae sp. (mean RA = 1.66%, appeared only in sample 150.6L). The genera *Streptomyces* (Actinobacteria) and *Bacillus* (Bacilli) belong to the bigger groups, too, with 8.71% and 8.77% (mean RA). Other present genera but in lower relative abundance were *Staphylococcus* (mean RA = 2.81%), *Pedobacter* (mean RA = 0.5%, but only present in sample 150.6L), Comamonadaceae sp. (only assigned up to family level; mean RA = 5.33%) and *Bradyrhizobium* (mean RA = 1.55%).

References

Callahan, B. J., McMurdie, P. J., and Holmes, S. P. (2017). Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J.* 11, 2639–2643. doi:10.1038/ismej.2017.119.

Döring, H., Clerc, P., Grube, M., and Wedin, M. (2000). Mycobiont-Specific PCR Primers for the Amplification of Nuclear its and LSU rDNA from Lichenized Ascomycetes. *Lichenol.* 32, 200–204. doi:10.1006/lich.1999.0250.

Dreaden, T. J., Davis, J. M., de Beer, Z. W., Ploetz, R. C., Soltis, P. S., Wingfield, M. J., et al. (2014). Phylogeny of ambrosia beetle symbionts in the genus Raffaelea. *Fungal Biol.* 118, 970–978. doi:10.1016/j.funbio.2014.09.001.

Edgar, R. (2016a). SINTAX: a simple non-Bayesian taxonomy classifier for 16S and ITS sequences. *bioRxiv*, 074161. doi:10.1101/074161.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi:10.1093/bioinformatics/btq461.

Edgar, R. C. (2016b). UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *bioRxiv*, 081257. doi:10.1101/081257.

Kawasaki, Y., Ito, M., Miura, K., and Kajimura, H. (2010). Superinfection of five Wolbachia in the alnus ambrosia beetle, Xylosandrus germanus (Blandford) (Coleoptera: Curculionidae). *Bull. Entomol. Res.* 100, 231–239. doi:10.1017/S000748530999023X.

Kawasaki, Y., Schuler, H., Stauffer, C., Lakatos, F., and Kajimura, H. (2016). Wolbachia endosymbionts in haplodiploid and diploid scolytine beetles (Coleoptera: Curculionidae: Scolytinae). *Environ. Microbiol. Rep.* 8, 680–688. doi:10.1111/1758-2229.12425.

Keller, A., Hohlfeld, S., Kolter, A., Schultz, J., Gemeinholzer, B., and Ankenbrand, M. (2019). BCdatabaser: on-the-fly reference database creation for (meta-)barcoding. doi:10.32942/osf.io/cmfu2.

Kostovcik, M., Bateman, C. C., Kolarik, M., Stelinski, L. L., Jordal, B. H., and Hulcr, J. (2015). The ambrosia symbiosis is specific in some species and promiscuous in others: Evidence from community pyrosequencing. *ISME J.* 9, 126–138. doi:10.1038/ismej.2014.115.

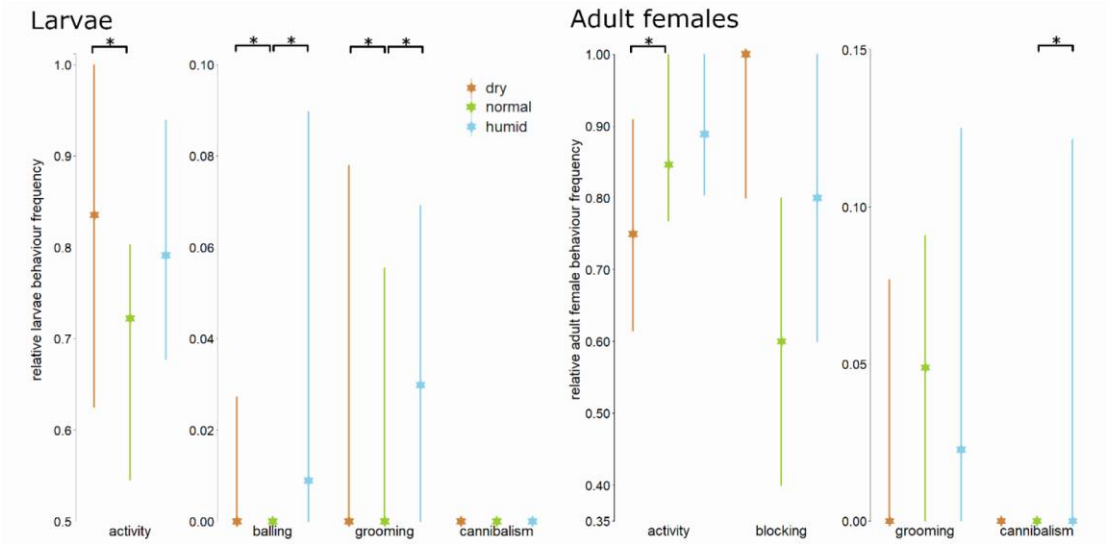
Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., and Schloss, P. D. (2013). Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. *Appl. Environ. Microbiol.* 79, 5112–5120. doi:10.1128/AEM.01043-13.

Malacrinò, A., Rassati, D., Schena, L., Mehzabin, R., Battisti, A., and Palmeri, V. (2017). Fungal communities associated with bark and ambrosia beetles trapped at international harbours. *Fungal Ecol.* 28, 44–52. doi:10.1016/j.funeco.2017.04.007.

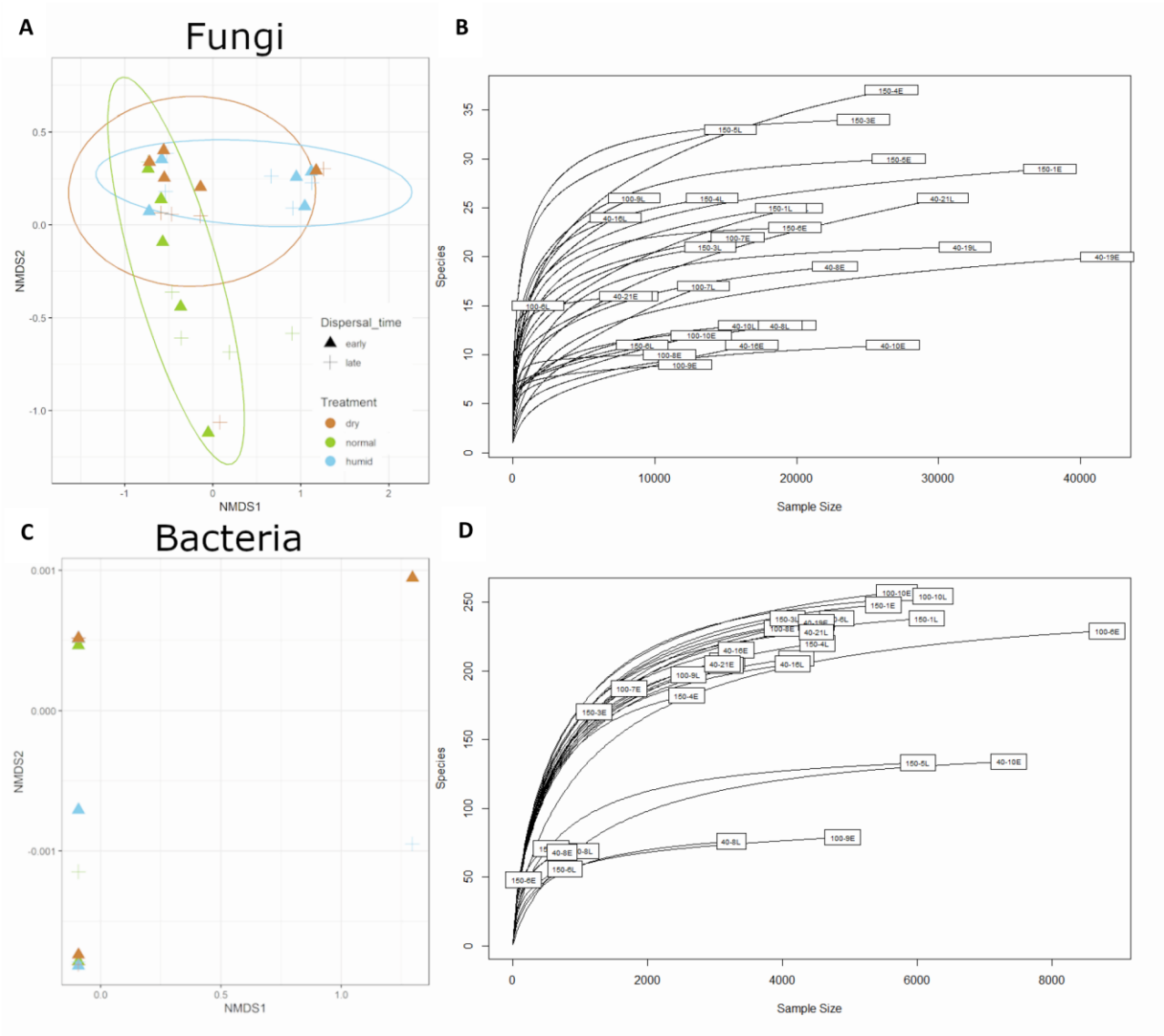
Miadlikowska, J., McCune, B., and Lutzoni, F. (2002). Pseudocyphellaria perpetua, a New Lichen from Western North America. *Bryologist* 105, 1–10. doi:10.1639/0007-

2745(2002)105[0001:PPANLF]2.0.CO;2.
Skelton, J., Jusino, M. A., Carlson, P. S., Smith, K., Banik, M. T., Lindner, D. L., et al. (2019). Relationships among wood-boring beetles, fungi, and the decomposition of forest biomass. *Mol. Ecol.* 28, 4971–4986. doi:10.1111/mec.15263.

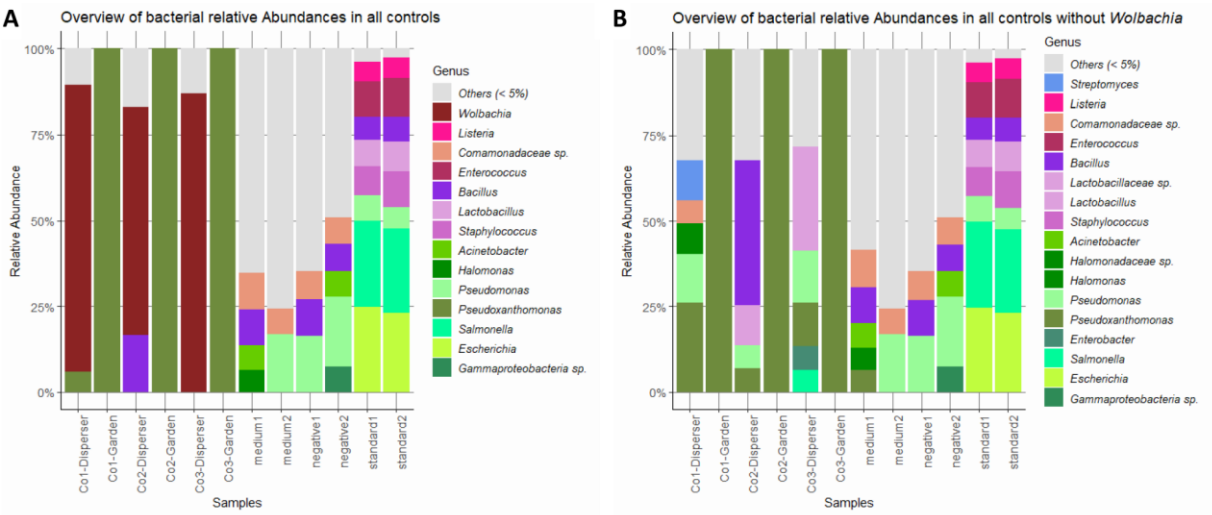
Supplementary Figures



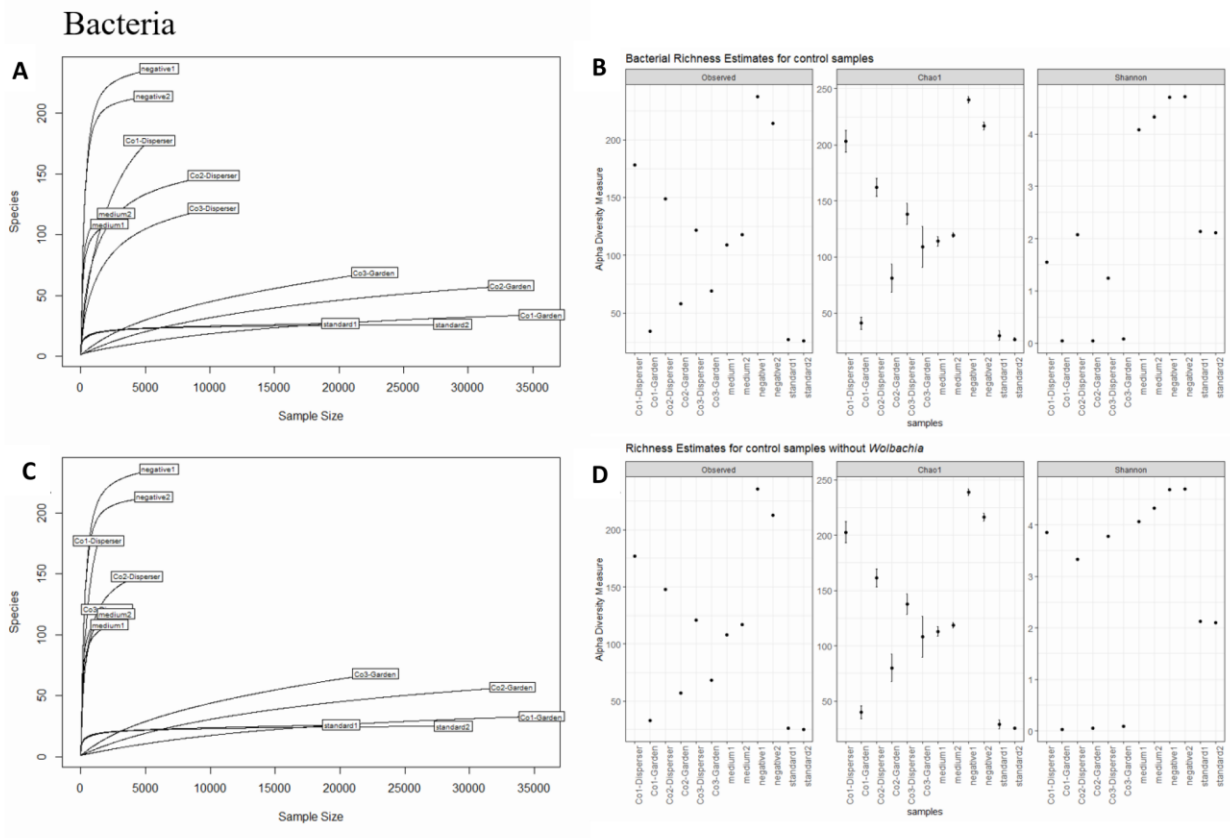
Supplementary Figure 1 | Relative frequencies of larvae and adult female behaviours recorded during the 'humidity experiment'. Medians and interquartile ranges of behavioural frequencies relative to all displayed behaviours are displayed; black stars in the top line indicate statistically significant differences between the humidity treatments within each behaviour of an age class (GLMM; $P<0.05$).



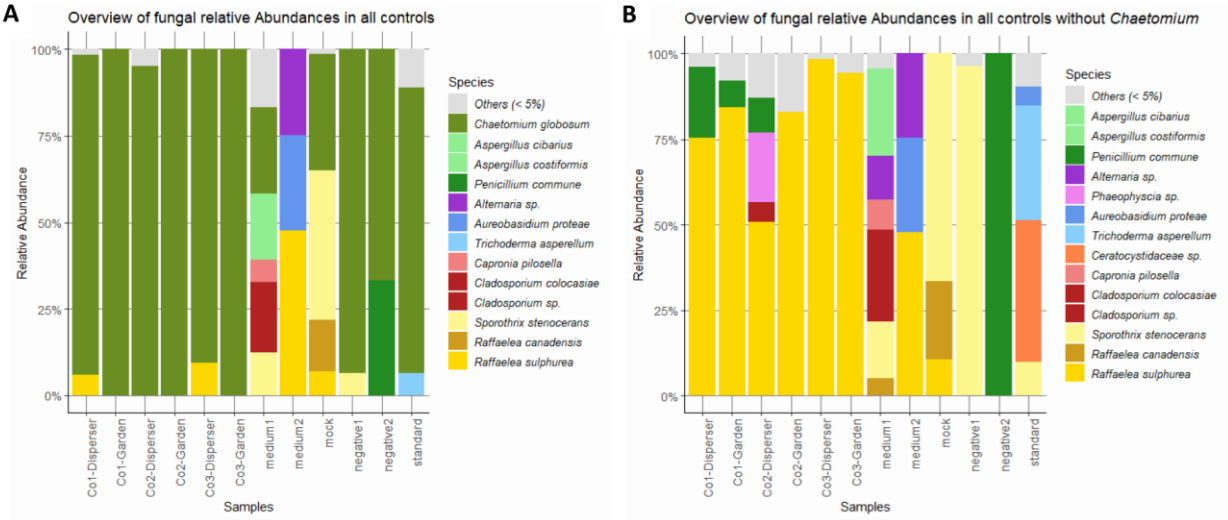
Supplementary Figure 2 | NMDS plots in **A** (fungi) and **C** (bacteria) do not reveal any obvious overall functional differences between the treatments regarding the respective microbial diversity (PERMANOVA: P>0.1). Rarefaction curves for **B** (fungi) and **D** (bacteria) indicate that most samples were sequenced to saturation.



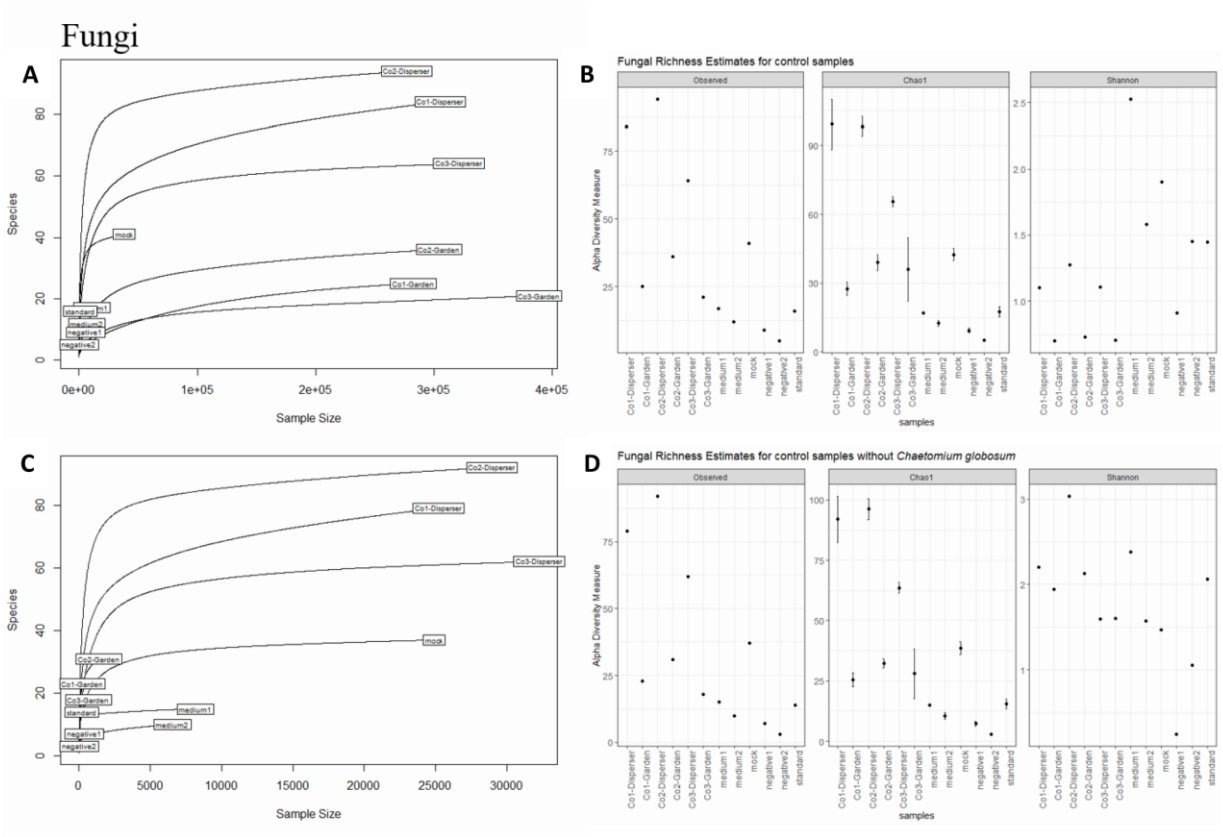
Supplementary Figure 3 | Relative read abundance of the major bacterial taxa **(A)** and the same for bacterial taxa without *Wolbachia sp.* **(B)** from all control samples used in this project. "Medium" represents the pure beetle rearing substrate which is sterile (autoclaved) before beetles are introduced. The "negative control" represents the PCR product of amplified PCR water used in the amplifications. The "positive control" represents ZymoBiomics Microbial Community Standard ("standard"). All bacterial species included in this standard were successfully sequenced. To directly compare a dispersing female ("Co-Disperser"), with their originating fungus garden ("Co-Garden"), we added additionally three of these controls. Note that these beetle nests were not from the same breeding line that was used for the present study. They were added later from a breeding stock using the 'normal' standard substrate formula at the University of Würzburg, where the microbial community analysis was conducted.



Supplementary Figure 4 | Rarefaction curves in **A** (bacterial controls) and **C** (bacterial controls, *Wolbachia* excluded) indicate that positive controls (standard 1 + 2) and control fungus garden samples (Co-Garden) were sequenced to saturation. Negative controls (negative 1 + 2, medium 1 + 2) and control samples of dispersing females (Co-Disperser) showed no clear asymptote, especially after removal of *Wolbachia sp.* reads. Richness estimates for **B** (bacterial controls) and **D** (bacterial controls, *Wolbachia* excluded) indicate that positive control samples and control fungus garden samples were similarly abundant (Shannon index), whereas negative controls were unequally abundant and more concentrated to single zOTUs. After removal of *Wolbachia sp.* reads, control samples of dispersing females changed from a similar abundance to an unequal one. The observed species richness and Chao1 indices did not change after the removal.



Supplementary Figure 5 | Relative read abundance of the major fungal taxa **(A)** and fungal taxa without *Chaetomium globosum* **(B)** from all control samples used in this project. "Medium" is the pure beetle rearing medium which is supposed to be sterile (autoclaved) before beetles are introduced. The "negative control" is the PCR product of amplified PCR water used in the amplifications. The first "positive control" represents the ZymoBiomics Microbial Community Standard. The two yeast species included in this standard were not sequenced and shows a lack of the new primers in amplifying yeasts. The second "positive control" is represented by a self-created mock community from known fungal species of ambrosia beetle gardens (*R. sulphurea, R. canadensis, C. globosum, Ophiostoma stenocerans* and *Pichia sp.*). The yeast (*Pichia sp.*) was again not successfully sequenced. To directly compare a dispersing female (Co-Disperser) with her originating nest chambers (Co-Garden), we additionally analysed three beetles and their corresponding nest chamber material from three different nests. As noted in the legend of **Supplementary Figure 3**, these beetle nests were not from the same breeding line that was used for the present study. They were added later from a breeding stock using the 'normal' standard substrate formula at the University of Würzburg, where the microbial community analysis was conducted.



Supplementary Figure 6 | Rarefaction curves in **A** (fungal controls) and **C** (fungal controls, *Chaetomium globosum* excluded) indicate that the fungal mock community, control samples of dispersing females (Co-Disperser) and control fungus garden samples (Co-Garden) were sequenced to saturation. In contrast, the negative controls (negative 1 + 2, medium 1 + 2) and the microbial community standard (ZymoBiomics) show no clear asymptote. After removal of *Chaetomium globosum* reads, this affects the control fungus garden samples as well. Richness estimates for **B** (fungal controls) and **D** (fungal controls, *Chaetomium globosum* excluded) indicate that overall all control samples were similarly abundant (Shannon index), whereas negative control medium 1 was more unequally abundant and more concentrated to single zOTUs. After removal of *Chaetomium globosum*, the control sample of dispersing female 2 changed from a similar abundance to a more unequal one, opposite to the medium 1 sample. The observed species richness and Chao1 indices did not change after the removal.

Supplementary Tables

Supplementary Table 1 | zOTU richness of fungal 28S ribosomal RNA and bacterial 16S ribosomal RNA. Sample ID coding: the number before the dot indicates the water content relative to the 'normal' treatment in %; the number after the dot indicates the respective nest from where the sampled beetle dispersed from either 'early' (E=first disperser) or 'late' (L=last disperser).

	# unique zOTUs in 'normal'			**# unique zOTUs in 'humid'**			**# unique zOTUs in 'dry'**	
Nest ID & dispersal time	**Bacteria 16S**	**Fungi 28S**	**Nest ID & dispersal time**	**Bacteria 16S**	**Fungi 28S**	**Nest ID & dispersal time**	**Bacteria 16S**	**Fungi 28S**
100.10E	257	12	150.1E	248	29	40.10E	134	11
100.10E	254	13	150.1L	238	25	40.10L	207	13
100.6E	229	16	150.3E	170	34	40.16E	215	11
100.6L	238	15	150.3L	238	21	40.16L	205	24
100.7E	187	22	150.4E	182	37	40.19E	235	20
100.7L	209	17	150.4L	220	26	40.19L	204	21
100.8E	231	10	150.5E	71	30	40.21E	205	16
100.8L	69	25	150.5L	133	33	40.21L	228	26
100.9E	79	9	150.6E	48	23	40.8E	68	19
100.9L	197	26	150.6L	56	11	40.8L	76	13
mean +SD	**195** ± 67.68	**16.5** ± 6.02	mean +SD	**160.4** ± 78.95	**26.9** ± 7.53	mean +SD	**177.7** ± 61.94	**17.4** ± 5.40

Supplementary Table 2 | Means ± SD of relative abundance (RA) of the different fungal taxa that composed more than 0.5% of the total fungal abundance within our samples.

Genus	Treatment	Mean RA (%)
Alternaria sp.	dry	1.18 ± 3.64
	normal	0.05 ± 0.17
	humid	0.33 ± 0.96
Aspergillus flavus	dry	2.08 ± 5.18
	normal	5.67 ± 14.25
	humid	0.03 ± 0.04
Aurebasidium leucospermi	dry	0.88 ± 2.68
	normal	0.36 ± 1.52
	humid	0.001 ± 0.006
Cladosporium sp.	dry	0.43 ± 0.81
	normal	0.79 ± 1.14
	humid	0.39 ± 0.81
Nectriaceae sp.	dry	44.94 ± 31.96
	normal	44.18 ± 31.69
	humid	36.56 ± 32.39
Petriella sp.	dry	22.31 ± 38.47
	normal	6.71 ± 14.50
	humid	47.39 ± 39.29
Raffaelea canadensis	dry	9.21 ± 12.84
	normal	2.01 ± 3.38
	humid	1.14 ± 1.19
Raffaelea sulphurea	dry	4.33 ± 14.92
	normal	13.99 ± 23.20
	humid	2.65 ± 4.06
Sporothrix sp.	dry	2.09 ± 3.02
	normal	3.65 ± 5.11
	humid	1.12 ± 1.68
Talaromyces rugulosus	dry	0.01 ± 0.03
	normal	0.08 ± 0.21
	humid	1.54 ± 4.75

Supplementary Table 3 | Means ± SD of relative abundance (RA) of the different bacterial taxa that composed more than 5% of the total bacterial abundance within our samples.

Genus	Treatment	Mean RA (%)
Acinetobacter	dry	4.79 ± 2.25
	normal	6.91 ± 4.91
	humid	5.62 ± 1.70
Bacillus	dry	9.68 ± 19.89
	normal	7.41 ± 17.16
	humid	9.22 ± 16.31
Bradyrhizobium	dry	1.07 ± 0.74
	normal	1.32 ± 0.83
	humid	2.25 ± 2.70
Comamonadaceae sp.	dry	5.27 ± 2.17
	normal	5.57 ± 1.38
	humid	5.16 ± 1.73
Enterobacteriaceae sp.	dry	1.11 ± 0.39
	normal	0.91 ± 0.45
	humid	2.96 ± 6.23
Halomonadaceae sp.	dry	6.09 ± 4.21
	normal	6.02 ± 3.42
	humid	4.48 ± 3.87
Halomonas	dry	3.90 ± 1.73
	normal	3.75 ± 1.47
	humid	3.47 ± 1.60
Moraxellaceae sp.	dry	1.77 ± 1.29
	normal	1.06 ± 0.55
	humid	1.44 ± 1.19
Pedobacter	dry	0.07 ± 0.09
	normal	0.13 ± 0.27
	humid	1.29 ± 3.81
Pseudomonas	dry	21.02 ± 23.01
	normal	11.83 ± 3.56
	humid	15.14 ± 9.22
Pseudoxanthomonas	dry	2.21 ± 3.72
	normal	8.95 ± 13.99
	humid	3.06 ± 2.48
Staphylococcus	dry	2.59 ± 1.39
	normal	2.90 ± 1.60
	humid	2.93 ± 1.54
Streptomyces	dry	8.51 ± 5.86
	normal	9.83 ± 5.41
	humid	7.78 ± 6.73

Supplementary Table 4 | Generalised linear mixed model outputs of the most common **larvae** behaviours (response variables). Effects of humidity treatment ('normal' (reference) vs 'dry' vs 'humid'), nest age and their interaction on the behaviours are shown. R code for a maximal model: glmer(behaviour ~ treatment * age + (1|nestID), family = binomial). §: Model corrected for overdispersion. Significant P-values are printed in bold.

	Parameter		Estimate±SE	Z	P
Activity (§)	intercept		1.438±0.354	4.061	**<0.001**
	nest age		-0.014±0.006	-2.499	**0.012**
	treatment	normal	reference		
		dry	1.037±0.360	2.879	**0.004**
		humid	0.463±0.367	1.261	0.207
Allogrooming	intercept		4.068±2.288	1.778	0.075
	nest age		-0.188±0.062	-3.030	**0.002**
	treatment	normal	reference		
		dry	-5.501±2.331	-2.360	**0.018**
		humid	-5.259±2.540	-2.071	**0.038**
	interaction	normal	reference		
		dry	0.165±0.062	2.645	**0.008**
		humid	0.148±0.068	2.191	**0.028**
Cannibalism (§)	intercept		-8.247±1.073	-7.686	**<0.001**
Balling	intercept		-3.827±0.621	-6.164	**<0.001**
	nest age		-0.025±0.009	-2.812	**0.005**
	treatment	normal	reference		
		dry	1.338±0.651	2.055	**0.040**
		humid	1.635±0.644	2.539	**0.011**

Supplementary Table 5 | Generalised linear mixed model outputs of the most common **adult female** behaviours (response variables). Effects of humidity treatment ('normal' (reference) vs 'dry' vs 'humid'), nest age and their interaction on the behaviours are shown. R code for a maximal model: glmer(behaviour ~ treatment * age + (1|nestID), family = binomial). §: Model corrected for overdispersion. Significant P-values are printed in bold.

	Parameter		Estimate±SE	Z	P
Activity (§)	intercept		0.140±0.775	0.181	0.857
	nest age		0.035±0.019	1.870	0.062
	treatment	normal	reference		
		dry	2.189±0.803	2.726	**0.006**
		humid	0.871±1.153	0.756	0.450
	interaction	normal	reference		
		dry	-0.049±0.019	-2.570	**0.010**
		humid	-0.018±0.026	-0.709	0.478
Allogrooming	intercept		-1.896±0.198	-9.568	**<0.001**
	nest age		-0.015±0.003	-4.503	**<0.001**
Cannibalism (§)	intercept		-3.911±0.960	-4.074	**<0.001**
	nest age		-0.046±0.015	-3.191	**0.001**
	treatment	normal	reference		
		dry	0.441±0.766	0.575	0.565
		humid	2.046±0.779	2.626	**0.009**
Blocking (§)	intercept		1.894±1.142	1.659	0.097
	nest age		-0.034±0.025	-1.346	0.178
	treatment	normal	reference		
		dry	-0.934±1.483	-0.630	0.529
		humid	1.755±2.002	0.877	0.381
	interaction	normal	reference		
		dry	0.048±0.029	1.666	0.096
		humid	-0.027±0.041	-0.658	0.511

Additional Protocols

ZymoBIOMICS DNA Miniprep Kit (adapted Protocol)

1. Add sample to **Micro Tubes 2 ml with cap and one ceramic bead.** Add 750 µl **ZymoBIOMICS Lysis Solution** to the tube and cap tightly.
2. Secure in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed (28s) for 6 minutes.
3. Centrifuge the **Micro Tubes** in a microcentrifuge at ≥ 10,000 x g for 1 minute.
4. Transfer all of the supernatant to another **Micro Tube 2ml with cap and 1 spoon of 0.1 mm and two spoons of 0.5 mm glass beads** and cap tightly.
5. Secure in a **Vortex Genie 2** and process for 30 minutes. Centrifuge at ≥10.000 x g for 1 minute.
6. Transfer up to 400 µl supernatant to the **Zymo-Spin III-F Filter** in a **Collection Tube** and centrifuge at 8,000 x g for 1 minute. Discard the Zymo-Spin III-F Filter.
7. Add 1,200 µl of **ZymoBIOMICS DNA Binding Buffer** to the filtrate in the Collection Tube from Step 6. Mix well.
8. Transfer 800 µl of the mixture from Step 7 to a **Zymo-Spin IIC-Z Column** in a Collection Tube and centrifuge at 10,000 x g for 1 minute.
9. Discard the flow through from the Collection Tube and repeat Step 8 with rest of mixture.
10. Add 400 µl **ZymoBIOMICS DNA Wash Buffer 1** to the Zymo-Spin IIC-Z Column in a new Collection Tube and centrifuge at 10,000 x g for 1 minute. Discard the flow-through.
11. Add 700 µl **ZymoBIOMICS DNA Wash Buffer 2** to the Zymo-Spin IIC-Z Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute. Discard the flow-through.
12. Add 200 µl **ZymoBIOMICS DNA Wash Buffer 2** to the Zymo-Spin IIC-Z Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute.
13. Transfer the Zymo-Spin IIC-Z Column to a clean 1.5 ml microcentrifuge tube and add 70 µl (60°C) **ZymoBIOMICS DNase/RNase Free Water** directly to the column matrix and incubate for 1 minute. Centrifuge at 10,000 x g for 1 minute to elute the DNA.
14. Reuse the column and add the same 70 µl again directly to the column matrix and incubate for 3 minutes. Centrifuge at 10,000 x g for 1 minute.
15. Place a **Zymo-Spin III-HRC Filter** in a new Collection Tube and add 600 µl **ZymoBIOMICS HRC Prep Solution**. Centrifuge at 8,000 x g for 3 minutes.
16. Transfer the eluted DNA (Step 11) to a prepared Zymo-Spin III-HRC Filter in a clean 1.5 ml microcentrifuge tube and centrifuge at exactly 16,000 x g for 3 minutes.

The filtered DNA is now suitable for PCR and other downstream applications.

Normalization

Binding step:

- Transfer desired volume of PCR product (25 µl) from the PCR plate into the wells of the SequelPrep Normalization plate
- Add an equivalent volume of SequelPrep Normalization Binding Buffer (25 µl PCR Product + 25 µl Binding Buffer)
- Mix completely by pipetting up and down or seal the plate with foil tape and vortex to mix and briefly centrifuge
- Incubate the plate for 1h at RT to allow binding of DNA to the surface (overnight)

Wash step:

- Throw away the liquid in the sink and dry the plate on a paper towel
- Add 50 µl washing buffer, mix carefully using heat block rotator
- Throw away washing buffer in sink and dry plate on a paper towel

Elution step:

- Add 20 µl elution buffer to each well
- Mix by pipetting up and down 5 times or seal the plate with foil tape and vortex to mix and briefly centrifuge
- Incubate at RT for 5 minutes
- After elution put 5 µl of each well all together in one Eppi
    - 4 x 96 (5 µl) ➔ 480 µl per plate pool

Measure DNA amount with Qubit

➔ 3 measurements of each pool (2 µl, 5 µl & 10 µl → calculate mean
    - 3 x 4 = 12 samples + 2 standards (14 eppis, better prepare two more
➔ Qubit working solution preparation (1:200)
    - 16 µl ds DNA HS Reagent
    - 3,814 µl Qubit ds DNA HS Buffer = 3,2 ml working solution
➔ For the measurements: Qubit assay tubes (14)
    - 2 µl measurement → 2 µl of the pool + 198 µl working solution
    - Standard: 10 µl + 190 µl working solution (ds DNA Standard 1 + 2)
    - Vortex and incubate for 2 min
    - Ready to measure

Magnetic bead clean up

Devices needed:
Ampure beads (30 min at RT at least)
Magnetic stand
Heat block (37°C)
70% ethanol freshly made

Protocol:

- Mix 180 µl of Ampure beads to the sample (100 µl)
    - Pipetting up and down
    - Allow beads to sit for at least 5 min
- Quick spin in centrifuge
- Transfer to the magnetic bead stand 3-5 min to bind tightly
    - Wait until supernatant is absolutely clear
- Pipette out supernatant carefully without disturbing the beads
- first wash of ethanol (fill above beads) 1 min
- Carefully go in and take out ethanol
- Second wash
- Afterwards remove as much as possible of the ethanol
- Remove eppi from the stand
    - Quick spin in the centrifuge
    - Beads are on the bottom
    - Back on the magnet (beads go on the wall)
- Remove the small volume carefully which is left (don't disturb beads)
- Dry the beads
- Remove the tube and set it to the heat block for 3-5 min
    - Look that the beads are completely flat or have a fine hairline crack
- Add a volume of water/ elution buffer to the beads (e.g. 70 µl)
    - Resuspend 3-5 times until everything is homogenized
    - Quickly vortex
- Allow to set down at RT for 2-3 min
    - Centrifuge quick spin
- Place back on the magnetic stand, let sit there for 3-5 min (beads on the stand)
- Use fresh tube and transfer the eluted volume

After cleaning:

Measurements on Qubit again (1 µl, 2 µl & 5 µl)

➔ After that we want to have the same amount of DNA for each pool in one final pool

You take the lowest concentration of one of the 4 Pool and calculate the others

For example:

pool 1: V1 x C1 = X
8 µl x 1.3 DNA/ µl = 10.4 (DNA) ng
pool 2: c2 = 1.8
10.4 / 1.8 = v2 (v2 < 8)

➔ calculate the concentrations you want to have for every pool and you have the final pool

➔ put all the volumes together and gain the final library

total pool

➔ make a 1:10 dilution

Bioanalyser

Preparing gel dye for the chip:

Priming statica (aparatus to press gel into the chamber)

- Button has to be set on 1 ml and pushed down carefully (feel soft pressure)
- After 60 sec open and let the pipette come up again (until 0.8 ml), afterwards pull the last bit until 1 ml carefully
- Following the protocol:
    - Loading the chip, pipette directly one each well (go with tip as deep as possible inside)
- Samples:

Pool 1	Pool 1 cleaned	Total Pool
Pool 2	Pool 2 cleaned	Total Pool 1:10
Pool 3	Pool 3 cleaned	
Pool 4	Pool 4 cleaned	

11 slots + 1 for the ladder

- Start the computer and the Bioanalyser
- Change the names of the samples
- Start run
- After starting Bioanalyser continue with the preparation of the MiSeq cassette
- Post run throw chip away and put cleaning chip with 350 µl PCR water inside for 30 sec

Measure total pool on the Qubit (2x 2 µl & 2x 5 µl)

Library preparation

0.15 N NaOH
15 µl 1N NaOH
+ 85 µl H2O
100 µl

5 µl 2 nM Library
+ 5 µl 0.15 N NaOH
10 µl vortex 280 x g 1 min

5 min at RT
+ 990 µl HT1
Denat. Library 10 pM

Dilution to 8 pM
480 µl 10 pM Library
+ 120 µl HT1
600 µl invert

PhiX Control
1 µl 10 nM PhiX
+ 1.5 µl H2O
2.5 µl

these 2.5 µl
+2.5 µl 0.15 N NaOH
5 µl vortex
centrifuge 280 x g 1 min

5 min at RT
+ 495 µl HT1
denat. PhiX 20 pM

dilution to 12.5 pM
375 µl 20 pM PhiX
+ 225 µl HT1
600 µl invert

570 µl Library 8 pM
+ 30 µl PhiX 12.5 pM
600 µl ready Library with PhiX
➔ Ready for cassette

Bring for sequencing on ice and give everything on the cassette in well 17

CHAPTER 5

Diehl J.M.C., Keller A., Biedermann P.H.W. (in review)

Succession of ambrosia beetle microbial community structure throughout development in field and laboratory galleries.

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Succession of ambrosia beetle microbial community structure throughout development in field and laboratory galleries

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Abstract

Some fungus farming ambrosia beetles rely on multiple nutritional cultivars (Ascomycota: Ophiostomatales and/or yeasts) that seem to change in relative abundance over time. The succession could benefit beetle hosts by optimal consumption of the substrate and improve the longevity of the nest. However, abundances of fungal symbionts are poorly studied and culture-independent quantification of symbiont communities over time has been applied in only a single species. Here, for the first time, we compared the diversity and succession of both fungal and bacterial communities in ambrosia beetle fungus gardens from field and laboratory nests over time. By amplicon sequencing of probed fungus gardens of both nest types at three development phases we could show an extreme reduction of diversity in both bacterial and fungal symbionts in laboratory nests. Furthermore, we observed a general transition of fungal symbionts during beetle development. Both nutritional mutualists occur in succession and communities were later dominated by fungal competitors and pathogens. The quicker the succession proceeded, the slower nests were developing. Finally, we found signs of transgenerational costs for delayed

dispersing daughters, as early dispersing daughters were more likely to transmit the more beneficial fungal communities for their developing offspring. Future studies should focus on the functional roles of the core bacterial taxa that were present in both field and laboratory nests.

1 Introduction

Advanced fungus-farming for nourishment is an ecological feature that evolved only a few times in insects, namely one lineage of termites and several lineages of both ants and wood-boring weevils (Mueller et al., 2005; Biedermann and Vega, 2020). In all these insect systems fungal ectosymbionts are grown within social societies and are consumed as the major food-source. The insects inoculate plant substrate with the mutualistic fungi, which are grown in so-called “fungus gardens”, always dominated by specific, nutritional fungi and protected by the insects from fungal competitors and pathogens by various means, comprising sequestration from the environment, active monitoring and behavioural and/or antibiotic treatment (e.g. Currie and Stuart 2001; Fernández-Marín et al. 2015; Van Arnam et al. 2018; Nuotclà et al. 2019; Diehl et al. 2022; Schmidt et al. 2022). Importantly, the nutritional fungi are not exhausted by the feeding insects, but rather facilitated the more (tending) insect specimen there are (Biedermann and Rohlfs, 2017). Thus, fungus gardens can provide food within one nest for multiple generations of insects (Mueller et al., 2005).

Despite some common features in the fungiculture of ants, termites and weevils, there are also major differences especially regarding the substrate supply for the fungi and the homogeneity of the nest environment. In contrast to ants and termites that actively forage for plant substrate for consecutive provisioning of their fungi (e.g. Wisselink et al. 2020; Römer et al. 2022), fungus farming weevils, also termed ambrosia beetles (Curculionidae: Scolytinae & Platypodinae), live and breed inside the wood-substrate they use for growing fungi (Kirkendall et al., 2015; Birkemoe et al., 2018). This has major impacts on the substrate conditions the fungus gardens are exposed to and the abundance and diversity of microbial competitors and pathogens inside fungus gardens. First, substrate quality for the nutritional fungi quickly deteriorates in ambrosia

beetles, because it is not replaced and essential nutrients are consumed by the fungi over time (Nuotclà et al., 2021). Beetles can counteract this effect to a limited degree by recycling their feces (Abrahamson and Norris, 1970; De Fine Licht and Biedermann, 2012) and expansion of their tunnel systems inside the wood, but latter is strongly constrained by intraspecific competition and general degradation of the wood (which is typically colonized shortly after the death of the host tree) (Kirkendall et al., 2015; Birkemoe et al., 2018). Second, while fungus gardens of ants and termites are very exposed to microorganisms that are constantly brought in with the new plant substrate and the surrounding soil (Pagnocca et al., 2012; Estrada et al., 2014; Chiri et al., 2020, 2021; Chen et al., 2021), gardens of ambrosia beetles are only exposed to microorganisms (i) already present in the wood at colonization (i.e., endosymbionts), (ii) initially brought in with the nest-founding beetle(s) or (iii) entering gardens from the surrounding wood over time. In relation, this massively reduces the exposure of ambrosia beetles to microbial threats and also explains their comparatively less advanced techniques of pathogen control (Mighell and Van Bael, 2016; Diehl et al., 2022b).

As outlined above termites and ants grow their fungi on a more or less homogenous substrate in a more or less homeostatic nest (Hughes et al., 2008), which allows them to grow a single dominant fungus species over multiple generations (Shinzato et al., 2005; Mehdiabadi et al., 2006; Mueller et al., 2010). By contrast, some ambrosia beetle species in the genera *Xyleborus* and *Xyleborinus* (Scolytinae) seem to rely on multiple nutritional cultivars, *Raffaelea* fungal mutualists (Ascomycota: Ophiostomatales) and/or yeasts (Ascomycota) that seem to change in abundance over time (Cruz et al., 2019; Ibarra-Juarez et al., 2020; Nuotclà et al., 2021; Diehl et al., 2022b). Such a succession could benefit their beetle hosts possibly by optimal consumption of the substrate, because of variation in enzymatic capabilities of cultivars (De Fine Licht and Biedermann, 2012; Ibarra-Juarez et al., 2020). However, abundances of fungal symbionts are poorly studied and despite a diversity of >3000 species of ambrosia beetles, culture-independent quantification of symbiont communities over time has been applied in only a single species (*Xyleborus affinis*; Ibarra-Juarez et al. 2020).

Xyleborus and *Xyleborinus* ambrosia beetles construct their nests as tunnel systems in the xylem of trees (typically weakened or recently dead) (Beaver, 1989). *Raffaelea* fungi and in some cases ascomycete yeasts serve as exclusive food source and provide their hosts with essential vitamins, amino acids and sterols (Kok et al., 1970; Beaver, 1989; Saucedo-Carabez et al., 2018; Cruz et al., 2019; Ibarra-Juarez et al., 2020). These species-specific food fungi are taken up by adult females inside their guts and/or oral or elytral mycetangia (= selective spore-carrying organs) within their natal nest, before they disperse and established their own nest and fungus garden (Francke-Grosmann, 1956, 1967, 1975; Mayers et al., 2022). Other unspecific Ophiostomatales fungi, yeasts and various groups of filamentous fungal saprophytes and plant pathogens (Hypocreales (e.g. *Fusarium, Beauveria*), Eurotiales (e.g. *Penicillium, Aspergillus, Paecilomyces, Talaromyces),* Botryosphaeriales (e.g. *Diplodia*), Dothideales (e.g. *Aureobasidium*), Pleosporales (e.g. *Alternaria*) and Cladosporiales (e.g. *Cladosporium*)) are typically co-transmitted from natal nests in low abundances, probably mostly unintentionally on beetle surfaces (Batra and Batra, 1979; Biedermann et al., 2013; Kostovcik et al., 2015; Saucedo-Carabez et al., 2018; Cruz et al., 2019; Biedermann, 2020; Ibarra-Juarez et al., 2020; Nuotclà et al., 2021). Apart from some obvious pathogens (e.g. *Aspergillus, Beauveria*) the functional roles of these fungi have not been determined, but given their mostly infrequent occurrence they are regarded non-beneficial for beetle fitness. The fact that these fungal weeds increase in abundance the older a nest gets (e.g. Biedermann 2020; Ibarra-Juarez et al. 2020) may, however, affect what and how many fungal weeds dispersing daughter females transmit when they leave their natal nest. The timing of daughter dispersal may thus potentially have transgenerational effects on beetle fitness, but this has not been determined yet.

Recent experimental evidence suggests that beetle promotes the dominance of *Raffaelea* nutritional mutualists over weeds in the fungus gardens of *Xyleborinus saxesenii* (Ratzeburg) (Diehl et al., 2022b). Similar removal of fungal pathogens has been repeatedly observed in other *Xyleborus* and *Xyleborinus* ambrosia beetles (Kingsolver and Norris, 1977; Biedermann et al.,

2013; Nuotclà et al., 2019; Biedermann, 2020). The mechanisms underlying this selective exclusion and promotion of nutritional fungi are unknown, however. It is possible that bacteria are playing a role in this defense (Grubbs et al., 2020), similar to specific defenses by bacteria in fungus-farming ants and termites (e.g. Van Arnam et al. 2018). Although the functional role of bacteria in ambrosia beetle communities has not been experimentally determined, similar bacterial groups dominate in all fungus-farming insect groups (Aylward et al., 2014). In ambrosia beetles bacterial taxa mainly belong to the classes of Alpha- (e.g. *Ochrobactrum, Phyllobacterium, Sphingomonas*), Beta- (e.g. *Burkholderia*) and Gammaproteobacteria (e.g. *Pseudomonas, Pseudoxanthomonas, Erwinia, Stenotrophomonas, Pantoea*), Sphingobacteria (e.g. *Pedobacter, Olivibacter, Sphingobacterium*), Actinobacteria (e.g. *Streptomyces, Microbacterium*), Flavobacteria (e.g. *Chryseobacterium*), Bacilli (e.g. *Staphylococcus, Bacillus*) and Chitinophagia (e.g. *Niabella*) (Fabig, 2011; Aylward et al., 2014; Ibarra-Juarez et al., 2020; Nuotclà et al., 2021; Nones et al., 2022). In *X. affinis,* cellular pathway analyses suggest that its bacterial symbionts contribute in wood degradation, nitrogen fixation and nutritional provisioning (Ibarra-Juarez et al., 2020).

Most studies on fungus garden communities of insects are either done with material collected in the field or from laboratory nests. Both have their benefits and disadvantages. While laboratory rearing has little effects on traits in some invertebrates (Kölliker-Ott et al., 2003; Jong et al., 2017), traits in other species do no longer reflect the ones of natural populations (Meats et al., 2004; Liedo et al., 2007). Field studies offer more realistic conditions, but experimental manipulations and high sample sizes are often possible only in the laboratory (Calisi and Bentley, 2009). The development of laboratory rearing for ambrosia beetles was a breakthrough for research on this species, especially regarding behavioral studies, but also for studying the effects of microbial manipulations, because their wood-tunneling behavior did not allow observations of ambrosia beetles in the field (Saunders and Knoke, 1967; Biedermann et al., 2009). However, so far, we have no knowledge if and how much fungus garden microbial communities and their succession are influenced by the artificial rearing substrate. Due to the addition of sugars, fats and proteins, the latter is

more nutrient rich and lower in plant secondary metabolites (phenolics and terpenoides, which are destroyed by autoclaving) compared to wood. Nevertheless, brood sizes between field and laboratory are comparable even though development is much faster in the laboratory, probably due to higher and stable temperatures (Biedermann et al., 2009).

This is the first attempt to compare the diversity and succession of both fungal and bacterial communities in ambrosia beetle fungus gardens from field and laboratory nests (i.e., in artificial media) over time. All gardens are collected from nests of the fruit-tree pinhole borer, *X. saxesenii,* out of the same population, at the same time and within substrate of the same tree species (beech trees in the field *vs.* beech sawdust in the lab). In both field and lab, we probed fungus garden communities at three development phases of nests (immature brood *vs.* immature and adult brood *vs.* only adult brood present); laboratory nests allowed us to collect additional information on the speed of beetle development in relation to symbionts and transgenerational effects of early or late dispersal of females from their natal nests on their own fungus garden communities later on. Since rearing media is autoclaved we expect fungus gardens in the laboratory to host only the most relevant bacterial and fungal symbionts. Under natural conditions, one can expect a far more diverse and unstable community. This may result in lower abundances of the nutritional fungi in the field, caused by a higher competition with fungal weeds. Development speed should increase the more nutritional fungi there are and later dispersal of daughters from nests may lead to higher abundances of weeds within their gardens later on.

2 Material and Methods

Field collection of nests and beetle laboratory rearing

In this study all beetles and nests (= 'galleries') collected for both field and lab sampling originated from a population of *X. saxesenii* in the Steinbachtal near Wuerzburg (49.767500, 9.896770/49°46'03.0"N 9°53'48.4"E), Germany. We marked four recently dead and wind-thrown beech tree logs (*Fagus sylvatica*) that were colonized by *X. saxesenii* in spring 2018. The examination of field nests is destructive, so we repeatedly went there between July to October 2018 to collect field nests at different developmental stages.

From the same beetle population, females for laboratory rearing were collected in the field by using ethanol baited traps (70 % EtOH) during their dispersal flight in May 2018. After rinsing females first with 70% EtOH and then tap water, they were dried on cosmetic towel and individually put into transparent plastic tubes filled with – previously prepared – sterile artificial medium ('standard media' after (Biedermann et al., 2009). These wild-caught female foundresses build the parental generation (= F0) and were bred under standard conditions (20°C, complete darkness). They immediately start tunnelling and 4-7 days later fungal symbionts start to cover the tunnel walls (i.e., "fungus garden") (Biedermann et al., 2009). About 40 days later dispersal of sib-mated, adult daughters starts and 150 of these, from 18 different nests, were collected and after sterilization, again introduced onto new rearing medium. This F1 generation of lab-born foundresses was then used for the following detailed examinations of symbiont communities and development.

Fungus garden sampling

Field nest classification and fungus garden sampling

In the field we sampled fungus gardens out of 30 nests. Log parts were brought to the laboratory and nests were opened using a cleaver, chisel and hammer (Figure 1A). We classified the phase of development in (i) nests with eggs and larvae, (ii) nests with larvae, pupae and adult offspring, and (iii) nests with only adults present (Table 1). After aseptic removal of all individuals, fungus gardens of these nests were sampled by slicing off thin layers of the nest walls (near the

center of the nest) with a flame-sterilized scalpel. These slices were aseptically stored in 1.5 ml Eppendorf tubes at -20°C until DNA extraction.

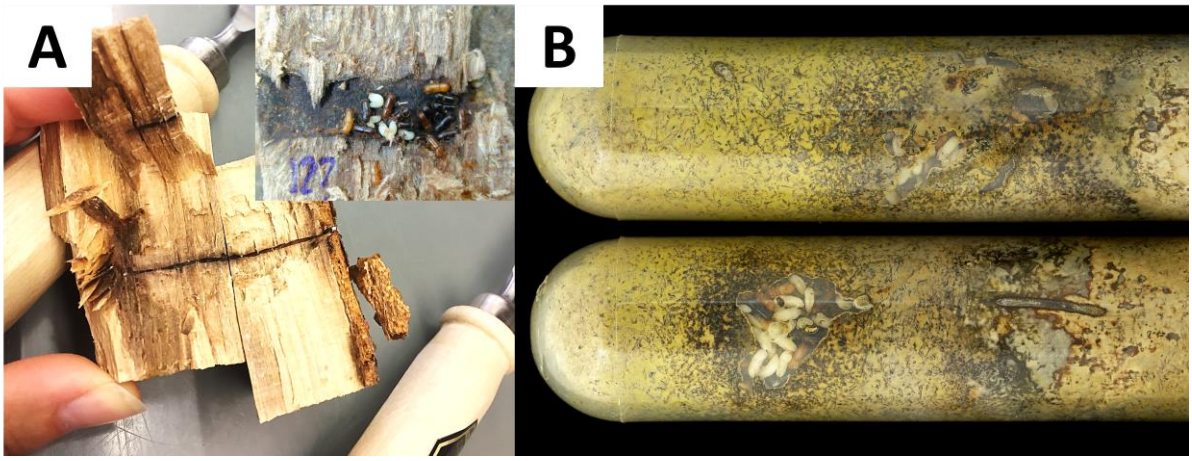

Figure 1. Two types of nests sampled for the study. (A) Opened *X. saxesenii* nests from *F. sylvatica* logs (field nests). (B) Laboratory nests in artificial medium. Yellow coloration of the medium due to growth of the nutritional mutualist *R. sulphurea*. White individuals are larvae or pupae, light-brown ones are teneral females and black ones are fully-sclerotized adult females (photo by Davide Vallotto).

Laboratory nest classification and fungus garden sampling

Laboratory rearing has the advantage that nest development can be directly monitored through the transparent walls of the rearing tubes (Figure 1B). Therefore, we were not only able to record the 'developmental phase' of the nest (see *field sampling*), but could distinguish also 'fast', 'medium' and 'slow' developing beetle nests (Table 1). Furthermore, we also had information about the timing of dispersal of the F1 foundresses from their natal nests – here classified as 'early', 'middle' or 'late' disperser. The timing of dispersal may have an influence on the symbiont community dispersed, as there is a succession of symbiont communities in nests over time (e.g. Ibarra-Juarez et al. 2020). For fungus garden sampling, we destructively dissected nests by first knocking out the solid rearing medium (containing the nest) out of the rearing tube, removal of all individuals and then collecting pieces of the nest walls from the nest centre under the sterile bench with a flame-sterilized sharp spoon (mean weight of pieces ± SD = 96.47 mg ± 34.34). Fungus garden samples were aseptically stored in 1.5 ml Eppendorf tubes at -20°C until DNA extraction.

Table 1. Classification of field and laboratory nests by their phase and speed of development. Numbers of nests (N) per classification are given.

DEVELOPMENT	N_{FIELD}	DEVELOPMENT SPEED	N_{LAB}
PHASE 1 LARVAE	13	fast (7-9 d)	6
		medium (10-12 d)	28
		slow (13-31 d)	16
PHASE 2 LARVAE – PUPAE - ADULTS	13	fast (25-26 d)	11
		medium (27-31 d)	25
		slow (32-43 d)	14
PHASE 3 ADULTS	4	fast (35-41 d)	16
		medium (42-47 d)	20
		slow (48-76 d)	14

DNA extraction and library preparation

DNA of all samples was extracted using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Germany) in accordance with the manufacturer's instructions and additional pre-processing steps with a ceramic bead and a mixer mill (Retsch MM400), followed by an additional step with glass beads (0.1 mm and 0.5 mm) vortexed on a Vortex Genie 2 (see Supporting Material in Nuotclà et al. 2021). The isolated DNA samples were stored at -20°C until the final amplification and sequencing.

PCRs and library preparation were performed as in previous projects (see Nuotclà et al. 2021a, Diehl et al. 2022) in triplicate reactions (each 10 µl) in order to avoid PCR bias. Bacteria 16S-rRNA gene libraries were constructed using the dual-indexing strategy described in Kozich et al. (2013) using the 515f and 806r primers that amplify amplicon sequences of a mean merged length of 246.17 bp_{field}/237.56 bp_{lab}, encompassing the full V4 region (modified from Caporaso et al. 2011). Conditions for the PCR were as follows: initial denaturation at 95°C for 4 min, 30 cycles of denaturation at 95°C for 40 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min, followed by a final extension step at 72°C for 5 min.

Fungal LSU (28S) rRNA gene libraries (mean merged length of 280.67 bp_{field}/276.63 $bp_{lab)}$ were constructed similarly from the same samples by using the dual-index primers of LIC15R and nu-LSU-355-3' (Nuotclà et al., 2021) to

amplify the large subunit (LSU) region. Conditions for the PCR were as follows: initial denaturation at 98°C for 30 sec, 35 cycles of denaturation at 98°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 15 sec, followed by a final extension step at 72°C for 10 min. Sample-specific labelling for both bacterial and fungal DNA was achieved by assigning each sample to a different forward/reverse index combination.

After both PCRs, triplicate reactions of each sample were combined per marker and further processed as described in Kozich et al. (2013), including between-sample normalization using the SequalPrep™ Normalization Plate Kit (Invitrogen GmbH, Darmstadt, Germany) and pooling of 96 samples. The pools were cleaned-up with the AMPure Beads Purification (Agilent Technologies, Inc. Santa Clara, CA, USA) and quality controlled using a Bioanalyzer High Sensitivity DNA Chip (Agilent Technologies, Santa Clara, CA, USA) and quantified with the dsDNA High Sensitivity Assay (Life Technologies GmbH, Darmstadt, Germany). Afterwards, pools were combined to a single library pool containing 384 samples in total. This library was diluted to 8 pM, denatured and spiked with 5% PhiX Control Kit v3 (Illumina Inc., San Diego, CA, USA) according to the Sample Preparation Guide (Illumina Inc. 2013). Sequencing was performed on an Illumina MiSeq using 2 × 250 cycles v2 chemistry (Illumina Inc., San Diego, CA, USA). Each marker was processed on a separate chip. See Supplements in Diehl et al. 2022) and GitHub Repository for further methods on sequencing controls and details on bioinformatics processing.

Statistical analysis of molecular data

All statistical analyses and visualisation of the sequence output were performed in RStudio (Version 1.4.1106) with R version 4.0.5 (R Core Team, 2021) using the phyloseq package (McMurdie and Holmes, 2013); see GitHub repository for information on the bioinformatic processing and R-script.

Field samples data preparation:

After excluding control samples, 30 out of the 36 field samples were left for further analyses. Further removal of Chloroplast genes, ASVs (amplicon sequence variants) that were only identified to domain level and running 'decontam' (Davis et al., 2018) for the 16S field data, left an average of 24,383 reads/sample for downstream analysis (range from 13,116 to 42,604). In total, 242 bacterial ASVs ran into the analysis. Bacterial composition was studied up to the genus level and their relative abundance (RA). For the LSU, only ASVs that were not further identified than to domain level and control samples were excluded and left an average of 24,702 reads/sample for downstream analysis [range from 4,830 to 40,839]. In total, 451 fungal ASVs ran into analysis. Fungal composition was studied up to the species level and their relative abundance (RA).

Laboratory samples data preparation:

82 out of the 151 samples (excluding 20 controls) showed infection with the endosymbiont *Wolbachia* or had low read numbers (≤ 500 reads). The ASVs identifying *Wolbachia* were excluded from further analyses, since insect related infection was not in the focus of our research on fungus garden material. It is worth mentioning, however, that *Wolbachia* has been present in several analyses of laboratory nests by now (this study and Nuotclà et al. 2021a; Diehl et al. 2022), whereas material of field nests never contained *Wolbachia*.

The further removal of ASVs and samples describes earlier left an average of 16,899 reads/sample for downstream analysis (range from 2,628 to 48,835). In total, 166 bacterial ASVs ran into the analysis. For the LSU, we ended up with an average of 21,245 reads/sample for downstream analysis [range from 2,190 to 55,630]. In total, 246 fungal ASVs ran into the analysis. Two out of the 150 samples (excluding 23 controls) had low read numbers (≤ 500 reads) and were therefore excluded from further analyses.

Rarefaction of sequence reads for the analysis of alpha diversity

For the analysis of the alpha diversity we rarefied the sequence reads of all samples depending on the quality of the datasets. For the field samples we decided to rarefy to a total of 10,000 reads/sample for the bacterial community and 4,000 reads/sample for the fungal community. Rarefaction removed two ASVs from the bacterial and 32 ASVs from the fungal dataset. The laboratory samples were rarefied to a total of 2,500 reads/sample for the bacterial community and 2,000 reads/sample for the fungal community. Rarefaction removed 60 ASVs from the bacterial and 32 ASVs from the fungal dataset.

Analysis of alpha diversity

We applied the chi-square tests on both total numbers of ASVs in field and laboratory community data. To investigate the microbial diversity and richness of fungus gardens, we calculated the observed estimate of taxa richness (OR) and Shannon's diversity index (SDI) ('microbiome' package: Lahti and Shetty, 2019). For both measures we ran a generalized linear mixed-effects model (GLMM) with 'tree origin' and 'lineage' (F1 females originated from different F0 families) as random variable, assuming a normal distribution ('glmmTMB' package: Brooks et al. 2017) to test for the influence of the developmental phase on the microbial community. Previous analyses (Diehl et al., 2022b) showed strong heritable effects of lineage and tree identity on symbiont communities in *X. saxesenii*. For laboratory samples we further ran linear mixed models (LMMs) to test the additional influence of dispersal time of the foundress ('*early*' vs '*middle*' vs '*late*') and development speed of the nest ('*fast*' vs '*medium*' vs '*slow*') on the microbial community. We implemented mixed models using the 'lme' function ('nlme' package: Pinheiro et al. 2021) and used the 'transformTukey' function (rcompanion package; Mangiafico 2021) to find the power transformation that brought the alpha diversity effects closest to a normal distribution.

All LMMs were initially fitted with all interaction terms. Best-fitting models were selected by the following procedure: First, we used the Akaike information criterion (AIC) to select an appropriate variance structure (using the weights-

argument in the 'lme' function), when residual plots indicated a deviation from homogeneity (Zuur et al., 2009). Second, we simplified the fixed component by dropping non-significant interaction terms ($p > 0.05$). In a last step, we used – if necessary – the AIC to select the appropriate transformation method to produce a more-normally distributed vector (using squared- or tukey-transformed response variables with the 'transformTukey' function of the 'rcompanion' package, Mangiafico 2021b).

We obtained the p-values of effects in these models using the Anova function (which uses type II sums of squares by default; Fox and Weisberg 2019). Significant models were further analysed using a pairwise post-hoc test (tukey method; 'emmeans' package: Lenth 2021). The package 'ggplot2' (Wickham, 2016) was used to build the figures for alpha diversity.

Analysis of beta diversity

To visualize differences in microbial composition (beta diversity), we applied non-metric multidimensional scaling (NMDS, 'phyloseq' package: McMurdie and Holmes 2013) on Bray Curtis dissimilarities derived from proportion transformed data, which consider presence/absence as well as abundance of ASVs (Clarke et al., 2006). To compare the microbial communities between the '*developmental phase*' and the '*tree origin*' for the field data, we performed a permutational ANOVA test (PERMANOVA) on Bray-Curtis distance matrices of the proportion data using the R package 'vegan' (Oksanen et al., 2020). Significant results were examined in more detail with a pairwise comparison of adjusted p-values ('pairwiseAdonis' package: Martinez Arbizu 2020). The homogeneity of multivariate dispersions was tested with a permutation test ('vegan' package: (Oksanen et al., 2020) applied on each the '*development phase*' and '*tree origin*'. Since we were able to collect more metadata in the laboratory bred nests we tested in the PERMANOVA the variables '*development phase*', '*development speed*' and '*dispersal time*', nested in the variable '*family lineage*'. With heatmaps of the microbial composition ('microbiome' package: (Lahti and Shetty, 2019), we concluded the overview of the beta diversity.

Closer look on fungal core taxa of field and laboratory galleries

We ran another set of LMMs on subsets of the most abundant fungi to test whether relative abundances of these specific taxa differed between the development phases. For example, we compared the relative abundances of the two ambrosia fungi, *R. sulphurea* and *R. canadensis*, and the commensal fungus *C. globosum* in laboratory galleries. Here, the relative abundances (RA) of the fungi were set as the response variables, and the phases and speed of development as well as the timing of maternal dispersal served as explanatory variables. The family lineage was included as a random factor. Core taxa chosen for the field galleries were, next to the ambrosia fungi, *Graphium sp.* and Sordariomycetes (unknown). RA of the taxa were set as response and development phase as explanatory variable. Tree origin of sampled galleries was included as a random factor. The analysis followed the same procedure of fitting and selection as in the previous LMMs.

Analysis of correlation between bacterial and fungal communities

To investigate the correlation between the bacterial and fungal communities in our field and laboratory samples, we employed the Bray-Curtis method using the vegdist() function from the 'vegan' package (Oksanen et al., 2020) to create matrices of dissimilarity indices based on the relative abundances of each community. To ensure a fair comparison, we made subsets of our laboratory dataset to 87 matching samples for both communities. The correlation was then determined using the Mantel statistic (also from the 'vegan' package) with 999 permutations.

Additional packages used

The packages 'fitdistrplus' (Delignette-Muller and Dutang, 2015), 'performance' (Lüdecke et al., 2021) and 'Dharma' (Hartig, 2021) were applied in testing for the best distribution, as well as model fit. 'ggplot2' (Wickham, 2016), 'scales' (Wickham and Seidel, 2020), and 'ggpubr' (Kassambara, 2020), 'ggrepel' (Slowikowski, 2021), 'lattice' (Sarkar, 2008) and 'cowplot' (Wilke, 2020) were

used to build the figures. ‘dplyr’ (Wickham et al., 2021) was used for data manipulation.

3 Results

Bacterial diversity of fungus gardens in field and laboratory nests

In general, both diversity and richness of bacteria was much higher in field (242 ASVs) than laboratory (155 ASVs) nests (chi-square test: χ^2 = 19.07, *df* = 1, *p* = <0.001). In both groups, bacterial diversity did not change over the course of nest development (SDI_{field}: GLMM: χ^2 = 1.48, *p* = 0.477; SDI_{lab}: LMM: χ^2 = 4.10, *p* = 0.129; Figure 2) and effects of development phase on richness were apparent only in lab nests (OR_{field}: χ^2 = 0.113, *p* = 0.945; OR_{lab}: χ^2 = 15.94, *p* = <0.001; Supplementary Figure 5). Development phases of field and laboratory nests slightly affected bacterial beta diversity in fungus gardens (see details in Supplementary Material). There was no effect of timing of foundress dispersal on bacterial beta diversity of lab nests (PERMANOVA: R^2 = 0.043, *F* = 1.95, *p* = 0.652). The tree, the field nests originated from, had a strong effect on the bacterial community composition (R^2 = 0.236, *F* = 2.85, *p* = 0.001), however.

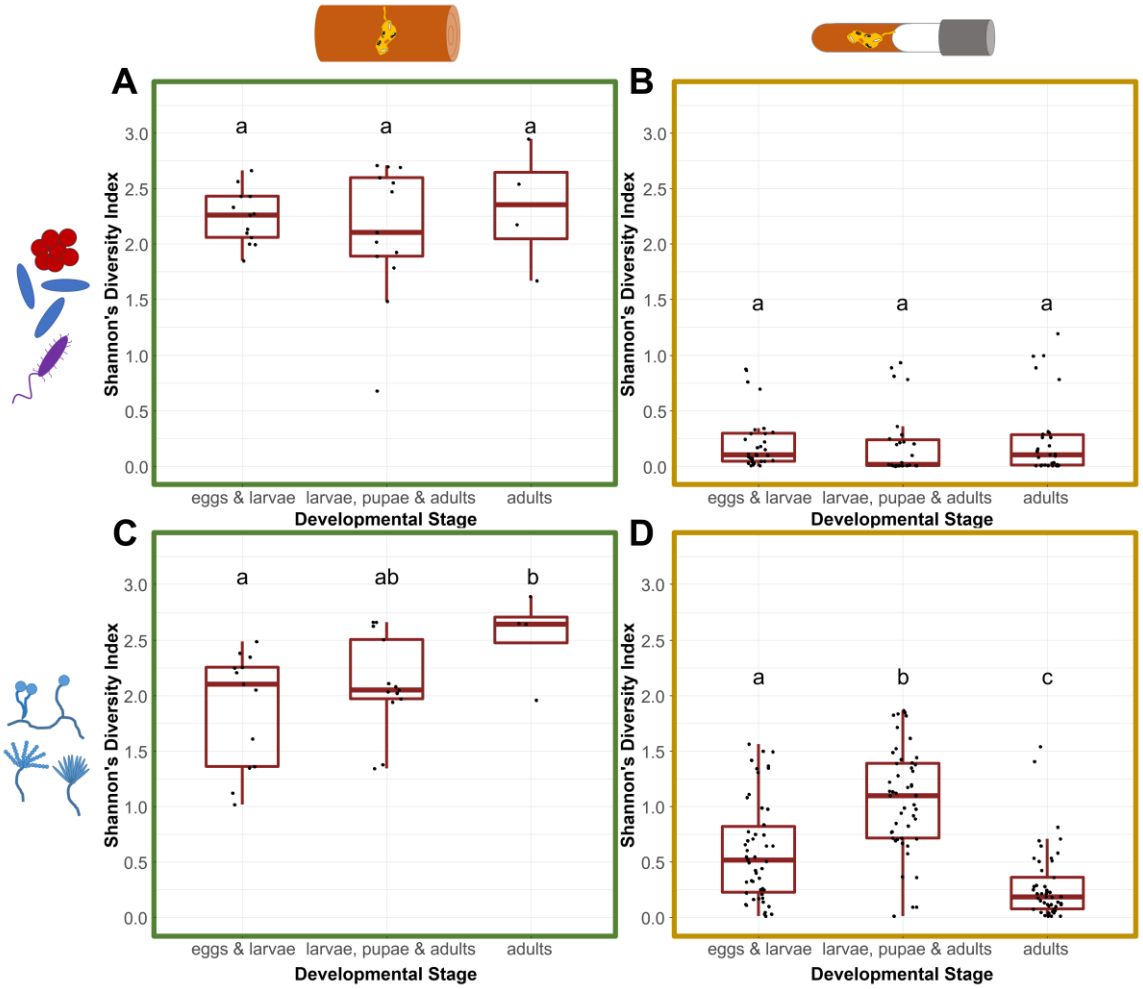

Figure 2. Symbiont diversity of field and laboratory fungus gardens in relation to the nest development phases of *X. saxesenii* nests. Box-plots for Shannon's diversity index of bacterial (A-B) and fungal (C-D) communities of fungus gardens in field (A, C) and laboratory (B, D) nests. Lowercase letters indicate significant differences between groups (Tukey's HSD test: $p < 0.05$).

Fungal diversity of fungus gardens in field and laboratory nests

Similar to the observation for bacteria, also the diversity and richness of the fungal community was much higher in field (451 ASVs) than laboratory (246 ASVs) nests (chi-square test: $\chi^2 = 60.29$, $df = 1$, $p = <0.001$). Fungal diversity increased over the course of nest development (SDI_{field}: GLMM: $\chi^2 = 6.65$, $p = 0.036$; SDI_{lab}: LMM: $\chi^2 = 133.2$, $p = <0.001$; Figure 2). While field fungus gardens had the highest diversity when only adults were present (phase1 vs. 3: $t = -2.55$, $p = 0.044$), lab-garden fungal diversity peaked earlier during the presence of immature and adult offspring (phase 1 vs. 2: $t = -5.86$, $p = <0.001$) and had the lowest diversity in later stages when only adults were present

(phase 1 vs. 3 / phase 2 vs. 3; p <0.001, Figure 2). Slow-developing nests tended to have lower SDI than medium (t = 2.42, p = 0.045). This was not the case for fast developing nests which showed neither a difference compared to medium nor to the slow developing nests ('fast-medium' p = 0.697, 'fast-slow' p = 0.247) (Supplementary Figure 4). The factor development only affected fungal richness of laboratory nests, with the highest OR during the presence of immature and adult offspring (OR_{field}: χ^2 = 0.372, p = 0.830; OR_{lab}: χ^2 = 15.41, p = <0.001; Supplementary Figure 5).

For the fungal beta diversity of field gardens, there was a stronger effect of development phase (PERMANOVA: R^2 = 0.148, F = 2.61, p = 0.007) than for the tree of origin (R^2 = 0.175, F = 2.06, p = 0.028). Both phase and speed of development influenced fungal beta diversity of lab gardens (PERMANOVA: R^2 = 0.056, p = 0.05) (for more details see Supplementary Material).

The core microbial community of fungus gardens in field and laboratory nests

Altogether 13 bacterial classes were detected across field samples. Among these, Actinobacteria, Chitinophagia, Flavobacteriia, Sphingobacteriia Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria were most abundant (>0.5% mean RA) and accounted for approximately 90% of total sequences (Figure 3A; Supplementary Table 2). Gammaproteobacteria comprised ASVs of the genera *Pseudoxanthomonas* (mean + s.d. = 12.43% ± 7.25 RA), *Erwinia* (9% ± 13.49) and *Xanthomonas* (0.7% ± 2.58). Betaproteobacteria were mostly represented by *Burkholderia* (0.76% ± 3.03). Alphaproteobacteria were dominated by *Phyllobacterium* (13.14% ± 10.66), *Ochrobactrum* (4.11% ± 5.15), *Pseudochrobactrum* (0.56% ± 2.15), *Mesorhizobium* (0.53% ± 0.72) and *Roseomonas* (0.51% ± 0.95). Four ASVs of Sphingobacteriia appeared frequently in the nests. Most abundant were *Sphingobacterium* (13.56% ± 12.83) and *Olivibacter* (10.62% ± 10.41), followed by *Pedobacter* (6.8% ± 8.23) and an unknown Sphingobacteriia (4.55% ± 9.59). Chitinophagia were represented by the genus *Taibaiella* (1.36% ± 2.36) an Actinobacteria by *Demetria* (0.52% ± 2.3). Another more abundant class included the Flavobacteriia with the genera *Chryseobacterium* (3.54% ± 5.09)

and *Flavobacterium* (0.98% ± 2.55). Lastly, an ASV of the phylum Bacteriodetes (not specified) was found in almost half of the nests (1.42% ± 6.04). Bacilli, Cytophagia, Deinococci, Thermoleophilia and Verrucomicrobia were detected in relative abundances less than 0.5% of mean total reads.

Laboratory samples covered 16 classes but only four ASVs with higher relative abundance (Figure 3B; Supplementary Table 3). The most dominant class here was the Gammaproteobacteria with its highly abundant *Pseudoxanthomonas* (83.32% ± 30.65) and *Erwinia* (7.56% ± 21.72), *Pantoea* (0.81% ± 7.35) and *Yersinia* (5.18% ± 20.2). Alphaproteobacteria were almost exclusively represented by *Ochrobactum* (1.64% ± 11.57). We also found Actinobacteria in an abundance of over 0.5% mean RA. Acidobacteria, Bacilli, Bacteroidia, Betaproteobacteria, Chitinophagia, Clostrida, Deinococci, Deltaproteobacteria, Flavobacteriia, Mollicutes, Negativicutes, Planctomycetacia and Sphingobacteriia were observed in relative abundances less than 0.5% of mean total reads.

The analyses of the field dataset yielded 15 fungal orders. Among these, Ophiostomatales, Microascales, Xylariales, Chartothyriales, Hypocreales and Togniniales were most abundant (>0.5% mean RA; Figure 3C; Supplementary Table 2). The highest relative abundance came from an unknown Sordariomycetes (46.6% ± 24.13). The most abundant order was the Ophiostomatales with the ambrosia fungi *R. sulphurea* (9.33% ± 8.0), *R. candensis* (10.34% ± 12.13) and *R. aff. canadensis* (0.78% ± 1.34). Microascales were represented by a *Graphium sp*. (9.95% ± 11.94). The order Hypocreales (11.87% ± 16.72) included two ASVs of the genus *Clonostachys*, *Nectria balansae*, a *Neonectria sp.* and *Trichoderma deliquescens* (Supplementary Material Table 2). Other ASVs with a relative abundance greater than 0.5% total mean RA were *Phaeoacremonium sp.* (Togniniales; 1.75% ± 5.20), a Diatrypcaeae (unknown) (Xylariales; 1.24% ± 4.47) and a Herpotrichiellaceae (unknown) (Chaetothyriales, 0.72% ± 2.84) (Supplementary Material Table 2). Moreover, additional fungi in the orders Eurotiales, Sordariales, Capnodiales, Helotiales, Coniochaetales, Saccharomycetales, Pyxidiophorales, Pleosporales and Orbiliales were successfully amplified, but

below the threshold of 0.5% total mean RA.

Less diversity was found in the laboratory dataset. Here, we detected 11 fungal orders, but only three higher abundant taxa (Figure 3D; Supplementary Table 3). Again, the two ambrosia fungi, *R. sulphurea* (38.61% ± 38.11) and *R. canadensis* (6.67% ± 15.49) were identified. Further, *Chaetomium globosum* (Sordariales; 52.16% ± 41.33) was detected in all nests and about a third of the nests contained some Eurotiales (0.58% ± 5.65) (Supplementary Material Table 3). Additional fungi in the orders Capnodiales, Chaetothyriales, Dothideales, Saccharomycetales, Hypocreales, Pleosporales, Microascales and Togniniales were exposed, but below the threshold of 0.5% total mean RA.

In the sequence output of the positive controls we were only able to detect four out of the present six fungal genera. While we got sequence results from some taxa in the Saccharomycetales order in the experimental samples, the primers failed to amplify the yeasts *Pichia* sp. & *Candida* sp. in the mock and Zymo control (Supplementary Figure 1C+E & 2C+E).

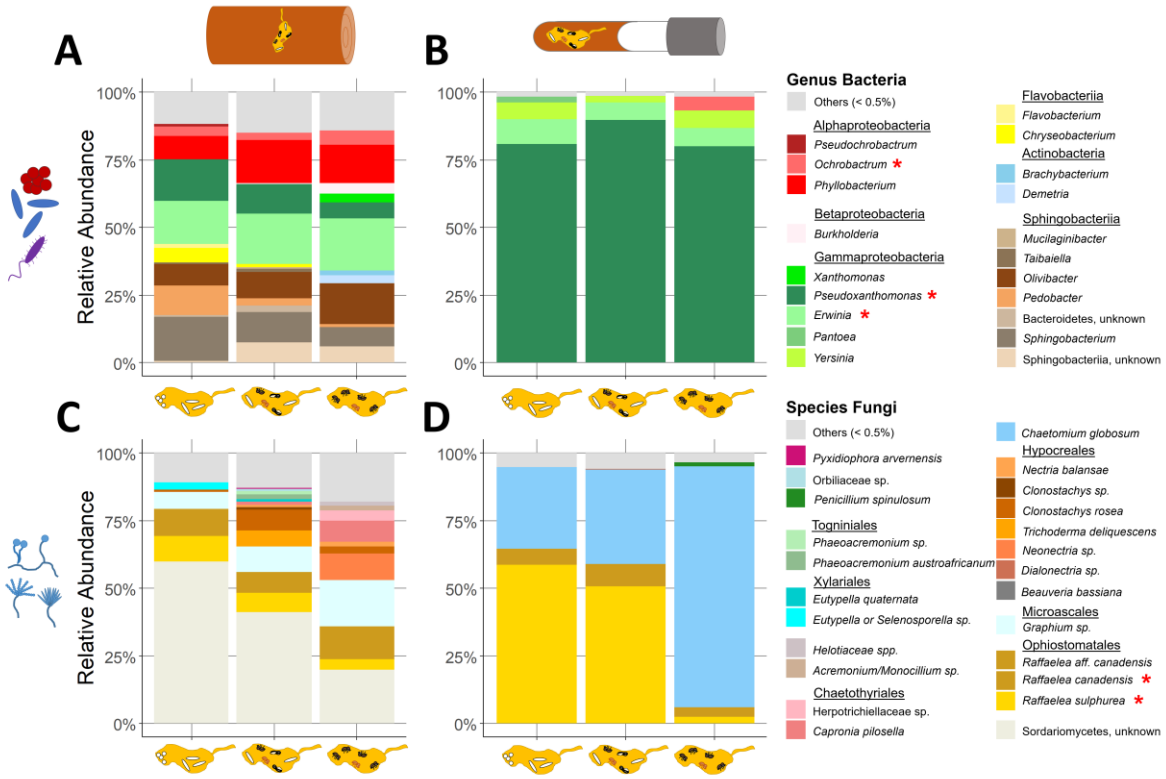

Figure 3. Relative abundance of symbiont taxa in field and laboratory fungus gardens in relation to the development phases of *X. saxesenii* nests. Community of bacterial genera (A, B) and fungal species (C, D) in fungus gardens of field (A, C) and laboratory (B, D) nests with a relative abundance of at least 0.5% (all else is combined in "others"). Taxa marked with (*) were found in both field and laboratory communities.

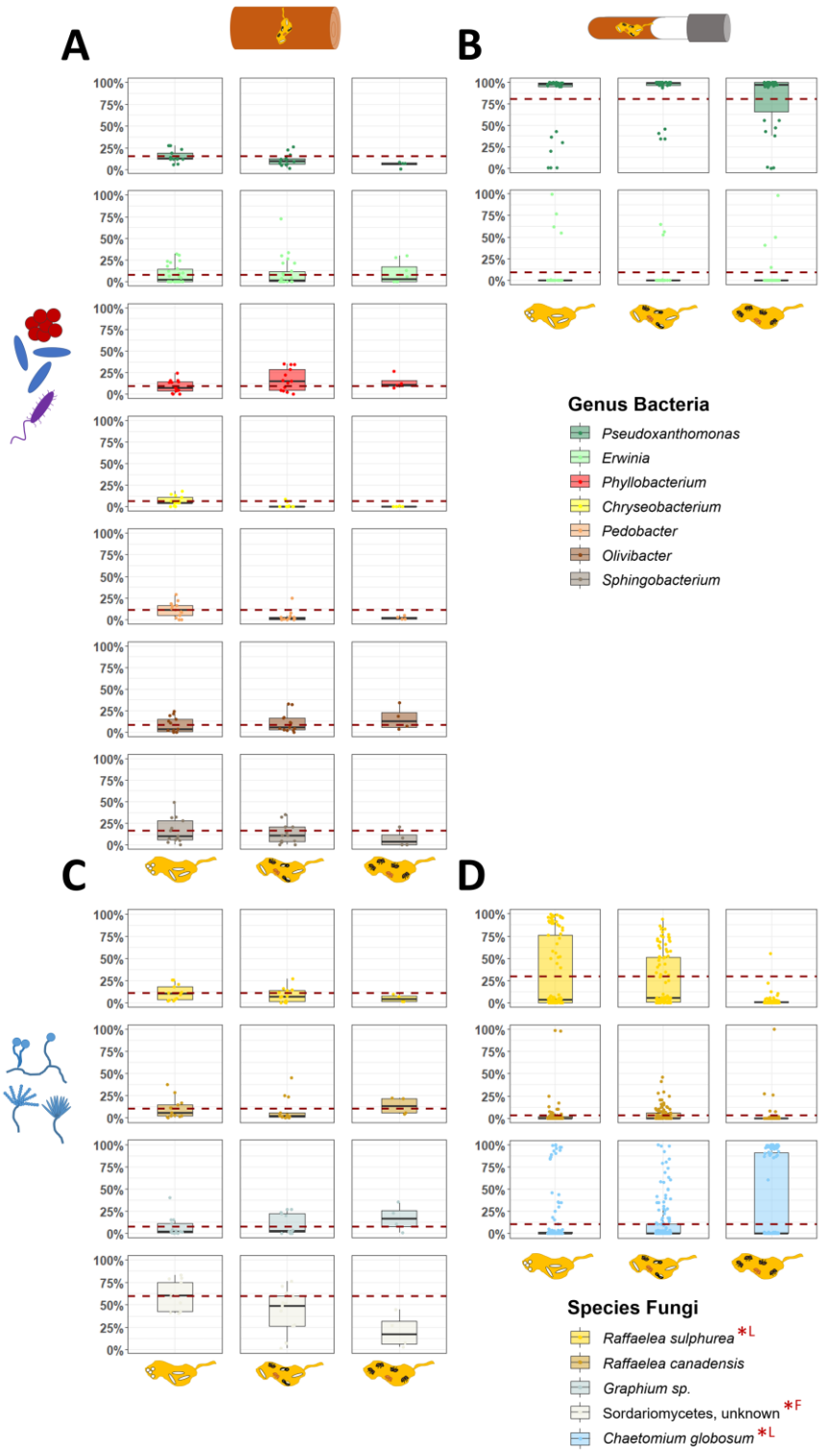

Figure 4. Comparison of the relative abundances of core symbionts in field and laboratory fungus gardens in relation to the development phases of *X. saxesenii* nests. Box-plots with the relative abundance of dominant bacterial genera (A, B) and fungal species (C, D) in fungus gardens of field (A, C) and laboratory (B, D) nests. Dashed red line represents mean relative abundance of the first developmental phase. Statistical differences ($p < 0.05$) of abundances between development phases are marked with (*).

The abundance of core taxa within fungus gardens of field and laboratory nests

Only two bacterial taxa, a *Pseudoxanthomonas* and an *Erwinia*, and the two ambrosia fungus species, *Raffaelea sulphurea* and *R. canadensis*, occurred in considerable abundance both in field and laboratory nests (Figure 4). Fungus gardens from the field additionally harboured several other bacterial taxa and two more fungi, a *Graphium* sp. and an unknown Sordariomycetes, while laboratory gardens only harboured *Chaetomium globosum*, which did not occur in the field.

Changes in relative abundances of these core taxa over the development of nests could hardly been detected, but some effects were observed. First, within field fungus gardens, the unknown Sordariomycetes decreased in abundance in the course of development (LMM: $\chi^2 = 8.34$, $p = 0.015$; EMM: 'P1-P3' $\chi^2 = 2.82$, $p = 0.034$) (Figure 4; Supplementary Figure 12). Such a decrease of abundance was also found for the main nutritional mutualist of *X. saxesenii*, *R. sulphurea*, in laboratory nests (LMM: $\chi^2 = 772.47$, $p = <0.001$; EMM: all contrasts $p = <0.001$; Figure 4 + 5). Interestingly, the two "extreme" nests that developed very quickly or very slowly showed lower abundances of this fungus (LMM: $\chi^2 = 40.55$, $p = <0.001$; EMM: all contrasts $p = <0.001$; Figure 5). The abundance of the second mutualist, *R. canadensis*, is highest in nests of early dispersing foundresses (EMM: 'early–late' $p = 0.008$; 'early-middle' $p = 0.031$; Supplementary Material Figure 13). Additionally, we found significant lower abundance of *R. canadensis* in the third phase of late dispersing females (EMM: 'early–late' $p = <0.001$; 'late-middle' $p = 0.001$; Supplementary Figure 13). Finally, within laboratory fungus gardens, relative abundance of *C. globosum* increased over the course of development (LMM: $\chi^2 = 13.98$, $p = 0.001$) and there was a strong interaction between development phase and speed (LMM: 'phase/speed' $\chi^2 = 74.90$, $p = <0.001$; Figure 5). Fast developing nests had significantly less *C. globosum* present, then medium and slow developing nests both during the presence of only immatures or immature and adult offspring (phase 1 and 2) (phase 1: EMM: 'medium-fast' $p = 0.023$; 'fast-slow' $p = 0.004$; phase 2: 'medium-slow' and 'fast-slow' $p = <0.001$; 'medium-

fast' p = 0.001). This effect disappeared when only adults were present within nests, since *C. globosum* was the most abundant taxon in almost all galleries (Figure 5).

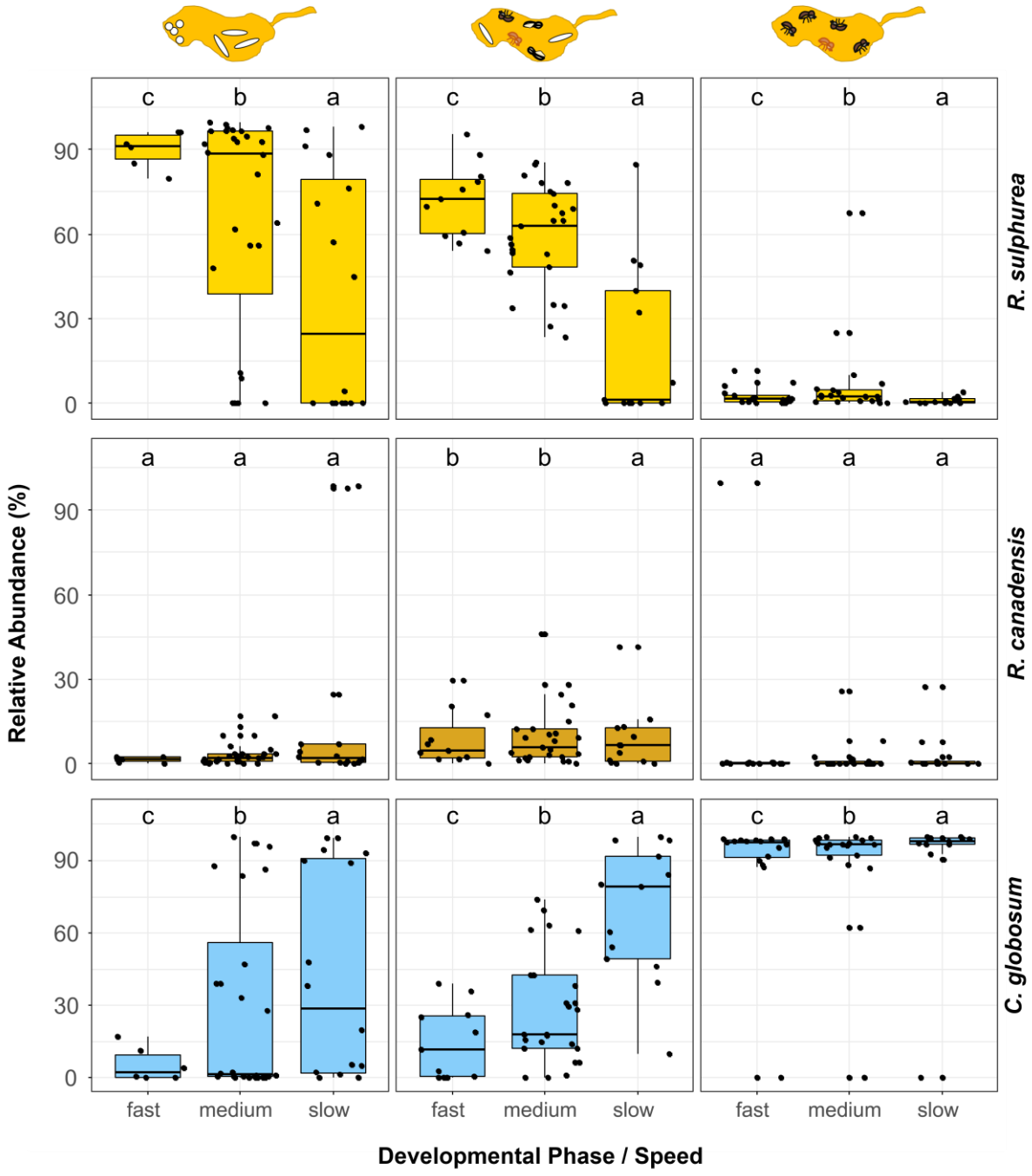

Figure 5. Comparison of the relative abundances of the three core fungi in laboratory fungus gardens in relation to the phases and speed of development of *X. saxesenii* nests. Box-plots with the relative abundance of the fungal mutualists *R. sulphurea* and *R. canadensis* as well as the fungal weed *C. globosum* for the different phases and speeds of nest development are given. Lowercase letters indicate significant differences between groups (Tukey's HSD test: $p < 0.05$).

Influence of bacterial and fungal communities on each other

Field samples revealed a positive linear correlation between the dissimilarity matrices of bacterial and fungal communities (r = 0.271; p = 0.002), but this correlation was not found in the laboratory samples (r = 0.047; p = 0.130).

4 Discussion

The analysis of the fungal symbionts in fungus gardens during *X. saxesenii* development in both field and laboratory originating nests provided us with several new insights. As expected, we detected a much higher diversity of both bacteria and fungi in the field compared to the laboratory. Fungus gardens in the laboratory are reduced to the core community of bacteria and fungi necessary for beetle nutrition and development (Figure 3). Besides these in lab nests present core taxa, field samples included additional taxa, which can be classified as plant saprophytes, endophytes or pathogens, as well as beetle endosymbionts. While the diversity and abundance of bacterial communities of fungus gardens were relatively stable (in both lab and field) over developmental time, fungal communities shifted quite a bit. Especially in the laboratory nests, a turnover from nutritional fungal mutualists to a putative lab contaminant (*C. globosum*) was observed. We could observe functional beetle-symbiont interactions with our data showing that fast development is linked with a higher abundance of *Raffaelea* fungi at least during the first phases of nest development (i.e., when immature brood was still present).

An interesting observation was that the bacterial and fungal communities in field fungus gardens had a mutual influence on each other, while laboratory gardens did not exhibit this relationship. We posit that this difference is due to the greater diversity of bacteria in the field, which enabled more flexibility to adapt to the changing conditions caused by the developing fungal community. In contrast, the laboratory conditions represent a more stable, closed environment which is based on sterile, semi-natural medium limiting the microbial community diversity and leading therefore to a dominance of few core taxa an few change

over time. This finding suggests that in natural environments, the overall bacterial community within ambrosia beetle nests may be shaped by the dominant fungal species. By changing the environment with their enzymatic activity, fungi could influence, positively or negatively, the bacterial symbionts as the study by Zhang et al. (Zhang et al., 2022) reported in the context of composting. Here, the bacterial genera, *Flavobacterium* and *Pseudomonas* showed a positive correlation to the fungal genus *Aspergillus*, but a negative one with *Myceliophthora*.

Overall bacterial communities of fungus gardens resembled taxa found in other studies, especially for ambrosia beetles associated with *Raffaelea* fungal symbionts (i.e., *Xyleborus*, Xyleborinus, Platypodinae; (Fabig, 2011; Aylward et al., 2014; Nuotclà et al., 2021; Nones et al., 2022). These are taxa in the Alpha- Beta- and Gammaproteobacteria, as well as Actinobacteria and Bacteroidetes (Ibarra-Juarez et al., 2020; Joseph and Keyhani, 2021). Focusing only on the changes in bacterial-community composition in the field, we found a decrease of Sphingobacteria and Flavobacteria (Bacteroidetes) over development phases; Sphingobacteria can exhibit xylanolytic activity (Zhou et al., 2009). Actinobacteria in return, happened to be more abundant in some galleries with only adult individuals present; they are known for their antimicrobial metabolites (Van Arnam et al., 2018; Grubbs et al., 2020). It is unclear if they are absent in the laboratory. By comparing field with laboratory nests, we found a shift from relatively heterogenous and balanced bacterial communities to ones dominated by Gammaproteobacteria. Specifically, a *Pseudoxanthomonas* sp. showed a mean relative abundance above 80% in all three development phases. The exact role of this specific bacterium in the context of bark beetles is still unknown, but it can be often found within the communities (Nuotclà et al., 2021; Nones et al., 2022). Another Gammaproteobacterium, *Erwinia*, was also both present in the field and laboratory; it might contribute to nitrogen fixation in the system (Papen and Werner, 1979). Future work needs to address potential functional roles of bacteria in the fungus gardens of *X. saxesenii* and this study points out the few taxa present in both lab and field that should be considered first.

While bacterial communities were relatively stable, both field and laboratory galleries showed strong shifts of fungal communities in the course of nest development. This change was manifested mostly in abundances but not diversities. In the field, the strongest shifts of abundances were observed for an unknown Sordariomycetes, which decreased with nest development, and a *Graphium* sp., which increased (Figure 3, 4). Both taxa are known as symbionts of some ambrosia beetles (Harrington, 2005; Kolařík et al., 2015), but for *X. saxesenii* both species are probably not essential, because they were missing in laboratory nests. By contrast only the two *Raffaelea* nutritional mutualists occurred both in the field and in the laboratory. Changes in abundances in relation to nest development was relatively equal between lab and field, showing both a relatively stable abundance of *R. canadensis* and a decrease of *R. sulphurea* over development phases (Figure 4, 5). This corresponds with the preference of *R. sulphurea* for moister conditions (i.e., substrates dry out over time) and its function as larval food (Nuotclà et al., 2021). It is quite likely that these fungi jointly complement the diet of the beetles also as they can co-occur on agar plates with no sign of inhibition (Biedermann, unpubl. data). A similar co-occurrence of two mutualists has been observed for the bark beetle *Dendroctonus ponderosae* (Six and Bentz, 2007).

Apart from *C. globosum*, which may be a laboratory contaminant because it occurred only in laboratory nests, fungus garden symbionts in laboratory nests were reduced to the core community of bacteria and fungi that are necessary for beetle nutrition. This bottleneck effect can inform us about the functional relevance of certain bacterial and fungal taxa for beetle fitness and shows that the majority of microbial symbionts in the field are possibly transient hitchhikers on beetles' surfaces (Birkemoe et al., 2018; Seibold et al., 2019). Metabarcoding studies of symbiont communities of ambrosia beetles in the field (e.g. Kostovcik et al., 2015; Malacrinò et al., 2017; Rassati et al., 2019) have revealed hundreds of potential beetle associates, but our outcome shows that the majority of them are not essentially needed by the beetles. Claims that culture-dependent studies are outdated and invalid, because they reveal only a fraction of the beetle symbionts (e.g. Kostovcik et al., 2015) should therefore be withdrawn as the required core community detected by our study has been detected by isolation studies before (Batra, 1966; Francke-Grosmann, 1975;

Biedermann et al., 2013). Nevertheless, as the laboratory conditions cannot mirror all possible abiotic and biotic scenarios that the beetles may face in nature, for example due to a lack of plant secondary compounds or the need for breakdown of recalcitrant plant polymers in the artificial substrate (Biedermann et al. 2009), it is possible that some microbial mutualists got lost under laboratory conditions while actually playing important roles in nature. Finally, more homogenous temperature and humidity conditions in the laboratory could have also led to the competitive exclusion of some taxa (e.g. Hibbing et al. 2010).

Using our laboratory nests, we could show that fungal communities had a strong influence on the speed of nest development (Supplementary Figure 4, 5). There is a clear succession from a *R. sulphurea* dominated first nest phase (with immature brood) to a mixed *R. sulphurea*, *R canadensis* and *C. globosum* community in the second nest phase (with immature and adult brood) and a last phase dominated by *C. globosum*. Interestingly, the earlier this succession moves away from *R. sulphurea*, the slower the beetle development is. Fast developing nests were characterized by higher fungal richness (Supplementary Figure 4, 5), lower relative abundance of the weedy fungus *C. globosum* and a higher abundance of the nutritional *Raffaelea* species, in particular *R. sulphurea* (Figure 5). This is certainly an effect of the better food supply for the developing offspring, which is further corroborated by the observation that there is no correlation between speed of development and relative abundance of nutritional fungi in the third nest phase with only adults present that finished development.

This study is the first to proof a microbe-mediated transgenerational effect of female dispersal time on development of the subsequent generation in *X. saxesenii*. Interestingly, effects of dispersal were not significant in the first phase of development, but appeared only in the second phase (with immature and adult brood). In particular, the earlier a female dispersed, the lower was the fungal diversity during the second phase. The same effect was also found for the relative abundance of *R. canadensis* in the second and third phase (Supplementary Figure 13). This suggests that an early dispersal of females from their maternal nests seems to benefit the beetles by a higher abundance of the second food fungus, *R. canadensis*, which is most abundant during the

second phase with first adult offspring present. This could give the freshly emerged immature females an additional nutritional benefit, as well as, advantage for a successful nest establishment in the following generation. As found by Nuotclà et al. (2021), *R. canadensis* enables long-lasting nests and increases offspring numbers. Therefore, delayed dispersal, which is found in many adult daughters of *X. saxesenii* that remain and socially care for brood and fungus (Peer and Taborsky, 2007; Biedermann and Taborsky, 2011), may come with a transgenerational cost of less beneficial symbiont communities later on. This can be easily explained by looking again at the above-mentioned succession from nutritional fungi to fungal weeds over nest development and the fact that adult females most likely fill mycetangia and guts for fungal spore transmission just before their own dispersal from the maternal nest (Francke-Grosmann, 1975; Mayers et al., 2022).

Our study shows that the fungus gardens of ambrosia beetles, at least the ones from *X. saxesenii*, are very different from the ones of farming ants and termites. While the latter live in long-lived eusocial societies that maintain growth conditions for their fungal mutualists very stable by progressive provisioning of substrate, dead-wood substrate is not replenished by the beetles and deteriorates relatively quickly (Biedermann and Vega, 2020). Hence, we find a single cultivar dominating typical fungus gardens of ants and termites, while more and more studies in facultative eusocial ambrosia beetles (the Xyleborini genera *Xyleborus* and *Xyleborinus*, possibly also Platypodinae) show that there is succession of different fungi (or yeasts) and at least two, possibly even more, can serve as food sources (this study, Ibarra-Juarez et al. 2020; Diehl et al. 2022). Alternatively, other less social ambrosia beetles have relatively short-lived nests (only one generation per nest) and rely on only single fungal cultivars (e.g. the Xyloterini and the Xyleborini genera *Xylosandrus* and *Anisandrus*; (Kostovcik et al., 2015; Mayers et al., 2015, 2020). Overall, ambrosia beetles are unable to stabilize the community, such as other farmers do because they cannot replenish the fungal substrate. The only exception might be the few Platypodinae ambrosia beetles that colonize living trees (without killing them; Kirkendall et al. 2015), in which trees may replenish the nutrients used by the growing fungi. Communities of their fungus gardens have not been studied, so far. What also remains unclear is if and how multiple

cultivars in ambrosia beetle nests respond to changing temperatures, moisture and switches of tree hosts (most of these ambrosia beetle species are tree-host generalists).

The first direct comparison of symbiont communities in fungus gardens of field and laboratory nests revealed a strong reduction of both bacterial and fungal symbionts in laboratory nests. We argue that these few taxa are the core mutualists, needed for *X. saxesenii* reproduction and development. Furthermore, we observed in both, field and laboratory, a succession of fungal symbionts during the course of beetle development, in which both nutritional mutualists occur in succession and communities get later dominated by fungal competitors and pathogens. The quicker the succession proceeds, the slower nests are developing, which certainly relates to the diminishing food supply. Finally, fungal succession might also have transgenerational costs for delayed dispersing daughters, as early dispersing daughters transmit the more beneficial fungal communities for their developing offspring. Future studies should focus now on revealing the functional roles of the core bacterial taxa that were present in both field and laboratory nests.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

JD and PB conceived and designed the experiments. JD carried out the study and analysed the data. AK helped in the bioinformatic and statistical processing. JD, AK, and PB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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9 Data Availability Statement

The data and scripts that support the findings of this study are openly available in the GitHub Repository (https://github.com/janinad88/microbial-succession-of-ambrosia-beetle-galleries). Raw sequence data are uploaded on the NCBI database under the BioProject ID: PRJNA915190.

10 References

Abrahamson, L. P., and Norris, D. M. (1970). Symbiontic interrelationships between microbes and ambrosia beetles (Coleoptera: Scolytidae). V. Amino acids as a source of nitrogen to the fungi in the beetle. Ann. Entomol. Soc. Am. 63, 177–180. doi: 10.1093/aesa/63.1.177.

Aylward, F. O., Suen, G., Biedermann, P. H. W., Adams, A. S., Scott, J. J., Malfatti, S. A., et al. (2014). Convergent bacterial microbiotas in the fungal agricultural systems of insects. MBio. 5, e02077-14. doi: 10.1128/mBio.02077-14.

Batra, L. R., and Batra, S. W. T. (1979). “Termite-fungus mutualism.,” in Insect-Fungus Symbiosis, ed. L. R. Batra (Allanheld, Osmun, Montclair, New Yersey), 117–163.

Batra, L. R. R. (1966). Ambrosia fungi: extent of specificity to ambrosia beetles. Science. 153, 193–195. doi: 10.1126/science.153.3732.193.

Beaver, R. A. (1989). “Insect-fungus relationships in the bark and ambrosia beetles,” in Insect-fungus interactions, eds. N. Wilding, N. M. Collins, P. M. Hammond, and J. F. Webber (London, UK: Academic Press), 121–143. doi: 10.1016/B978-0-12-751800-8.50002-1.

Biedermann, P. H. W. (2020). Cooperative breeding in the ambrosia beetle *Xyleborus affinis* and management of its fungal symbionts. Front. Ecol. Evol. 8, 1–12. doi: 10.3389/fevo.2020.518954.

Biedermann, P. H. W., Klepzig, K. D., and Taborsky, M. (2009). Fungus cultivation by ambrosia beetles: behavior and laboratory breeding success in three Xyleborine species. Environ. Entomol. 38, 1096–1105. doi: 10.1603/022.038.0417.

Biedermann, P. H. W., Klepzig, K. D., Taborsky, M., and Six, D. L. (2013). Abundance and dynamics of filamentous fungi in the complex ambrosia gardens of the primitively eusocial beetle *Xyleborinus saxesenii* Ratzeburg (Coleoptera: Curculionidae, Scolytinae). FEMS Microbiol. Ecol. 83, 711–723. doi: 10.1111/1574-6941.12026.

Biedermann, P. H. W., and Rohlfs, M. (2017). Evolutionary feedbacks between insect sociality and microbial management. Curr. Opin. Insect Sci. 22, 92–100. doi: 10.1016/j.cois.2017.06.003.

Biedermann, P. H. W., and Taborsky, M. (2011). Larval helpers and age polyethism in ambrosia beetles. Proc. Natl. Acad. Sci. 108, 17064–17069. doi: 10.1073/pnas.1107758108.

Biedermann, P. H. W., and Vega, F. E. (2020). Ecology and evolution of insect-fungus mutualisms. Annu. Rev. Entomol. 65, 431–455. doi: 10.1146/annurev-ento-011019-024910.

Birkemoe, T., Jacobsen, R. M., Sverdrup-Thygeson, A., and Biedermann, P. H. W. (2018). “Insect-fungus interactions in dead wood systems,” in Saproxylic insects (Springer), 377–427. doi: 10.1007/978-3-319-75937-1_12.

Brooks, M. E., Kristensen, K., van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., et al. (2017). glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. R J. 9, 378–400.

Calisi, R. M., and Bentley, G. E. (2009). Lab and field experiments: Are they the same animal? Horm. Behav. 56, 1–10. doi: 10.1016/J.YHBEH.2009.02.010.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. 108, 4516–4522. doi: 10.1073/PNAS.1000080107.

Chen, Q. L., Hu, H. W., Yan, Z. Z., Li, C. Y., Nguyen, B. A. T., Zheng, Y., et al. (2021). Termite mounds reduce soil microbial diversity by filtering rare microbial taxa. Environ. Microbiol. 23, 2659–2668. doi: 10.1111/1462-2920.15507.

Chiri, E., Greening, C., Lappan, R., Waite, D. W., Jirapanjawat, T., Dong, X., et al. (2020). Termite mounds contain soil-derived methanotroph communities kinetically adapted to elevated methane concentrations. ISME J. 14, 2715–2731. doi: 10.1038/s41396-020-0722-3.

Chiri, E., Nauer, P. A., Lappan, R., Jirapanjawat, T., Waite, D. W., Handley, K. M., et al. (2021). Termite gas emissions select for hydrogenotrophic microbial communities in termite mounds. Proc. Natl. Acad. Sci. U. S. A. 118, e2102625118. doi: 10.1073/pnas.2102625118.

Clarke, K. R., Somerfield, P. J., and Chapman, M. G. (2006). On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray–Curtis coefficient for denuded assemblages. J. Exp. Mar. Bio. Ecol. 330, 55–80. doi: 10.1016/J.JEMBE.2005.12.017.

Cruz, L. F., Menocal, O., Mantilla, J., Ibarra-Juarez, L. A., and Carrillo, D. (2019). *Xyleborus volvulus* (Coleoptera: Curculionidae): Biology and fungal associates. Appl. Environ. Microbiol. 85. doi: 10.1128/AEM.01190-19.

Currie, C. R., and Stuart, A. E. (2001). Weeding and grooming of pathogens in agriculture by ants. Proc. R. Soc. London. Ser. B Biol. Sci. 268, 1033–1039. doi: 10.1098/rspb.2001.1605.

Davis, N. M., Proctor, Di. M., Holmes, S. P., Relman, D. A., and Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome. 6(1). doi: 10.1186/S40168-018-0605-2.

De Fine Licht, H. H., and Biedermann, P. H. W. (2012). Patterns of functional enzyme activity in fungus farming ambrosia beetles. Front. Zool. 9, 13. doi: 10.1186/1742-9994-9-13.

Delignette-Muller, M. L., and Dutang, C. (2015). fitdistrplus: An R package for fitting distributions. J. Stat. Softw. 64, 1–34. doi: 10.18637/jss.v064.i04.

Diehl, J. M. C., Kowallik, V., Keller, A., and Biedermann, P. H. W. (2022). First experimental evidence for active farming in ambrosia beetles and strong heredity of garden microbiomes. Proc. R. Soc. B Biol. Sci. 289. doi: 10.1098/rspb.2022.1458.

Estrada, C., Rojas, E. I., Wcislo, W. T., and Van Bael, S. A. (2014). Fungal endophyte effects on leaf chemistry alter the in vitro growth rates of leaf-cutting ants' fungal mutualist, *Leucocoprinus gongylophorus*. Fungal Ecol. 8, 37–45. doi: 10.1016/J.FUNECO.2013.12.009.

Fabig, W. (2011). The microbial community associated with the ambrosia beetle *Xyleborinus saxesenii* (Coleoptera: Curculionidae) and its influence on the growth of the mutualistic fungus. master's thesis. Bayreuth: University of Bayreuth

Fernández-Marín, H., Nash, D. R., Higginbotham, S., Estrada, C., Zweden, J. S. van, d'Ettorre, P., et al. (2015). Functional role of phenylacetic acid from metapleural gland secretions in controlling fungal pathogens in evolutionarily derived leaf-cutting ants. Proc. R. Soc. B Biol. Sci. 282. doi: 10.1098/RSPB.2015.0212.

Fox, J., and Weisberg, S. (2019). An R companion to applied regression. Thousand Oaks, CA: Sage Publications Inc.

Francke-Grosmann, H. (1956). Hautdrüsen als Träger der Pilzsymbiose bei Ambrosiakäfern. Zeitschrift für Morphol. und Ökologie der Tiere. 45, 275–308. doi: 10.1007/BF00430256.

Francke-Grosmann, H. (1967). "Ectosymbiosis in wood-inhabiting insects," in symbiosis, ed. S. M. Henry (New York: Academic Press), 141–205.

Francke-Grosmann, H. (1975). Zur epizoischen und endozoischen Übertragung der symbiotischen Pilze des Ambrosiakäfers *Xyleborus saxeseni* (Coleoptera: Scolytidae). Entomol. Ger. 1, 279–292.

Grubbs, K. J., Surup, F., Biedermann, P. H. W., McDonald, B. R., Klassen, J. L., Carlson, C. M., et al. (2020). Cycloheximide-producing *Streptomyces* associated with *Xyleborinus saxesenii* and *Xyleborus affinis* fungus-farming ambrosia beetles. Front. Microbiol. 11, 1–12. doi: 10.3389/fmicb.2020.562140.

Harrington, T. C. (2005). "Ecology and evolution of mycophagous bark beetles and their fungal partners.," in Insect-fungal associations: ecology and evolution, eds. F. E. Vega and M. Blackwell (New York: Oxford University Press), 257–291.

Hartig, F. (2021). DHARMa: Residual diagnostics for hierarchical (multi-level / mixed) regression models. Available at: https://cran.r-project.org/package=DHARMa.

Hibbing, M. E., Fuqua, C., Parsek, M. R., and Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. Nat. Rev. Microbiol. 8, 15. doi: 10.1038/NRMICRO2259.

Hughes, D. P., Pierce, N. E., and Boomsma, J. J. (2008). Social insect symbionts: evolution in homeostatic fortresses. Trends Ecol. Evol. 23, 672–677. doi: 10.1016/j.tree.2008.07.011.

Ibarra-Juarez, L. A., Burton, M. A. J., Biedermann, P. H. W., Cruz, L., Desgarennes, D., Ibarra-Laclette, E., et al. (2020). Evidence for succession and putative metabolic roles of fungi and bacteria in the farming mutualism of the ambrosia beetle *Xyleborus affinis*. mSystems. 5, e00541-00520. doi: 10.1128/MSYSTEMS.00541-20/ASSET/527FF6DB-D4E5-4920-8F5B-3208E1531817/ASSETS/GRAPHIC/MSYSTEMS.00541-20-F0010.JPEG.

Jong, Z. W., Kassim, N. F. A., Naziri, M. A., and Webb, C. E. (2017). The effect of inbreeding and larval feeding regime on immature development of *Aedes albopictus*. J. Vector Ecol. 42, 105–112. doi: 10.1111/JVEC.12244.

Joseph, R., and Keyhani, N. O. (2021). Fungal mutualisms and pathosystems: life and death in the ambrosia beetle mycangia. Appl. Microbiol. and Biotechnol. 105, 3398-3410. https://doi.org/10.1007/s00253-021-11268-0

Kassambara, A. (2020). ggpubr: “ggplot2” based publication ready plots. Available at: https://cran.r-project.org/package=ggpubr.

Kingsolver, J. G., and Norris, D. M. (1977). The interaction of *Xyleborus ferrugineus* (Coleoptera: Scolytidae) behavior and intial reproduction in relation to its symbiotic fungi. Ann. Entomol. Soc. Am. 70, 1–4.

Kirkendall, L. R., Biedermann, P. H. W., and Jordal, B. H. (2015). “Evolution and diversity of bark and ambrosia beetles,” in Bark beetles: Biology and ecology of native and invasive species (Elsevier Academic Press), 85–156. doi: 10.1016/B978-0-12-417156-5.00003-4.

Kok, L. T., Norris, D. M., and Chu, H. M. (1970). Sterol metabolism as a basis for a mutualistic symbiosis. Nature 225, 661–662. doi: 10.1038/225661b0.

Kolařík, M., Hulcr, J., and Kirkendall, L. R. (2015). New species of *Geosmithia* and *Graphium* associated with ambrosia beetles in Costa Rica. Czech Mycol. 67, 29–35.

Kölliker-Ott, U. M., Bigler, F., and Hoffmann, A. A. (2003). Does mass rearing of field collected *Trichogramma brassicae* wasps influence acceptance of European corn borer eggs? Entomol. Exp. Appl. 109, 197–203. doi: 10.1046/J.0013-8703.2003.00104.X.

Kostovcik, M., Bateman, C. C., Kolarik, M., Stelinski, L. L., Jordal, B. H., and Hulcr, J. (2015). The ambrosia symbiosis is specific in some species and promiscuous in others: Evidence from community pyrosequencing. ISME J. 9, 126–138. doi: 10.1038/ismej.2014.115.

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., and Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl. Environ. Microbiol. 79, 5112–5120. doi: 10.1128/AEM.01043-13.

Lahti, L., and Shetty, S. (2019). microbiome R package. Available at: http://microbiome.github.io.

Lenth, R. V. (2021). emmeans: Estimated marginal means, aka least-squares means. Available at: https://cran.r-project.org/package=emmeans.

Liedo, P., Salgado, S., Oropeza, A., and Toledo, J. (2007). Improving mating performance of mass-reared sterile mediterranean fruit flies (Diptera: Tephritidae) through changes in adult holding conditions: Demography and mating competitiveness. Florida Entomol. 90, 33–40. doi: 10.1653/0015-4040(2007)90[33:IMPOMS]2.0.CO;2.

Lüdecke, D., Ben-Shachar, M. S., Patil, I., Waggoner, P., and Makowski, D. (2021). performance: An R package for assessment, comparison and testing of statistical models. J. Open Source Softw. 6, 3139. doi: 10.21105/joss.03139.

Malacrinò, A., Rassati, D., Schena, L., Mehzabin, R., Battisti, A., and Palmeri, V. (2017). Fungal communities associated with bark and ambrosia beetles trapped at international harbours. Fungal Ecol. 28, 44–52. doi: 10.1016/j.funeco.2017.04.007.

Mangiafico, S. (2021). rcompanion: Functions to support extension education program evaluation. Available at: https://cran.r-project.org/package=rcompanion.

Martinez Arbizu, P. (2020). pairwiseAdonis: Pairwise multilevel comparison using adonis.

Mayers, C. G., Harrington, T. C., and Biedermann, P. H. W. (2022). “Mycangia define the diverse ambrosia beetle–fungus symbioses,” in The convergent evolution of agriculture in humans and insects (The MIT Press). doi: 10.7551/mitpress/13600.003.0013.

Mayers, C. G., Harrington, T. C., Mcnew, D. L., Roeper, R. A., Biedermann, P. H. W., Masuya, H., et al. (2020). Four mycangium types and four genera of ambrosia fungi suggest a complex history of fungus farming in the ambrosia beetle tribe Xyloterini. https://doi.org/10.1080/00275514.2020.1755209 112, 1104–1137. doi: 10.1080/00275514.2020.1755209.

Mayers, C. G., McNew, D. L., Harrington, T. C., Roeper, R. A., Fraedrich, S. W., Biedermann, P. H. W., et al. (2015). Three genera in the Ceratocystidaceae are the respective symbionts of three independent lineages of ambrosia beetles with large, complex mycangia. Fungal Biol. 119, 1075–1092. doi: 10.1016/j.funbio.2015.08.002.

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217. doi: 10.1371/journal.pone.0061217.

Meats, A., Holmes, H. M., and Kelly, G. L. (2004). Laboratory adaptation of *Bactrocera tryoni* (Diptera: Tephritidae) decreases mating age and increases protein consumption and number of eggs produced per milligram of protein. Bull. Entomol. Res. 94, 517–524. doi: 10.1079/BER2004332.

Mehdiabadi, N. J., Hughes, B., and Mueller, U. G. (2006). Cooperation, conflict, and coevolution in the attine ant-fungus symbiosis. Behav. Ecol. 17, 291–296. doi: 10.1093/BEHECO/ARJ028.

Mighell, K., and Van Bael, S. A. (2016). Selective elimination of microfungi in leaf-cutting ant gardens. Fungal Ecol. 24, 15–20. doi: 10.1016/J.FUNECO.2016.08.009.

Mueller, U. G., Gerardo, N. M., Aanen, D. K., Six, D. L., and Schultz, T. R. (2005). The evolution of agriculture in insects. Annu. Rev. Ecol. Evol. Syst. 36, 563–595. doi: 10.1146/annurev.ecolsys.36.102003.152626.

Mueller, U. G., Scott, J. J., Ishak, H. D., Cooper, M., and Rodrigues, A. (2010). Monoculture of leafcutter ant gardens. PLoS One 5, e12668. doi: 10.1371/JOURNAL.PONE.0012668.

Nones, S., Fernandes, C., Duarte, L., Cruz, L., and Sousa, E. (2022). Bacterial community associated with the ambrosia beetle *Platypus cylindrus* on declining *Quercus suber* trees in the Alentejo region of Portugal. Plant Pathol. 71, 966–979. doi: 10.1111/ppa.13536.

Nuotclà, J. A., Biedermann, P. H. W., and Taborsky, M. (2019). Pathogen defence is a potential driver of social evolution in ambrosia beetles. Proc. R. Soc. B Biol. Sci. 286, 1–9. doi: 10.1098/rspb.2019.2332.

Nuotclà, J. A., Diehl, J. M. C., and Taborsky, M. (2021). Habitat quality determines dispersal decisions and fitness in a beetle – fungus mutualism. Front. Ecol. Evol. 9, 1–15. doi: 10.3389/fevo.2021.602672.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2020). vegan: Community ecology package.

Pagnocca, F. C., Masiulionis, V. E., and Rodrigues, A. (2012). Specialized fungal parasites and opportunistic fungi in gardens of attine ants. Psyche (London). doi: 10.1155/2012/905109.

Papen, H., and Werner, D. (1979). N2-fixation in *Erwinia herbicola*. Arch. Microbiol. 120, 25–30. doi: 10.1007/BF00413267.

Peer, K., and Taborsky, M. (2007). Delayed dispersal as a potential route to cooperative breeding in ambrosia beetles. Behav. Ecol. Sociobiol. 61, 729–739. Available at: http://link.springer.com/article/10.1007/s00265-006-0303-0.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., and R Core Team (2021). _nlme: Linear and nonlinear mixed effects models_. Available at: https://cran.r-project.org/package=nlme.

R Core Team (2021). R: A language and environment for statistical computing.

Rassati, D., Marini, L., and Malacrinò, A. (2019). Acquisition of fungi from the environment modifies ambrosia beetle mycobiome during invasion. PeerJ 2019, e8103. doi: 10.7717/PEERJ.8103/SUPP-5.

Römer, D., Aguilar, G. P., Meyer, A., and Roces, F. (2022). Symbiont demand guides resource supply: leaf-cutting ants preferentially deliver their harvested fragments to undernourished fungus gardens. Sci. Nat. 109, 1–5. doi: 10.1007/S00114-022-01797-7/TABLES/1.

Sarkar, D. (2008). Lattice: Multivariate data visualization with R. New York: Springer.

Saucedo-Carabez, J. R., Ploetz, R. C., Konkol, J. L., Carrillo, D., and Gazis, R. (2018). Partnerships between ambrosia beetles and fungi: Lineage-specific promiscuity among vectors of the laurel wilt pathogen, *Raffaelea lauricola*. Microb. Ecol. doi: 10.1007/s00248-018-1188-y.

Saunders, J. L., and Knoke, J. K. (1967). Diets for rearing the ambrosia beetle *Xyleborus ferrugineus* (Fabricius) in vitro. Science. 157, 460–463. doi: 10.1126/science.157.3787.460.

Schmidt, S., Kildgaard, S., Guo, H., Beemelmanns, C., and Poulsen, M. (2022). The chemical ecology of the fungus-farming termite symbiosis. Nat. Prod. Rep. 39, 231–248. doi: 10.1039/D1NP00022E.

Seibold, S., Müller, J., Baldrian, P., Cadotte, M. W., Šťursová, M., Biedermann, P. H. W., et al. (2019). Fungi associated with beetles dispersing from dead wood – Let's take the beetle bus! Fungal Ecol. 39, 100–108. doi: 10.1016/J.FUNECO.2018.11.016.

Shinzato, N., Muramatsu, M., Watanabe, Y., and Matsui, T. (2005). Termite-regulated fungal monoculture in fungus combs of a macrotermitine termite *Odontotermes formosanus*. Zoolog. Sci. 22, 917–922. doi: 10.2108/ZSJ.22.917.

Six, D. L., and Bentz, B. J. (2007). Temperature determines symbiont abundance in a multipartite bark beetle-fungus ectosymbiosis. Microb. Ecol. 54, 112–118. doi: 10.1007/S00248-006-9178-X/FIGURES/2.

Slowikowski, K. (2021). ggrepel: Automatically position non-overlapping text labels with "ggplot2". Available at: https://cran.r-project.org/package=ggrepel.

Van Arnam, E. B., Currie, C. R., and Clardy, J. (2018). Defense contracts: molecular protection in insect-microbe symbioses. Chem. Soc. Rev. 47, 1638–1651. doi: 10.1039/C7CS00340D.

Wickham, H. (2016). ggplot2: Elegant graphics for data analysis. New York: Springer-Verlag New York.

Wickham, H., François, R., Henry, L., and Müller, K. (2021). dplyr: A grammar of data manipulation. Available at: https://cran.r-project.org/package=dplyr.

Wickham, H., and Seidel, D. (2020). scales: scale functions for visualization. Available at: https://cran.r-project.org/package=scales.

Wilke, C. O. (2020). cowplot: Streamlined plot theme and plot annotations for "ggplot2". Available at: https://cran.r-project.org/package=cowplot.

Wisselink, M., Aanen, D. K., and van 't Padje, A. (2020). The longevity of colonies of fungus-growing termites and the stability of the symbiosis. Insects 11, 1–15. doi: 10.3390/INSECTS11080527.

Zhang, Y., Chen, M., Guo, J., Liu, N., Yi, W., Yuan, Z., et al. (2022). Study on dynamic changes of microbial community and lignocellulose transformation mechanism during green waste composting. Eng. Life Sci. 22, 376–390. doi: 10.1002/elsc.202100102.

Zhou, J., Huang, H., Meng, K., Shi, P., Wang, Y., Luo, H., et al. (2009). Molecular and biochemical characterization of a novel xylanase from the symbiotic *Sphingobacterium* sp. TN19. Appl. Microbiol. Biotechnol. 85, 323–333. doi: 10.1007/S00253-009-2081-X/FIGURES/6.

Zuur, A. F., Ieno, E. N., Walker, N., Saveliev, A. A., and Smith, G. M. (2009). Mixed effects models and extensions in ecology with R. New York, NY: Springer New York doi: 10.1007/978-0-387-87458-6.

ANNEX - CHAPTER 5

Supplementary Material

Supplementary Material

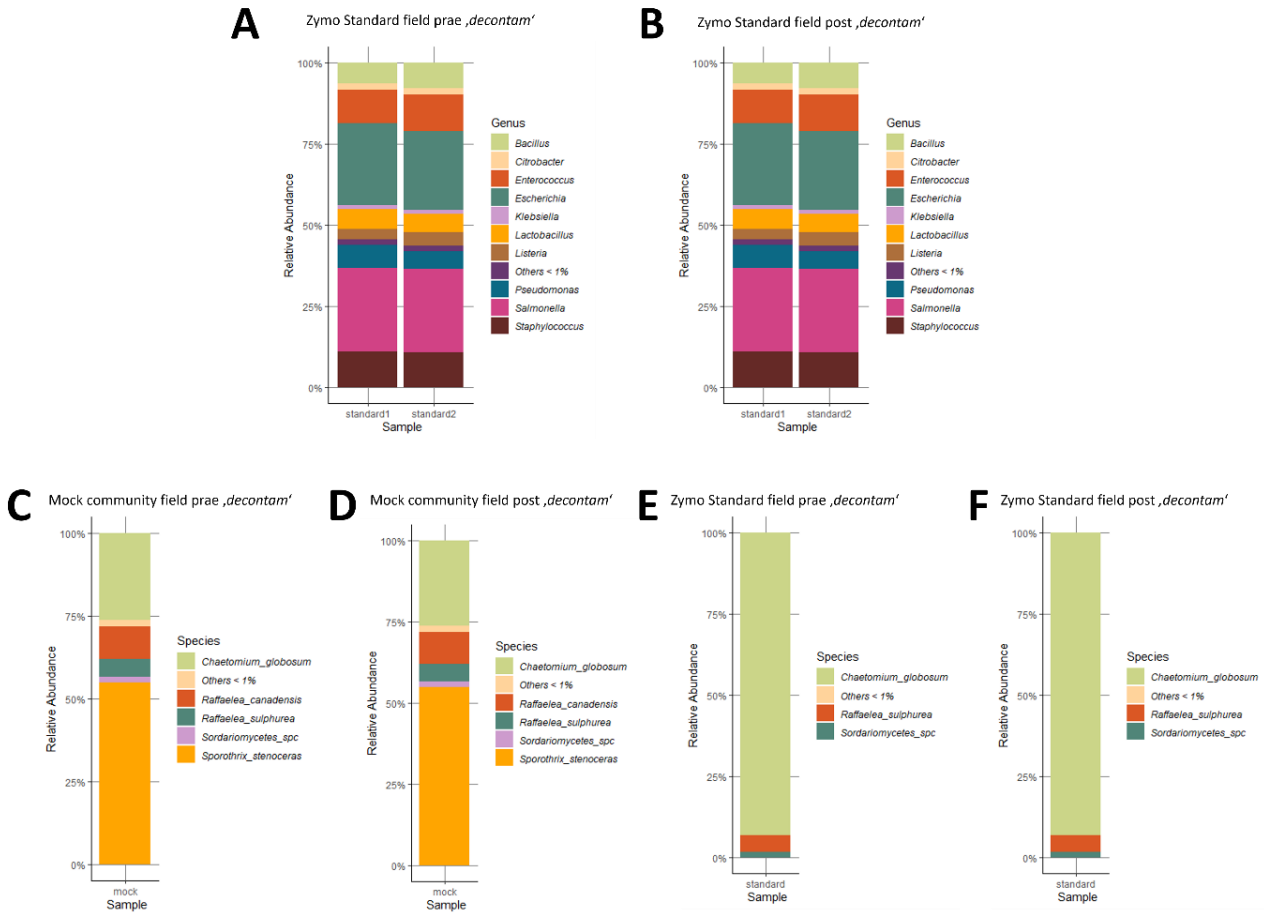
To publication **Succession of ambrosia beetle microbial community structure throughout development in field and laboratory galleries** by **JMC Diehl, A Keller & PHW Biedermann** in Front. Microbiol.

GitHub Repository: https://github.com/janinad88/microbial-succession-of-ambrosia-beetle-galleries
Raw Sequence Data available on NCBI SRA under BioProjectID: PRJNA915190

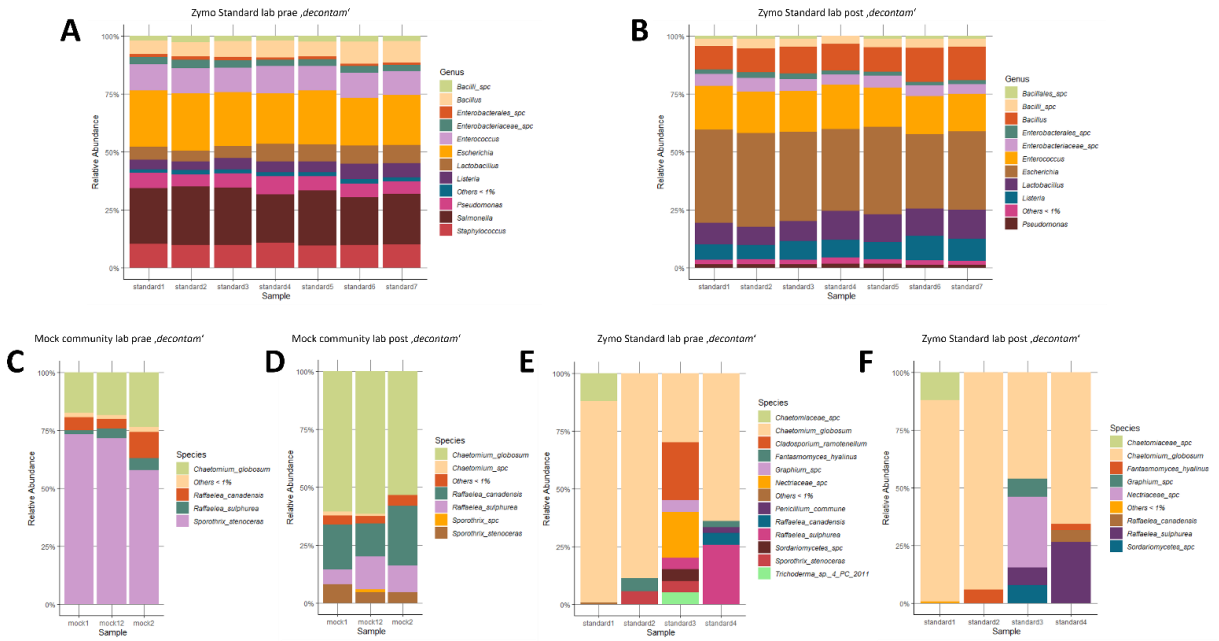
Sequencing controls

Controls showed a sufficient sequencing result of the microbial community standard from ZymoBiomics. All bacterial species contained in the standard are represented in the bar graph of relative bacterial taxa abundance (Supplementary Figure 1A & Supplementary Figure 2A) and appeared rarely in the other samples (≤105 reads/sample, only for *Staphylococcus aureus*). Negative controls (autoclaved rearing medium for beetle breeding and PCR water control) showed some bacterial species which can be neglected, since the first control of these samples with gel electrophoresis ahead to sequencing revealed no visible bands and rarefaction curves and richness estimates suggest a low input of single sequences due to possible cross-contamination. Due to a very low read number of these ASVs in our samples (≤ 480 reads/sample, *Streptomyces sp.*) we choose the low abundance filtering method. Similarly, a closer look on the fungal controls yielded important information on the quality of our 28S MiSeq primers. Our own mock community of known fungi associated with *X. saxesenni* (*R. sulphurea, R. canadensis, C. globosum, Ophiostoma stenosterans* and *Pichia sp.*) revealed like in Nuotcla et al. (2021) and Diehl et al. (2022) that the two symbionts *R. canadensis* and *R. sulphurea* can be distinguished, as well as other fungi of the orders Eurotiales, Sordariales, Hypocreales, Capnodiales, Onygenales and Dothideales, but again the yeasts including Saccharomycetales (e.g. *Pichia sp., Candida sp.*) were not differentiated (Supplementary Figure 1C & Supplementary Figure 2C). The negative controls showed only few reads (≤ 418 reads, *C. globosum*) of fungal taxa. We again decided to neglect this information since all negative controls (*N* = 6) revealed in gel electrophoresis ahead to sequencing no visible bands and

rarefaction curves as well as richness estimates again suggest a low input of single sequences due to potential cross-contamination.



Supplementary Figure 1. Relative Abundance of detected taxa in the sequencing control samples of the field samples prae und post filtering with the 'decontam' package. (A) ZymoBIOMICS™ Microbial Community Standard prae removal of bacterial contaminants (standard 1 = 12,661 reads; standard 2 = 14,118 reads). (B) ZymoBIOMICS™ Microbial Community Standard post removal of bacterial contaminants (standard 1 = 12,661 reads; standard 2 = 14,118 reads). The Zymo Microbial Community Standard should contain the following eight bacterial genera: *Pseudomonas, Escherichia, Salmonella, Lactobacillus, Enterococcus, Staphylococcus, Listeria, Bacillus* (ZymoBIOMICS™ Microbial Community Standard Instruction Manual). These could all be detected in our sequencing. (C) Mock community prae removal of fungal contaminants. Equal amounts of biomass were used to create the community. (32,739 reads) (D) Mock community post removal of fungal contaminants (32,674 reads). (E) ZymoBIOMICS™ Microbial Community Standard prae removal of fungal contaminants (59 reads). (F) ZymoBIOMICS™ Microbial Community Standard post removal of fungal contaminants (59 reads). These Mock communities should contain two fungal genera: *Cryptococcus* and *Saccharomyces* (Instruction Manual) which both could not be amplified with our primers. Instead, the Ambrosia beetle fungi appear, however the extremely low read numbers in these samples demonstrate that only neglectable cross contamination was sequenced.

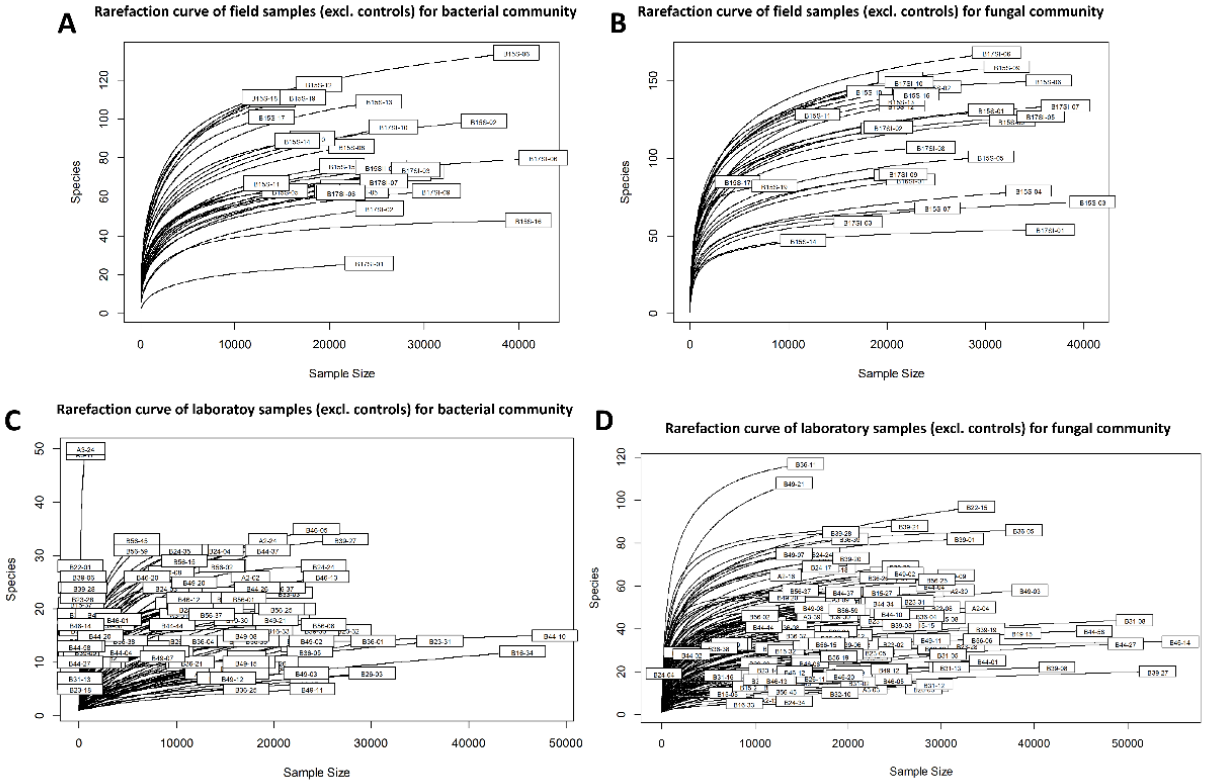


Supplementary Figure 2. Relative Abundance of detected taxa in the sequencing control samples of the laboratory samples prae und post filtering with the 'decontam' package. (A) ZymoBIOMICS™ Microbial Community Standard prae removal of bacterial contaminants (average 22,405 reads). (B) ZymoBIOMICS™ Microbial Community Standard post removal of bacterial contaminants (average 13,804 reads). The Zymo Microbial Community Standard should contain the following eight bacterial genera: *Pseudomonas, Escherichia, Salmonella, Lactobacillus, Enterococcus, Staphylococcus, Listeria, Bacillus* (ZymoBIOMICS™ Microbial Community Standard Instruction Manual). These could all be detected in our sequencing, however, the decontamination step removed *Staphylococcus* as this also appeared as a contaminant in the negative controls and could not be found in our true samples. (C) Mock community prae removal of contaminants. Equal amounts of biomass were used to create the community. (average 23,271 reads) (D) Mock community post removal of contaminants (average 7,638 reads). (E) ZymoBIOMICS™ Microbial Community Standard prae removal of fungal contaminants (average 627 reads). (F) ZymoBIOMICS™ Microbial Community Standard post removal of fungal contaminants (average 625 reads). These Mock communities should contain two fungal genera: *Cryptococcus* and *Saccharomyces* (Instruction Manual) which both could not be amplified with our primers. Instead, the Ambrosia beetle fungi appear, however the extremely low read numbers in these samples demonstrate that only neglectable cross contamination was sequenced.

After running the contaminant removal method with the package 'decontam' (Davis et al., 2018), 'negative' control samples (N_{field} = 4; N_{lab} = 14) identified 163 of the bacterial 16S ribosomal RNA and 3 of the fungal 28S ribosomal RNA ASVs as external contaminants from the field samples. Whereas, 430 in the laboratory nests of the bacterial 16S ribosomal RNA and 16 of the fungal 28S ribosomal RNA ASVs were found. Overall the quality of field samples was better and showed less contaminations. The filtering process for 'decontam' reduces the complexity of microbiome data while preserving its integrity in downstream analysis. By reducting the classification methods' sensitivity and technical variability, it allows researchers to generate more reproducible and better comparable results in microbiome data analysis (Cao et al., 2021).

Species accumulation curves (Supplementary Figure 3) showed that most samples were sequenced to saturation after approximately 20,000 high quality

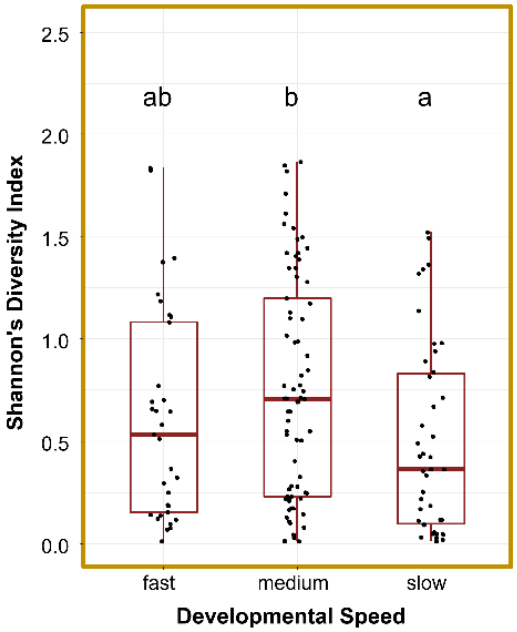
reads for 16S and 28S. The insufficient sequencing effort to represent the entire microbial communities in some of the samples could be due to a high amount of single read copies. Taxa that could not be assigned further than to Kingdom/Domain level, were removed prior to follow-up analyses.



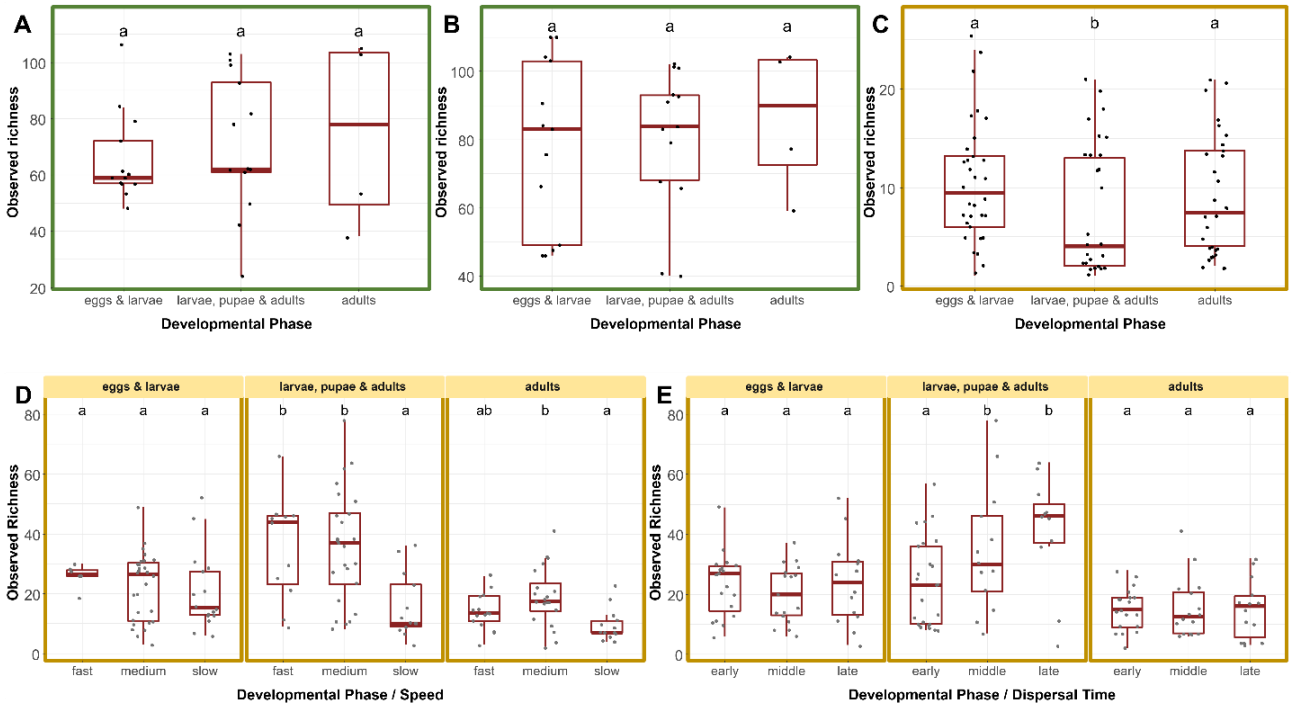
Supplementary Figure 3. Rarefaction curves of amplicon sequence variants in the datasets after running 'decontam' filtering step and excluding sequence control samples. (A) Samples of field nests from *X. saxesenii* in beech logs sequenced with 16S primers to detect the bacterial community. (B) Same samples of field nests sequenced with 28S primers to detect the fungal community. (C) Samples of laboratory nests from *X. saxesenii* in artificial beech rearing medium sequenced with 16S primers to detect the bacterial community. (D) Same samples of laboratory nests sequenced with 28S primers to detect the fungal community.

Supplementary Table 1. Number of replicates used for analysis in the different sequencing datasets.

Dataset	Replicates Phase 1	Replicates Phase 2	Replicates Phase 3
16S field	13	13	4
28S field	13	13	4
16S laboratory	32	27	30
28S laboratory	50	49	49



Supplementary Figure 4. Boxplots of Shannon's diversity indices in the three developmental speeds for the fungal communities in laboratory nests. Here, 'medium' developing nests had a higher Shannon's diversity index than 'slow' developing ones. Lowercase letters indicate significant differences between groups ($p < 0.05$; Tukey's test).



Supplementary Figure 5. Boxplots of observed richness estimates in the three developmental phases for the bacterial (A+C) and fungal (B, D & E) communities in field (green) and laboratory (yellow) nests. Field nests showed no significant differences between the developmental phases in both bacterial (A) and fungal (B) observed richness. Laboratory nests, on the other hand, had lower observed richness in the bacterial communities in the phase with larvae, pupae and adults present compared to only eggs and larvae or only adults (C). The fungal communities in laboratory nests, moreover, pointed out interactions where richness was as well influenced by the developmental speed (D), as well as the dispersal time of foundresses (E). In both interactions differences could be found in the second phase with larvae, pupae and adults present. Here, 'fast' and 'medium' developing nests or nests from 'middle' and 'late' dispersing foundresses had a higher fungal richness. Lowercase letters indicate significant differences between groups ($p < 0.05$; Tukey's test).

Bacterial beta diversity

Beta diversity slightly differed between phase 1, 2 and 3 ($PERMANOVA_{Field}$: phase1 vs. phase2: R^2 = 0.078, F = 2.43, p = 0.014; phase1 vs. phase3: R^2 = 0.115, F = 2.36, p = 0.010), but not between phase 2 and 3 (R^2 = 0.039, F = 0.714, p = 0.720) in the field. The NMDS plot of Bray-Curtis dissimilarity displayed some separation of the phases within the sampled trees (Supplementary Figure 6). The homogeneity groups between the developmental variable demonstrated an equal compositional variance (Betadisper: F = 1.64, p = 0.213), whereas the groups of the originating trees did not (Betadisper: F = 17.30, p = <0.001).

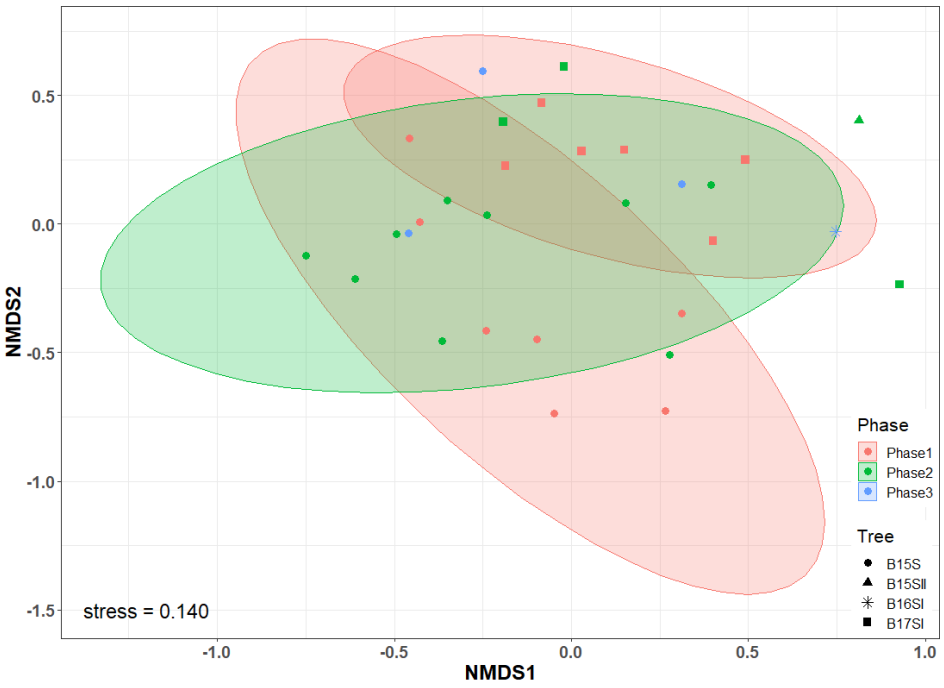
In laboratory nests pairwise comparison on the basis of PERMANOVA including the phase and development speed showed that dispersion of the developmental speed differed between the first and third phase (R^2 = 0.061, F = 1.89, p = 0.024). No statistically different dispersion was found between the first and second ('phase' p = 0.359, 'speed' p = 0.936) or second and third phase ('phase' p = 0.335, 'development' p = 0.843). The NMDS plot of Bray-Curtis dissimilarity displayed no separation of the phases or development speed (Supplementary Figure 9). The homogeneity groups between the developmental variables and dispersal time demonstrated an equal compositional variance (Betadisper: 'phase' F = 0.867, p = 0.424, 'speed' F = 1.46, p = 0.238, 'dispersal' F = 2.10, p = 0.129).

Fungal beta diversity

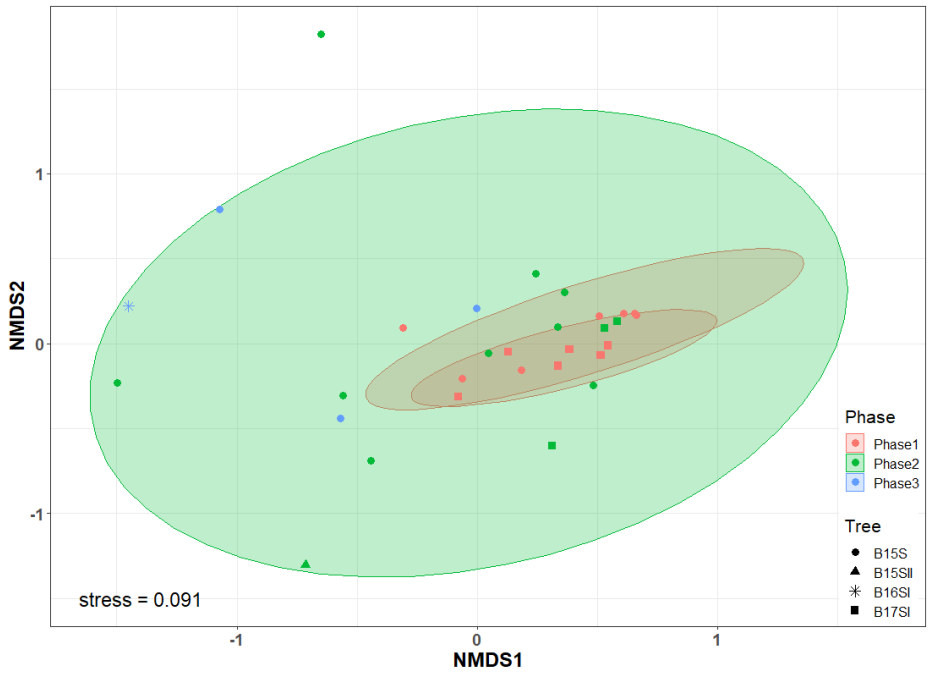
Adjusted p-values of pairwise PERMANOVA demonstrated significant variation between the first and third phase (R^2 = 0.237, F = 5.26, p = 0.001) and between the first and second (R^2 = 0.079, F = 2.24, p = 0.030) in the field. No separation was found for the second and third developmental phases (R^2 = 0.080, F = 1.47, p = 0.167). Instead, looking at these two groups, the pairwise PERMANOVA showed a marginal significant effect of the tree nests originated from (R^2 = 0.261, F = 1.59, p = 0.083), as well as in the first and second phase comparison (R^2 = 0.147, F = 2.08, p = 0.091). In the NMDS plot of Bray-Curtis dissimilarity some variation of the phases, where the dispersion of samples in

the groups gets bigger the older the fungus garden, is displayed. (Supplementary Figure 7). This heterogeneity of groups between the developmental phase demonstrates more compositional variance and is reflected by the significant test of homogeneity (Betadisper: F = 4.17, p = 0.026). As in the bacterial composition, we found this effect for the originating tree for the samples, too (Betadisper: F = 3.82, p = 0.022).

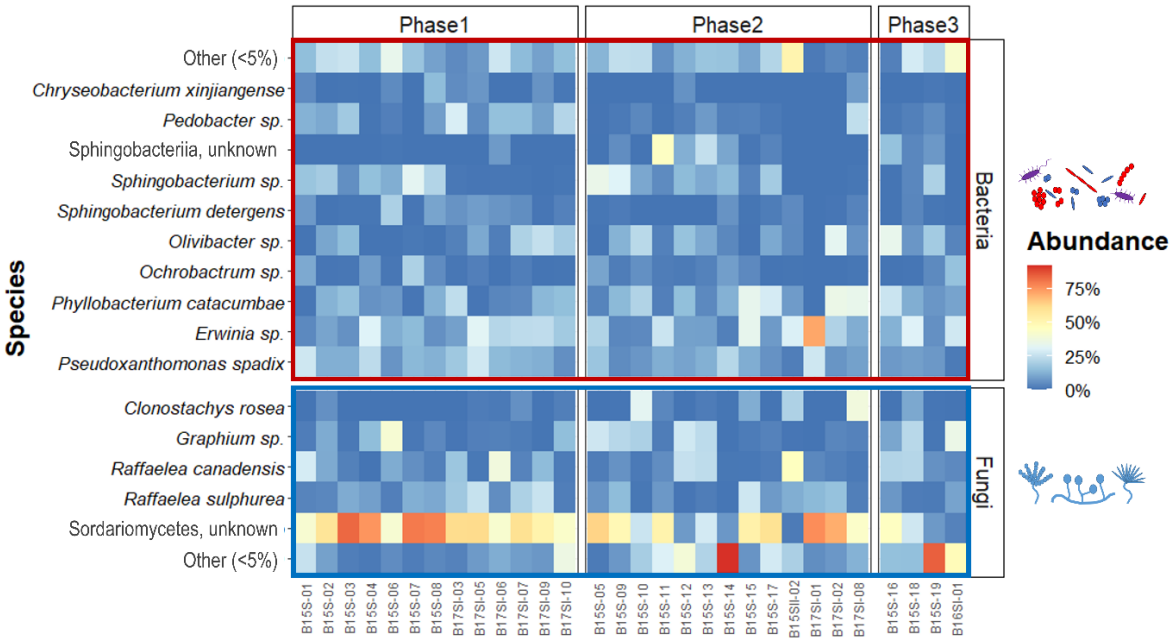
In lab fungus gardens, adjusted p-values of pairwise PERMANOVA demonstrated significant variation between the first and third phase (R^2 = 0.391, F = 63.51, p = 0.001) with no influence of the speed (R^2 = 0.025, F = 2.03, p = 0.246), and between the second and third ('phase' R^2 = 0.443, F = 87.68, p = 0.001, 'speed' R^2 = 0.083, F = 8.19, p = 0.006) (Supplementary Figure 10). No significant separation was found for the first and second developmental phases ('phase' R^2 = 0.022, F = 2.71, p = 0.194, 'speed' R^2 = 0.196, F = 11.90, p = 0.071). In the NMDS plot of Bray-Curtis dissimilarity some variation of the phases, where the dispersion of samples in the groups gets bigger for the second phase and a shift from first to third phase is displayed. (Supplementary Figure 10). This heterogeneity of groups between the developmental phase and speed demonstrates more compositional variance and is reflected by the significant test of homogeneity (Betadisper: 'phase' F = 18.91, p = <0.001, 'speed' F = 2.50, p = 0.086).



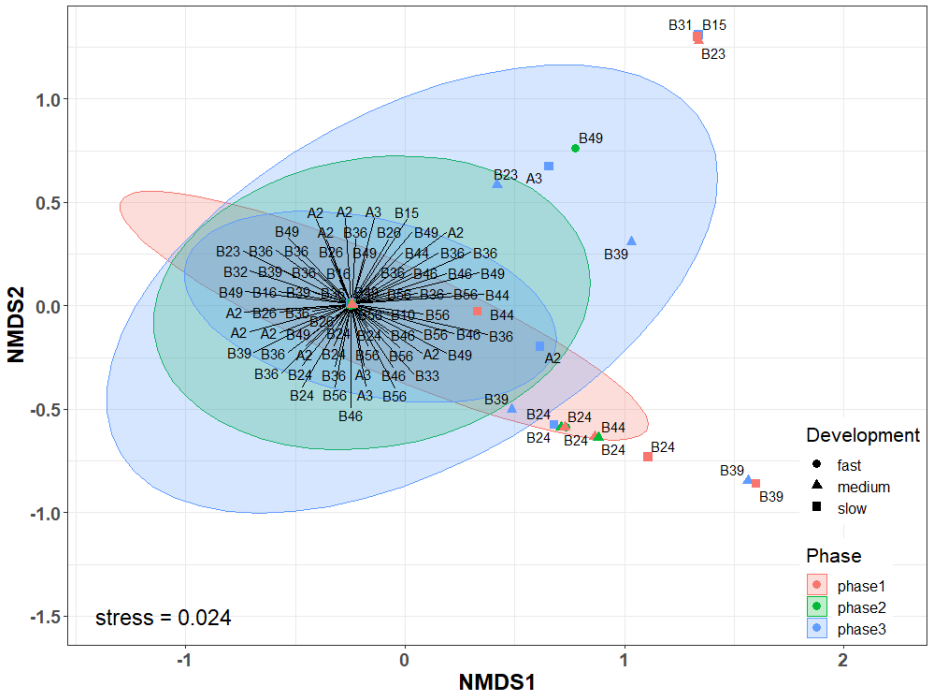
Supplementary Figure 6. The NMDS plot of Bray-Curtis dissimilarity in bacterial communities of field nests displayed some separation of the phases within the sampled tree logs.



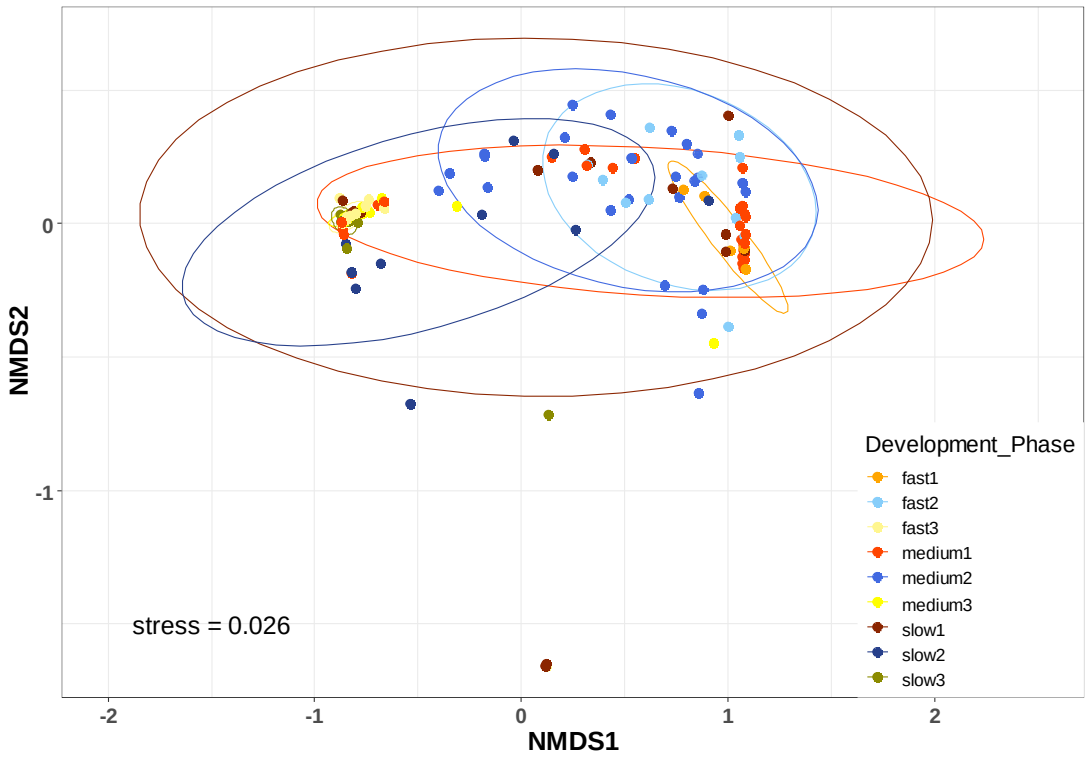
Supplementary Figure 7. The NMDS plot of Bray-Curtis dissimilarity in fungal communities of field nests displayed some variation of the phases, where dispersion of samples in the groups gets bigger the older the fungus garden.



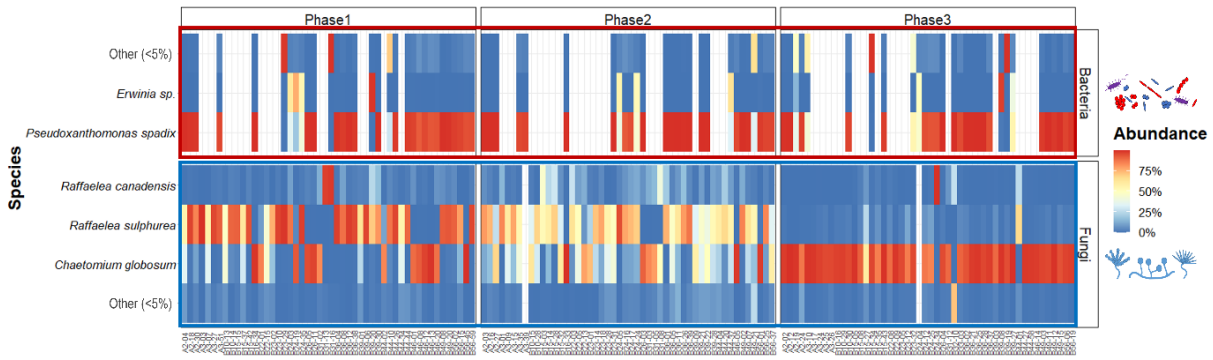
Supplementary Figure 8. Heatmap of most abundant taxa in the three developmental phases for both bacterial and fungal communities in field nests. Taxa under detection threshold of 5% relative abundance are combined into "Other".



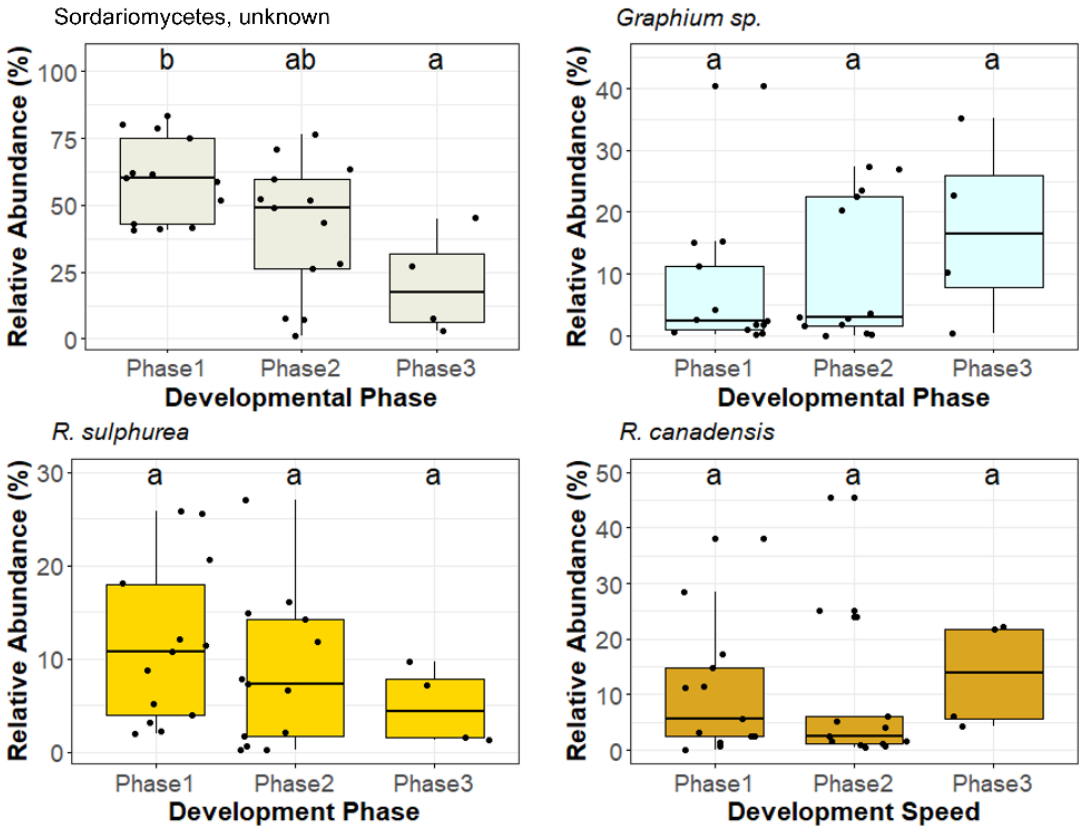
Supplementary Figure 9. The NMDS plot of Bray-Curtis dissimilarity displayed no separation of the phases or development speed in bacterial communities of laboratory nests.



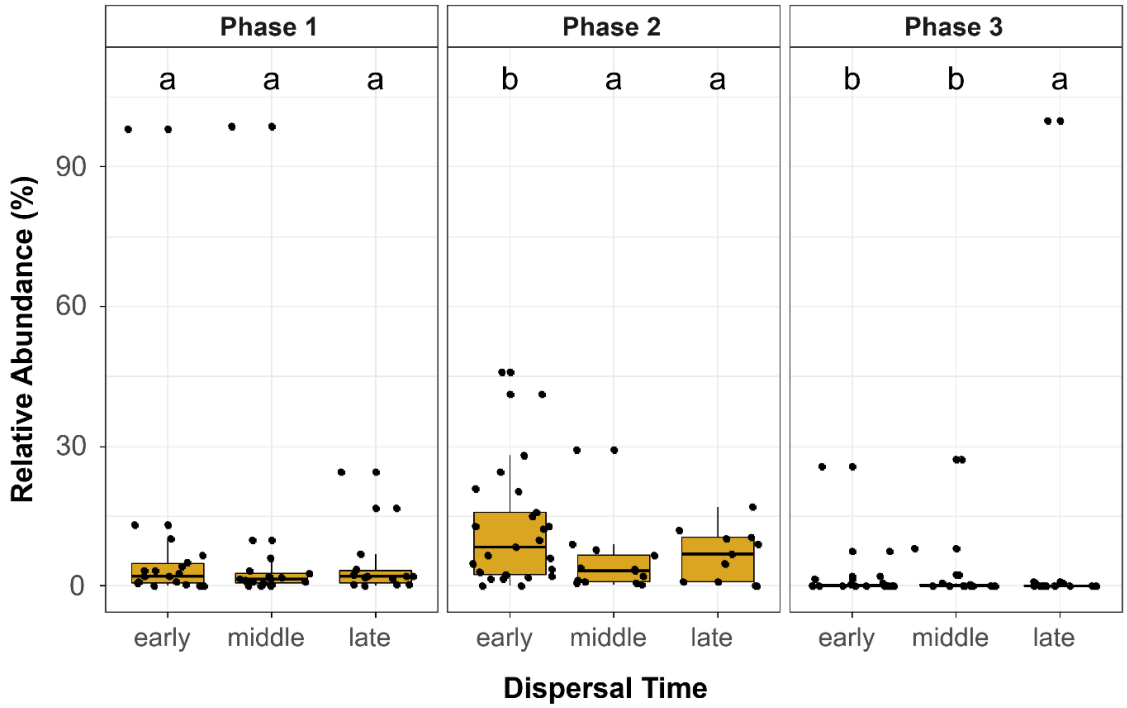
Supplementary Figure 10. NMDS of Bray-Curtis dissimilarity displayed separation by phases and development speed in fungal communities of laboratory nests.



Supplementary Figure 11. Heatmap of most abundant taxa in the three developmental phases for both bacterial and fungal communities in laboratory nests.



Supplementary Figure 12. Effects of the relative abundance of fungal core taxa in field nests. Direct comparison of the relative abundance of core taxa in field nests showed neither significant reduction of the primary food fungus, *R. sulphurea*, over nest development (bottom, left), nor increase of *R. canadensis* with developmental phases (bottom, right). *Graphium* sp. abundance seemed to have increased with nest age, but also revealed not significant difference between the phases (top, right). The abundance of the highly dominant unknown Sordariomycetes decreased between the first and third developmental phase significantly (top, left). Lowercase letters indicate significant differences between groups ($p < 0.05$; Tukey's test).



Supplementary Figure 13. Effect of the relative abundance of *R. canadensis* in laboratory nests. Direct comparison of the relative abundance of *R. canadensis* in laboratory nests showed an influence of the developmental phase with time of the foundress's dispersal, where nests of 'early' dispersing foundresses in the second developmental phase had a significant higher relative abundance compared to 'middle' and 'late' dispersing ones. Lowercase letters indicate significant differences between groups ($p < 0.05$; Tukey's test).

Supplementary Table 2. Mean abundance and standard deviation of fungal and bacterial taxa in field nests by order and species with number of nests detected.

Fungi							
Order	**mean**	**SD**	**N**	**Species**	**mean**	**SD**	**N**
Xylariales	2.56%	6.16%	28	Unknown Diatrypaceae	1.24%	4.47%	17
Microascales	9.96%	11.94%	30	*Graphium* sp.	9.95%	11.94%	29
Chaetothyriales	2.93%	8.80%	22	Unknown Herpotrichiellaceae	0.72%	2.84%	15
Hypocreales	11.87%	16.72%	28	*Clonostachys rosea*	2.37%	7.05%	35
				Clonostachys sp.	0.81%	1.81%	15
				Unknown Hypocreales	0.78%	1.06%	24
				Nectria balansae	0.71%	2.78%	8
				Neonectria sp.	1.45%	7.18%	13
				Trichoderma deliquescens	2.70%	10.99%	12
Togniniales	1.75%	5.20%	18	*Phaeoacremonium austroafrica*	0.96%	4.54%	9
				Phaeoacremonium sp.	0.79%	2.76%	14
Ophiostomatales	21.58%	14.62%	30	*Raffaelea aff. canadensis*	0.78%	1.34%	24
				Raffaelea canadensis	10.34%	12.13%	30
				Raffaelea sulphurea	9.33%	8.0%	30
				Unknown Sordariomycetes	46.6%	24.13%	30
Bacteria							
Class	**mean**	**SD**	**N**	**Genus**	**mean**	**SD**	**N**
				Unknown Bacteriodetes	1.42%	6.04%	12
Flavobacteriia	4.54%	5.44%	27	*Chryseobacterium*	3.54%	5.09%	26
				Flavobacterium	0.98%	2.55%	15
Actinobacteria	2.83%	4.59%	30	*Demetria*	0.52%	2.30%	7
Chitinophagia	1.36%	2.36%	19	*Taibaiella*	1.36%	2.36%	19
Alphaproteobacteria	20.34%	10.10%	30	*Mesorhizobium*	0.53%	0.72%	22
				Ochrobactrum	4.11%	5.15%	29
				Phyllobacterium	13.14%	10.66%	30
				Pseudochrobactrum	0.56%	2.15%	25
				Roseomonas	0.51%	0.95%	21
Betaproteobacteria	1.32%	3.32%	26	*Burkholderia*	0.76%	3.03%	11
Gammaproteobacteria	31.76%	17.71%	30	*Erwinia*	9.0%	13.49%	30
				Pseudoxanthomonas	12.43%	7.25%	30
				Xanthomonas	0.70%	2.58%	23
Sphingobacteriia	36.21%	15.25%	30	*Olivibacter*	10.62%	10.41%	29
				Pedobacter	6.80%	8.23%	28
				Unknown Sphingobacteriia	4.55%	9.59%	22
				Sphingobacterium	13.56%	12.83%	29

Supplementary Table 3. Mean abundance and standard deviation of fungal and bacterial taxa in laboratory nests by order and species with number of nests detected.

Fungi							
Order	***mean***	***SD***	***N***	***Species***	***mean***	***SD***	***N***
Eurotiales	0.58%	5.65%	43				
Ophiostomatales	45.70%	40.86%	148	*Raffaelea canadensis*	6.67%	15.49%	144
				Raffaelea sulphurea	38.61%	38.11%	147
Sordariales	52.96%	41.29&	148	*Chaetomium globosum*	52.16%	41.33%	148
Bacteria							
Class	***mean***	***SD***	***N***	***Genus***	***mean***	***SD***	***N***
Actinobacteriia	1.08%	1.60%	65				
Alphaproteobacteria	1.73%	11.56%	72	*Ochrobactrum*	1.64%	11.57%	45
Gammaproteobacteria	97.13%	11.56%	89	*Erwinia*	7.56%	21.72%	80
				Pantoea	0.81%	7.35%	42
				Pseudoxanthomonas	83.32%	30.65%	89
				Yersinia	5.18%	20.20%	43

References

Cao, Q., Sun, X., Rajesh, K., Chalasani, N., Gelow, K., Katz, B., et al. (2021). Effects of Rare Microbiome Taxa Filtering on Statistical Analysis. Front. Microbiol. 11, 3203. doi: 10.3389/FMICB.2020.607325/BIBTEX.

Davis, N. M., Proctor, Di. M., Holmes, S. P., Relman, D. A., and Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome 6. doi: 10.1186/S40168-018-0605-2.

Diehl, J. M. C., Kowallik, V., Keller, A., and Biedermann, P. H. W. (2022). First experimental evidence for active farming in ambrosia beetles and strong heredity of garden microbiomes. Proc. R. Soc. B Biol. Sci. 289. doi: 10.1098/rspb.2022.1458.

Nuotclà, J. A., Diehl, J. M. C., and Taborsky, M. (2021). Habitat quality determines dispersal decisions and fitness in a beetle – fungus mutualism. Front. Ecol. Evol. 9, 1–15. doi: 10.3389/fevo.2021.602672.

GENERAL DISCUSSION & CONCLUSION

A farmer cultivating a crop is not simply a relationship between a farmer and a cultivated crop, but rather a complex multipartite symbiosis. In agricultural ecosystems there are niches for pathogens that affect the farmer and the crop, as well as niches for pathogens of pathogens. For instance, some pests may infest stored products, while antagonistic 'weeds' may have adapted to coexist and exploit resources the farmers provide, such as cleared land, fertilizers and water. This understanding of agriculture as a complex symbiotic system highlights the importance of considering multiple factors when managing and optimising agricultural production (Fuller and Stevens, 2017; Schultz, 2022). Throughout the history of human agriculture, various techniques to manage and prevent the spread of unwanted symbiotic organisms have been implemented. These range from burning and intercropping to using biocontrol agents and genetic engineering in order to limit the impact of unwanted organisms (Denevan, 2001; Anderson and Wohlgemuth, 2012; Schultz, 2022). Comparable techniques are used in fungus-farming insects, such as ants, termites and ambrosia beetles, with the latter providing a special evolutionary perspective (Mueller et al., 2005). At least 13 independent origins of fungus farming have been observed in the polyphyletic group of ambrosia beetles, which comprises over 3,400 species globally (Hulcr and Stelinski, 2017; Biedermann and Vega, 2020). The aim of my doctoral thesis was to gain a more comprehensive understanding of the ecology and symbiosis of these small farmers. To this end, it was crucial to consider multiple key factors that play into these complex interactions, including habitat, pathogen pressure, sociality and the nests' microbial community.

Although it has always been assumed that ambrosia beetles actively manage their fungal gardens (Hubbard, 1897; Neger, 1908), it has never been experimentally tested. As such, my thesis assessed the ability of an established fungus garden to thrive in the presence or absence of its host. As expected, the presence of both adults and larvae had a significant effect on the microbial composition of ambrosia beetle fungus gardens. I observed a higher relative

abundance of ambrosia beetle fungi in the presence of adults and larvae compared to their absence, even though the beetles were feeding on the crop at the same time (Diehl et al., 2022b; Chapter 1). Although we could not pinpoint the underlying mechanism, it became clear that the crop depends not only on inoculation, but also on promotion and protection by the beetle host. To this end, recognition by the host of both beneficial and harmful symbionts is paramount.

The ability of ambrosia beetles to recognise and react to olfactory cues of their own food fungi has been demonstrated in studies such as that of Hulcr et al. (2011). However, this has not been reported for antagonistic and pathogenic microbes (c.f. Hanula et al., 2008; Hulcr et al., 2011; Luna et al., 2014). In **Chapter 2**, I investigated this question by confronting both adults and larvae with two antagonistic (*Aspergillus* sp. and *Penicillium commune*) and one entomopathogenic fungus (*Beauveria bassiana*). The individuals had the choice between their food fungi (*Dryadomyces sulphurous; syn. Raffaelea sulphurea* or *R. canadensis*) or a combination of one food fungus and one pathogen. I demonstrated the ability of ambrosia beetles to recognise other relevant symbionts. Additionally, I showed that larvae and adults react differently depending on the species they were confronted with (Diehl et al., 2022a; Chapter 2). Since larvae also play a significant role in this group of fungus farmers, it may be that physical constitution and pathogen susceptibility play a role in the division of labour.

With this knowledge, the question naturally arises as to how pathogens will influence beetle's behaviour. Many studies have investigated the response of ant and termites to introduced pathogens (Royce et al., 1991; Currie and Stuart, 2001; Rosengaus and Traniello, 2001; Pie et al., 2004; Konrad et al., 2012), but little attention has been paid to ambrosia beetles (e.g. Nuotclà et al., 2019). It is precisely here that there is great potential for research on the evolution of sociality in insects (Biedermann and Rohlfs, 2017; Nuotclà et al., 2019). By studying the response of facultatively eusocial ambrosia beetles, such as *X. saxesenii,* to pathogens, we can test the hypothesis of pathogens as drivers of the evolution of sociality (Biedermann and Rohlfs, 2017). By considering factors such as life-history traits, behavioural adaptations, as well as the associated

microbial community, I managed to get a glimpse of the complex trade-offs that accompany the introduction of a pathogen (**Chapter 3**). As seen in the individual response towards the fungal volatiles, injection of *Aspergillus* sp. or *B. bassiana* resulted in slightly different behaviour and developmental changes (Diehl & Biedermann, in prep.; Chapter 3). Individual and social immunity measures as well as a change in the bacterial and fungal communities were detected. The strength of the response was relatively low compared to previously known reactions by ants and termites (e.g. Currie and Stuart, 2001; Tranter et al., 2015). As ambrosia beetles have a different lifestyle than fungus-farming ants and termites, they are less exposed to sudden threats (Mueller et al., 2005) and their response to pathogens may be delayed and more long-term. Once again, I observed the ability of *X. saxesenii* to adapt its response to the specific pathogens it is confronted with, and it appears that there are different adaptation phases (Diehl & Biedermann, in prep.; Chapter 3). Drivers of adaptation phases are, among others, the dose and dosage of infection, as well as the general condition of the fungus garden, with beetles possibly evaluating the benefits and costs of the prospective adjustments to time their dispersal from the natal nest (e.g. Roy and Kirchner, 2000). The current level of sociality, observed behavioural and developmental measures, and the symbionts in this species allow for great flexibility and rapid adaptation to the environmental challenges that *X. saxesenii* may face.

The condition of the fungus garden is dependent upon the habitat and substrate quality. The habitat can influence the composition and presence of both antagonists and pathogens. Ambrosia beetles occupy a niche which undergoes changes depending on seasonality and nest age (Ulyshen, 2016). The strategy of *X. saxesenii* to establish two species of food fungi may be advantageous in this context. Deadwood loses moisture over the course of the year, therefore the nests of wood-dwelling insects must cope with increasingly dry substrate towards the end of their development. I suggest that the second food fungus of *X. saxesenii*, *R. canadensis*, although less productive, is drought resistant (Nuotclà et al., 2021; Chapter 4). Even if this slows down the development of the offspring, this symbiont enables the beetles to build longer-lasting nests and produce a higher number of offspring. In addition, the suboptimal conditions could limit the invasion of antagonists (Nuotclà et al., 2021; Chapter 4). In *X.*

saxesenii, the other mutualistic fungal species, *D. sulphureus*, typically appears during the initial phase of fungus gardens (Diehl et al., n.d., under review; Francke-Grosmann, 1975).This fast-growing and highly productive fungus has been observed to be the dominant species under standard breeding conditions (Nuotclà et al., 2021; Chapter 4). However, the success of this strategy depends on factors such as the availability of alternative nesting sites, conditions for dispersal and how far advanced the season is. In laboratory nests, the higher competitive pressure by *C. globosum* seems to make mature females more likely to decide to abandon the natal nests and start a new generation under better conditions (**Chapter 5**). Repeated transfer of beetles to artificial dry medium resulted in the loss of the main mutualist, *D. sulphureus* (**Chapter 4**). Thus, under natural conditions for dispersing females, the subsequent decision-making processes are paramount in the establishment of a new nest. The choice of a drier substrate, which may have already been colonised by conspecifics would be expected to have a direct effect on the cooperative investment of the daughters. In addition, however, a long-lasting effect could prevail for future generations. Through the induced selection of mutualists, only suitable wood conditions corresponding to those in the natal nest would thus be possible (Nuotclà et al., 2021; Chapter 4).

X. saxesenii can adapt to the changing substrate conditions and requirements of nest inhabitants by carrying two mutualistic food fungi (Nuotclà et al., 2021 (Chapter 4); Diehl et al., 2022b (Chapter 1)). The margin for rapid adaptation is evidenced by a succession of the mutualists with changing nest age and developmental stages (Diehl et al., n.d., under review; Chapter 5). Transgenerational and developmental costs are also evident in the context of a temporal succession of the fungal composition in the fungus gardens (Diehl et al., n.d., under review; Chapter 5). Accelerated succession slows down the rate of development, which is due to the lower nutrient supply by the other mutualists or competing antagonists (cf. Nuotclà et al., 2021; Chapter 4). Once again, the importance of choosing the right dispersal time and evaluating the demand for philopatry in congruence with the microbial community, becomes clear. Based on my research, I consider that the niche occupied by ambrosia beetles requires a combination strategy of both staying in the nest and helping, as well as early dispersing females, to be successful.

By comparing field and laboratory nests in **Chapter 5**, I can now draw parallels and make more informed statements about the potential functionally-significant fungus garden symbionts. Overall, I found a strong bottle-neck effect of species richness between both bacterial and fungal communities from field to laboratory nests. The symbionts that remain are possibly those that are crucial to the survival and success of beetles (e.g. *D. sulphureus, R. canadensis, Pseudoxanthomonas* sp. and *Erwinia* sp.; Diehl et al., n.d., under review; Chapter 5). Given the faster development of nests in the laboratory than in the field, and the greater alpha diversity under natural conditions, the interpretation of data should be made with caution and placed in the appropriate context. Laboratory studies are useful for identifying the core symbionts and disentangling complex interactions in a controlled environment. In this context, the effects of temperature, limited nutrient availability, and host tree defence mechanisms can be excluded. However, the natural environment allows for more flexibility, where other saprobionts may play additional beneficial roles and become more prominent if required (c.f. Diehl & Biederman, in prep.; Chapter 3).

Ants and termites have developed specific symbiotic relationships with certain types of fungi and microorganisms that are limited to certain habitats or geographic areas. In contrast, the specific microbial taxa associated with ambrosia beetles can vary depending on the geographic location of the beetle population (Baker, 1963; von Arx and Hennebert, 1965) and the environmental conditions in the area (see **Chapter 4**). While important mutualists are transported via the mycetangia or gut (Bateman et al., 2016; Mayers et al., 2018; Skelton et al., 2019a), the species composition of other symbionts can vary, as long as the functional role of the mutualistic microbes is fulfilled in facilitating the beetles' needs. Compared to ants and termites, the shorter life span and enduring fertility of ambrosia beetle ‘castes’ may be one of the reasons for their associated microbial community's high adaptability to environmental conditions. Furthermore, unlike other farmers, they are not able to stabilise the microbial community.

In general, *X. saxesenii* produces one or two generation(s) of offspring that disperses to found their own nests after successfully raising their siblings

(Biedermann et al., 2012). As such, there is no need for the fungus garden to last several years and remain healthy and productive during that time. Ambrosia beetles can reproduce successfully on a wide range of tree genera and families (Beaver, 1979), and the specificity of their host plant choice appears to be determined by the food fungus (Harrington 2005). Therefore, this generalist approach to functional symbiotic relationships appears to be advantageous, as it allows ambrosia beetles to adapt more quickly to shifting conditions and evolve in multiple directions, which is reflected in their flexible habitat selection and global distribution (Kirkendall et al., 2015).

The Microbial Community of Fungus Gardens

Several studies have sequenced material from galleries and beetles themselves in order to gain, in addition to the well-studied mutualistic crop fungi, more knowledge on symbionts involved in this farming lifestyle (Kajimura and Hijii, 1992; Fabig, 2011; Aylward et al., 2014; Kostovcik et al., 2015; Ibarra-Juarez et al., 2020; Nones et al., 2022). Fungal communities mostly consist of only a few taxa belonging to the orders of Microascales (e.g. *Ambrosiella*, Ceratocystis, Graphium, Petriella), Ophiostomatales (e.g. *Raffaelea, Sporothrix*), Saccharomycetales (e.g. *Candida*), Hypocreales (e.g. *Fusarium*), Eurotiales (e.g. *Penicillium, Aspergillus, Paecilomyces, Talaromyces)* and, in lower abundances, plant saprophytes, epiphytes, endophytes or pathogens belonging to the Botryosphaeriales (e.g. *Diplodia*), Dothideales (e.g. *Aureobasidium*), Pleosporales (e.g. *Alternaria*) and Cladosporiales (e.g. *Cladosporium*) (Kajimura and Hijii, 1992; Fabig, 2011; Kostovcik et al., 2015; Ibarra-Juarez et al., 2020; Nuotclà et al., 2021). Bacterial taxa mainly belong to the classes of Alpha- (e.g. *Ochrobactrum, Phyllobacterium, Sphingomonas*), Beta- (e.g. *Burkholderia*) and Gammaproteobacteria (e.g. *Pseudomonas, Pseudoxanthomonas, Erwinia, Stenotrophomonas, Pantoea*), Sphingobacteria (e.g. *Pedobacter, Olivibacter, Sphingobacterium*), Actinobacteria (e.g. *Streptomyces, Microbacterium*), Flavobacteriia (e.g. *Chryseobacterium*), Bacilli (e.g. *Staphylococcus, Bacillus*) and Chitinophagia (e.g. *Niabella*) (Fabig, 2011; Aylward et al., 2014; Ibarra-Juarez et al., 2020; Nuotclà et al., 2021; Nones et al., 2022). To date, only a few studies investigated their functional role, but some taxa provide antimicrobial or

fertilizing properties, which can be seen as beneficial for the crop (Baumann, 2016; Grubbs et al., 2020). In *X. affinis,* KEGG analyses suggest that its bacterial symbionts contribute to wood degradation, nitrogen fixation and nutritional provisioning (Ibarra-Juarez et al. 2020). The Gammaproteobacteria *Erwinia* was present both in the field and laboratory (Diehl et al., 2022b (Chapter 1), n.d., under review (Chapter 5)) and is a known potential pathogen in discoloured wood (Agrios, 2005). It is also known for its ability to fixate nitrogen (Papen and Werner, 1979).

Future work should investigate the potential contribution of bacterial communities in relationship to mutualism. While analysing community composition changes in the field, I observed a decrease in Sphingobacteriia and Flavobacteriia as nest age increased. Within the phylum of Bacteroidetes, Sphingobacteriaceae are known to exhibit xylanolytic activity. A *Sphingobacterium* with this ability was isolated from the gut of cerambycid larvae (Zhou et al., 2009). Taxa of the order Actinobacteria, which have the potential to produce antimicrobial metabolites, had in turn higher incidences in galleries where only adult individuals were present (Groth et al., 1997; Undabarrena et al., 2016). Clearly, the influence of the beetles is not the only factor changing the associated bacterial community, but progressive wood degradation also plays a role (Nuotclà et al., 2021; Diehl et al., 2022b). However, in wood excluded from beetles in the field, an increased bacterial richness has been observed in *Fagus sylvatica* and *Picea abies* (Hoppe et al., 2015), which I could not confirm in the current thesis. It is currently unclear whether this is due to the beetles' influence, or lack thereof, or whether the sampling period, environmental conditions and/or other organism might have played a role in the results.

Johnston et al. (2019), analysed bacterial-fungal interactions in decomposing beech wood and found that the bacterial community depended on the ecological strategy of dominating fungi and secondary colonizers, which could lead to shifts in bacterial diversity. Moreover, they found a clear effect of wood pH on bacterial richness at the community and family level and expected fungal pH manipulation to be one of the key factors for bacterial regulation. Therefore, I speculate that freshly excavated galleries with higher abundances of the

mutualist have a lower pH than older galleries with contaminating/decomposing fungi, as preferred by Actinobacteria (Lladó et al., 2017).

Additionally, in a laboratory setting, the mutualists were under higher competition pressure by *C. globosum*, a gallery contaminating mould (Diehl et al., 2022b (Chapter 1), n.d., under review (Chapter 5)). This mould proved to be a strong competitor for the ambrosia fungi and caused a compositional turnover from crop dominating gallery walls in the early developmental phase, towards mould dominating walls at the end of family life (Diehl et al., under review). Since in the third developmental phase the nest approaches the end of its life, with adult offspring about to disperse and start a new generation in a freshly excavated tunnel, I speculate that these adults do not invest into an intensive care of their crop fungi anymore. Another reason could be the stronger and more effective contribution of the larvae to fungal care. Our data could not pinpoint the exact mechanism behind this, but the findings from **Chapter 1** (Diehl et al., 2022b) show that beetle presence influences the fungal community, which points to active fungus farming in this species. It is well known that fungus gardens of ants quickly fail when farmers are removed and contaminants start to congest the nests (Batra and Batra, 1979; Wood and Thomas, 1989; Currie et al., 1999a; Currie, 2001; Currie and Stuart, 2001; Diehl et al., 2022b), but to which degree the beetles actively decide to abandon the nest is still unknown.

Comparison of Fungal Community Succession in Field and Laboratory Galleries

The comparison of the succession of microbial communities in field and laboratory nests provided new information on the bacterial and fungal composition throughout *X. saxesenii*'s nest life. While bacterial community structure stayed relatively stable during nest development, both field and laboratory galleries showed a change of the community for fungi (Diehl et al., under review). Interestingly, in galleries originating from beech trees in the field, I did not observe a change in fungal richness. However, the proportions of the taxa differed between the three phases of nest development. I found evidence

that the fungal community composition is different in nests where only larvae are present, compared to older nests with adult individuals (Diehl et al., under review). The strongest effect for the compositional change was due to a stepwise decrease of an unknown Sordariomycetes fungus and an increase of externally derived taxa unrelated to the fungus-beetle mutualism, which can be categorized as plant-pathogenic fungi, in older nests. Both field and laboratory communities contained the two mutualistic ambrosia fungi, *D. sulphureus* and *R. canadensis*, but we were able to demonstrate an extreme bottleneck effect of laboratory reared nests, with high abundances of these fungi (Diehl et al., under review).

For laboratory communities, I was able to demonstrate the expected succession of the two mutualists. In the second phase, before the contaminant completely dominated, I found a decrease of *D. sulphureus* relative abundance and an increase of *R. canadensis* (Diehl et al., under review). Experimental data by Nuotclà and Taborsky (unpublished data) suggests that *R. canadensis* is the slower growing mutualist, but it is better adapted to drier conditions than *D. sulphureus*. Therefore, a succession from one mutualist that is fast growing, highly productive and better adapted to the initial conditions, towards a second one complementing the main mutualist, is an advantage. The beetles can thus rely on a constant nutrient supply. This mutualist complementarity can also be found in the bark beetle *Dendroctonus ponderosae*. At least two associated ambrosia fungal species with varying abundances depending on temperature conditions are reported for *D. ponderosae* (Six and Bentz, 2007).

Comparing Fungal Metabarcoding Results Across the Chapters: A Comprehensive Evaluation

Most of my doctoral thesis (**Chapters 1, 3, 4 & 5**) has used next-generation sequencing technology to study the microbial communities in fungal gardens and in ambrosia beetles. As no suitable amplicon primers for the identification and differentiation of the mutualists of *X. saxesenii*, the Ophiostomataceae, were available at the beginning of my research, I designed my own primers according to my hypotheses and their application. After extensive literature

research and experimentation, the fungal large subunit (LSU) was chosen as the genetic marker. The primer pair LIC15R (Miadlikowska et al., 2014) and nu-LSU-355-3` (Döring et al., 2000) was first tested for fungal species identification based on Sanger sequencing. Due to their adequate differentiation performance, we expanded the primers in accordance with the overall oligo scaffold described in Kozich et al. (2013) for conformity to the Illumina MiSeq platform, choosing the dual-indexing design (Supplementary Material, Nuotclà et al., 2021).

My approach aligns with the efforts of Skelton et al. (2019b) and Ibarra-Juarez et al. (2020) in utilizing non-ITS (nuclear ribosomal internal transcribed spacer) primers for sequencing the fungal symbionts of ambrosia beetles and their fungus gardens. In all my studies, I effectively distinguished the closely related species of Ophiostomataceae. I was also able to identify the majority of other fungal symbionts (Ophiostomatales, Eurotiales, Sordariales, Hypocreales, Capnodiales, Helotiales and Xylariales). However, yeasts of the order Saccharomycetales, which were successfully amplified with the small subunit (SSU) primers used by Ibarra-Juarez (2020) and Basidiomycetes were not detected by my primers, as observed in the mock community outputs. This, once again, highlights the bias of the primers used for fungal metabarcoding. An important detail is that amplicon sequencing does not provide absolute abundance information of the phylotypes, but relates the amplified taxa to each other (Gloor et al., 2017). As a result, the inclusion of unamplified taxa may shift the relative composition of the community. I suggest that future studies of insect fungal microbiomes carefully select the metabarcoding primers that amplify the fungal groups of primary interest for testing the key hypothesis, while acknowledging their limitations (Ceballos-Escalera et al., 2022; Tedersoo et al., 2022).

A recurring pattern emerged when examining the sequencing outcomes from all the studies in my thesis - significant symbionts were identified in all the fungus gardens and in the beetles alike. The clear differences, especially in the alpha diversities, between the materials (laboratory nests, beetles and field nests) are conclusive and were to be expected. By employing sterilized breeding medium, saprobionts from the wood were precluded from the outset,

allowing only the symbionts introduced by the beetles to establish. The fungal microbiome of the beetles is comprised of mutualistic fungi as well as those with an antagonistic relationship, such as *Chaetomium globosum*, *Phaeoacremonium* sp., or other wood saprobionts. These latter fungi become increasingly dominant in the galleries during the final phase of nest development, and dispersing females are likely to encounter them as they exit the nests (Diehl et al., under review). By utilizing innovative primers, I uncovered transformations in the fungal communities within the nests. This allowed me to associate the abundance of the key fungal taxa with factors such as the presence of beetles, the nest's age and condition, and the various developmental stages present.

In conclusion, the newly designed primers proved to be a highly valuable tool in addressing my questions, although certain aspects should be approached with caution due to the exclusion of non-amplified taxa. The primers lay the foundation for a comprehensive understanding of fungal communities, including their composition and the identification of previously unknown functional symbionts. With respect to the bacterial communities, future studies can delve deeper into potential interactions with the mutualistic and antagonistic fungi of fungus gardens and the beetle hosts.

Suggested Improvements

Overall, my methods and designs were effective in answering my questions. Nonetheless, some parts of the studies could have yielded better results if the design was optimized, should I choose to conduct them again.

After my initial sequencing run, I optimized my design and ran the sequence again. For **Chapters 1, 4, and 5**, the samples were sequenced together, and I had to remove many reads from the dataset due to chloroplast contamination. This adversely affected the overall data quality. I attribute the high amplification rate of chloroplasts to the use of tap water in the preparation of beetle breeding medium. To avoid this problem, I chose to use Millipore water in the preparation of all media used for my study in **Chapter 3**. Laboratory nests were cultivated in centrifuge tubes (Sarstedt AG & CO. KG; Ref. 55.511). While this method

enabled space-efficient and abundant breeding, it posed challenges for accurate observation and recording of the beetles' behaviour and developmental stages. It was often difficult to obtain a clear view of successful nests, which only became apparent at a later stage, and the field of vision into the galleries was typically limited. As a result, gaining a precise understanding of the nest condition and individual activities was challenging and conducting focal observations was almost impossible. Ultimately, I had to exclude a significant number of these nests from the studies. Nuotclà (unpublished data) has developed a new breeding design that could address this issue and enable more precise manipulations. The design involves two plexiglass discs, between which the breeding medium is compressed to create a flat plane, allowing a clear view of the beetle tunnel passages. The structure is held together by a silicone frame and can be easily opened and closed. However, the extent to which this design impacts the risk of contamination is uncertain, and the number of replicas is limited by the need for a larger amount of storage space.

Collecting nests from the field was a time-intensive process, and although the entrance holes were marked at the beginning of the dispersal season, determining the precise start of the nests was not possible, as they often turned out to be unsuccessful after opening. Thus, I had to rely on successful nests with an unknown start date and determine their developmental stages to establish a temporal frame of reference. To standardize field collection, forthcoming studies could employ flight cages with a sufficient amount of unoccupied deadwood that is subsequently colonized by a large number of released ambrosia beetles. This approach would allow for additional system manipulations and precise assessments of colonization density and developmental time in the field. Nonetheless, the selection of location, such as shady and humid spots, must be considered.The analysis of behavioural data in **Chapter 3** proved to be partly problematic. A better approach would have been to observe the nests before and after the treatment to determine the exact effect and to incorporate individual nest variations as a random factor (e.g. Nuotclà et al., 2019). However, the early onset of female dispersal often allowed little room, making the injection of the solution necessary upon the emergence of the first adults. Observations before this point would not have been useful, as only larvae and pupae were present in the nests at that time. Therefore, improving

the data analysis would only have been possible with the help of an additional observation group without any treatment. This would be similar to the reference in the F0 generation but include generational changes. However, I do not necessarily think this would add significant value to the analysis, as a parallel comparison is possible through the control group, which also takes into account the effect of the solvent.

Finally, I want to give an outlook on the analyses still needed for **Chapter 3**. This chapter is still in preparation for submission to a peer-reviewed journal and needs some optimisation. In particular, I would like to take a closer look at the microbial communities and gain detailed information on the adaptation of the communities under the influence of the pathogens with the help of a Mantel test and a cooccurrence analysis. In terms of behavioural data, it is suggested that a revised classification of behaviours grouped as 'hygiene behaviour' should be considered. One particular behaviour, 'cleaning', lacks evidence and is considered by some researchers to be a problematic definition (Biedermann and Taborsky, 2011). By examining and isolating individual behaviours, it may be possible to identify patterns that help us understand their purpose and facilitate a more accurate definition.

Conclusion

In conclusion, this study presents crucial findings regarding the complex symbiotic relationships of ambrosia beetles and their fungus gardens, shedding light on their ability to recognize both beneficial and harmful symbionts and respond accordingly. The study indicates that the presence of both adults and larvae has a significant impact on the microbial composition of ambrosia beetle fungus gardens, emphasizing the need to promote and safeguard this crop. Additionally, the study demonstrates the ability of ambrosia beetles to recognize other relevant symbionts and make complex decisions based on their stage of development and the species they face. Moreover, these findings have implications for understanding how ambrosia beetles react to pathogen pressure. Additionally, they indicate that there are different stages of adaptation and that the response strength of *X. saxesenii* is relatively low compared to

other social insects. The current level of sociality and symbionts in this species allow for great flexibility and swift adaptation to environmental challenges, which is critical in the face of a changing environment.

Overall, this study enhances our comprehension of the complex relationships between ambrosia beetles and their symbiotic partners, offering a foundation for future research on the specific interactions within this insect-fungus mutualism.

REFERENCES

Aanen, D. K. (2006). As you reap, so shall you sow: coupling of harvesting and inoculating stabilizes the mutualism between termites and fungi. Biol. Lett. 2, 209–212. doi: 10.1098/RSBL.2005.0424.

Abrahamson, L. P., and Norris, D. M. (1970). Symbiontic interrelationships between microbes and ambrosia beetles (Coleoptera: Scolytidae). V. Amino acids as a source of nitrogen to the fungi in the beetle. Ann. Entomol. Soc. Am. 63, 177–180. doi: 10.1093/aesa/63.1.177.

Agrios, G. (2005). Plant Pathology. 5th ed. Cambridge, MA, USA: Elsevier Academic Press Available at: https://books.google.com/books?hl=de&lr=&id=CnzbgZgby60C&oi=fnd&pg=PP1&dq=Agrios,+G.+Plant+Pathology,+&ots=FrEmyh2Dkh&sig=WSVuY5M-EAg3k_m4sM0DDajTEFU.

Anderson, M. K., and Wohlgemuth, E. (2012). “California Indian proto-agriculture: Its characterization and legacy,” in Biodiversity in Agriculture: Domestication, Evolution, and Sustainability, eds. P. Gepts, T. R. Famula, R. L. Bettinger, S. B. Brush, A. B. Damania, P. E. McGuire, et al. (New York: Cambridge University Press), 190–224.

Arenas, A., and Roces, F. (2017). Avoidance of plants unsuitable for the symbiotic fungus in leaf-cutting ants: Learning can take place entirely at the colony dump. PLoS One 12, e0171388. doi: 10.1371/JOURNAL.PONE.0171388.

Aylward, F. O., Suen, G., Biedermann, P. H. W., Adams, A. S., Scott, J. J., Malfatti, S. A., et al. (2014). Convergent bacterial microbiotas in the fungal agricultural systems of insects. MBio 5, e02077-14. doi: 10.1128/mBio.02077-14.

Baker, J. M. (1963). Ambrosia beetles and their fungi, with particular reference to *Platypus cylindrus Fab*. Symp. Soc. Gen. Microbiol. 13, 232–264.

Bateman, C., Šigut, M., Skelton, J., Smith, K. E., and Hulcr, J. (2016). Fungal Associates of the *Xylosandrus compactus* (Coleoptera: Curculionidae, Scolytinae) Are Spatially Segregated on the Insect Body. Environ. Entomol. 45, 883–890. doi: 10.1093/ee/nvw070.

Batra, L. R., and Batra, S. W. T. (1979). “Termite-fungus mutualism” in Insect-Fungus Symbiosis, ed. L. R. Batra (Allanheld, Osmun, Montclair, New Yersey), 117–163.

Batra, L. R. R. (1966). Ambrosia fungi: extent of specificity to ambrosia beetles. Science (80-.). 153, 193–195. doi: 10.1126/science.153.3732.193.

Baumann, P. (2016). Bacterial induction of asexual fruiting in beetle-cultivated fungi.

Beaver, R. A. (1979). Host specificity of temperate and tropical animals. Nat. 1979 2815727 281, 139–141. doi: 10.1038/281139a0.

Beaver, R. A. (1989). “Insect-fungus relationships in the bark and ambrosia beetles,” in Insect-fungus interactions, eds. N. Wilding, N. M. Collins, P. M. Hammond, and J. F. Webber (London, UK: Academic Press), 121–143. doi: 10.1016/B978-0-12-751800-8.50002-1.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B 57, 289–300. doi: 10.1111/J.2517-6161.1995.TB02031.X.

Best, A. S., Johst, K., Mü, T., Travis, J. M. J., and Münkemüller, T. (2007). Which species will succesfully track climate change? The influence of intraspecific competition and density dependent dispersal on range shifting dynamics. Oikos 116, 1531–1539. doi: 10.1111/J.0030-1299.2007.16047.X.

Biedermann, P. H. W. (2007). Social behaviour in sib mating fungus farmers - Intra- and interspecific cooperation in ambrosia beetles. Master Thesis. Available at: http://behav.zoology.unibe.ch/sysuif/uploads/files/esh/pdf_online/biedermann/masterthesis_endversion2.pdf.

Biedermann, P. H. W. (2020). Cooperative breeding in the ambrosia beetle *Xyleborus affinis* and management of its fungal symbionts. Front. Ecol. Evol. 8, 1–12. doi: 10.3389/fevo.2020.518954.

Biedermann, P. H. W., Klepzig, K. D., and Taborsky, M. (2009). Fungus cultivation by ambrosia beetles: behavior and laboratory breeding success in three Xyleborine species. Environ. Entomol. 38, 1096–1105. doi: 10.1603/022.038.0417.

Biedermann, P. H. W., Klepzig, K. D., and Taborsky, M. (2011). Costs of delayed dispersal and alloparental care in the fungus-cultivating ambrosia beetle *Xyleborus affinis* Eichhoff (Scolytinae: Curculionidae). Behav. Ecol. Sociobiol. 65, 1753–1761. doi: 10.1007/s00265-011-1183-5.

Biedermann, P. H. W., Klepzig, K. D., Taborsky, M., and Six, D. L. (2013). Abundance and dynamics of filamentous fungi in the complex ambrosia gardens of the primitively eusocial beetle *Xyleborinus saxesenii* Ratzeburg (Coleoptera: Curculionidae, Scolytinae). FEMS Microbiol. Ecol. 83, 711–723. doi: 10.1111/1574-6941.12026.

Biedermann, P. H. W., and Nuotclà, J. A. (2020). Social Beetles. Encycl. Soc. Insects, 1–8. doi: 10.1007/978-3-319-90306-4_108-1.

Biedermann, P. H. W., Peer, K., and Taborsky, M. (2012). Female dispersal and reproduction in the ambrosia beetle *Xyleborinus saxesenii* Ratzeburg (Coleoptera; Scolytinae). Mitteilungen der Dtsch. Gesellschaft für Allg. und Angew. Entomol. 18, 231–235. Available at:

http://behav.zoology.unibe.ch/sysuif/uploads/files/esh/pdf_online/biedermann/Biedermann_et_al_MDGAAE2012.pdf.

Biedermann, P. H. W., and Rohlfs, M. (2017). Evolutionary feedbacks between insect sociality and microbial management. Curr. Opin. Insect Sci. 22, 92–100. doi: 10.1016/j.cois.2017.06.003.

Biedermann, P. H. W., and Taborsky, M. (2011). Larval helpers and age polyethism in ambrosia beetles. Proc. Natl. Acad. Sci. 108, 17064–17069. doi: 10.1073/pnas.1107758108.

Biedermann, P. H. W., and Vega, F. E. (2020). Ecology and evolution of insect-fungus mutualisms. Annu. Rev. Entomol. 65, 431–455. doi: 10.1146/annurev-ento-011019-024910.

Birkemoe, T., Jacobsen, R. M., Sverdrup-Thygeson, A., and Biedermann, P. H. W. (2018). “Insect-fungus interactions in dead wood systems,” in Saproxylic insects (Springer), 377–427. doi: 10.1007/978-3-319-75937-1_12.

Bischoff, L. L. (2004). The social structure of the haplodiploid bark beetle, *Xylosandrus germanus*.

Bodawatta, K. H., Poulsen, M., and Bos, N. (2019). Foraging *Macrotermes natalensis* Fungus-Growing Termites Avoid a Mycopathogen but Not an Entomopathogen. Insects 2019, Vol. 10, Page 185 10, 185. doi: 10.3390/INSECTS10070185.

Borden, J. H. (1988). “The Striped Ambrosia Beetle,” in Dynamics of Forest Insect Populations, ed. A. A. Berryman (New York: Springer Science and Business Media New York), 579–596. doi: 10.1007/978-1-4899-0789-9_27.

Bot, A. N. M., Ortius-Lechner, D., Finster, K., Maile, R., and Boomsma, J. J. (2002). Variable sensitivity of fungi and bacteria to compounds produced by the metapleural glands of leaf-cutting ants. Insectes Soc. 49, 363–370. doi: 10.1007/PL00012660.

Bot, A. N. M., Rehner, S. A., and Boomsma, J. J. (2001). Partial incompatibility between ants and symbiotic fungi in two sympatric species of Acromyrmex leaf-cutting ants. Evolution (N. Y). 55, 1980–1991. doi: 10.1111/J.0014-3820.2001.TB01315.X.

Bourke, A. F. G. (2011). Principles of social evolution. Oxford University Press Available at: https://research-portal.uea.ac.uk/en/publications/principles-of-social-evolution.

Bravo, I. S. J., and Zucoloto, F. S. (1998). Performance and feeding behavior of *Ceratitis capitata*: comparison of a wild population and a laboratory population. Entomol. Exp. Appl. 87, 67–72. doi: 10.1046/J.1570-7458.1998.00305.X.

Bresson, J., Varoquaux, F., Bontpart, T., Touraine, B., and Vile, D. (2013). The PGPR strain *Phyllobacterium brassicacearum* STM196 induces a reproductive delay and physiological changes that result in improved drought tolerance in Arabidopsis. New Phytol. 200, 558–569. doi: 10.1111/nph.12383.

Brooks, M. E., Kristensen, K., van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., et al. (2017). glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. R J. 9, 378–400.

Buresova, A., Kopecky, J., Hrdinkova, V., Kamenik, Z., Omelka, M., and Sagova-Mareckova, M. (2019). Succession of microbial decomposers is determined by litter type, but site conditions drive decomposition rates. Appl. Environ. Microbiol. 85, 1–16. doi: 10.1128/AEM.01760-19.

Calisi, R. M., and Bentley, G. E. (2009). Lab and field experiments: Are they the same animal? Horm. Behav. 56, 1–10. doi: 10.1016/J.YHBEH.2009.02.010.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. 108, 4516–4522. doi: 10.1073/PNAS.1000080107.

Carrias, J. F., Gerphagnon, M., Rodríguez-Pérez, H., Borrel, G., Loiseau, C., Corbara, B., et al. (2020). Resource availability drives bacterial succession during leaf-litter decomposition in a bromeliad ecosystem. FEMS Microbiol. Ecol. 96, 1–12. doi: 10.1093/femsec/fiaa045.

Ceballos-Escalera, A., Richards, J., Arias, M. B., Inward, D. J. G., and Vogler, A. P. (2022). Metabarcoding of insect-associated fungal communities: a comparison of internal transcribed spacer (ITS) and large-subunit (LSU) rRNA markers. MycoKeys 88, 1. doi: 10.3897/MYCOKEYS.88.77106.

Chen, Q. L., Hu, H. W., Yan, Z. Z., Li, C. Y., Nguyen, B. A. T., Zheng, Y., et al. (2021). Termite mounds reduce soil microbial diversity by filtering rare microbial taxa. Environ. Microbiol. 23, 2659–2668. doi: 10.1111/1462-2920.15507.

Chiri, E., Greening, C., Lappan, R., Waite, D. W., Jirapanjawat, T., Dong, X., et al. (2020). Termite mounds contain soil-derived methanotroph communities kinetically adapted to elevated methane concentrations. ISME J. 14, 2715–2731. doi: 10.1038/s41396-020-0722-3.

Chiri, E., Nauer, P. A., Lappan, R., Jirapanjawat, T., Waite, D. W., Handley, K. M., et al. (2021). Termite gas emissions select for hydrogenotrophic microbial communities in termite mounds. Proc. Natl. Acad. Sci. U. S. A. 118, e2102625118. doi: 10.1073/pnas.2102625118.

Choe, J. C., and Crespi, B. J. (1997). The evolution of social behaviour in insects and arachnids. Cambridge University Press Available at: https://books.google.de/books?hl=de&lr=&id=qkHHO_SmM_kC&oi=fnd&pg=PA9&ots=KJJ_I3Oa0N&sig=68LNG74Nz5czEFb-eiDiPK2F1H8&redir_esc=y#v=onepage&q&f=false.

Clarke, K. R., Somerfield, P. J., and Chapman, M. G. (2006). On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray–Curtis coefficient for denuded assemblages. J. Exp. Mar. Bio. Ecol. 330, 55–80. doi: 10.1016/J.JEMBE.2005.12.017.

Coleman-Derr, D., and Tringe, S. G. (2014). Building the crops of tomorrow: Advantages of symbiont-based approaches to improving abiotic stress tolerance. Front. Microbiol. 5, 1–6. doi: 10.3389/fmicb.2014.00283.

Costa, J. T. (2006). The other insect societies. Cambridge, Massachusetts and London, England: The Belknap Press of Havard University Press Available at: www.hup.harvard.edu/catalog.php?isbn=9780674021631.

Craven, S. E., Dix, M. W., and Michaels, G. E. (1970). Attine Fungus Gardens Contain Yeasts. Science (80). 169, 184–186. doi: 10.1126/science.169.3941.184.

Cruz, L. F., Menocal, O., Mantilla, J., Ibarra-Juarez, L. A., and Carrillo, D. (2019). *Xyleborus volvulus* (Coleoptera: Curculionidae): Biology and fungal associates. Appl. Environ. Microbiol. 85. doi: 10.1128/AEM.01190-19/ASSET/F3EC497A-78ED-4BF9-BFC2-B8886B43A50E/ASSETS/GRAPHIC/AEM.01190-19-F0004.JPEG.

Currie, C. R. (2001). A community of ants, fungi, and bacteria: A multilateral approach to studying symbiosis. Annu. Rev. Microbiol. 55, 357–380. doi: 10.1146/annurev.micro.55.1.357.

Currie, C. R., Mueller, U. G., and Malloch, D. (1999a). The agricultural pathology of ant fungus gardens. Proc. Natl. Acad. Sci. 96, 7998–8002. doi: 10.1073/PNAS.96.14.7998.

Currie, C. R., Scott, J. A., Summerbell, R. C., and Malloch, D. (1999b). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398, 701–704. doi: 10.1038/19519.

Currie, C. R., and Stuart, A. E. (2001). Weeding and grooming of pathogens in agriculture by ants. Proc. R. Soc. London. Ser. B Biol. Sci. 268, 1033–1039. doi: 10.1098/rspb.2001.1605.

Dahlsten, D. L. (1982). “Relationships between bark beetles and their natural enemies,” in Bark beetles in North American conifers. A system for the study of evolutionary biology. (The Corrie Herring Hooks Series, No.6), eds. J. B. Mitton and K. B. Sturgeon (Austin Texas: University of Texas Press), pp.140-182.

Davis, N. M., Proctor, Di. M., Holmes, S. P., Relman, D. A., and Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome 6. doi: 10.1186/S40168-018-0605-2.

De Fine Licht, H. H., and Biedermann, P. H. W. (2012). Patterns of functional enzyme activity in fungus farming ambrosia beetles. Front. Zool. 9, 13. doi: 10.1186/1742-9994-9-13.

De Roode, J. C., and Lefèvre, T. (2012). Behavioral Immunity in Insects. Insects 2012, Vol. 3, Pages 789-820 3, 789–820. doi: 10.3390/INSECTS3030789.

Delignette-Muller, M. L., and Dutang, C. (2015). fitdistrplus: An R package for fitting distributions. J. Stat. Softw. 64, 1–34. doi: 10.18637/jss.v064.i04.

Denevan, W. M. (2001). Cultivated Landscapes of Native Amazonia and the Andes, eds. G. Clark, A. Goudie, and C. Peach Oxford: Oxford University Press.

Diamantidis, A. D., Carey, J. R., Nakas, C. T., and Papadopoulos, N. T. (2011). Ancestral populations perform better in a novel environment: domestication of Mediterranean fruit fly populations from five global regions. Biol. J. Linn. Soc. 102, 334–345. doi: 10.1111/J.1095-8312.2010.01579.X.

Diehl, J. M. C., Kassie, D., and Biedermann, P. H. W. (2022a). Friend or Foe: Ambrosia beetle response to volatiles of common threats in their fungus gardens. bioRxiv, 2022.12.23.521835. doi: 10.1101/2022.12.23.521835.

Diehl, J. M. C., Keller, A., and Biedermann, P. H. W. (n.d.). Succession of ambrosia beetle microbial community structure throughout development in field and laboratory galleries. Front. Microbiol., 1–22.

Diehl, J. M. C., Kowallik, V., Keller, A., and Biedermann, P. H. W. (2022b). First experimental evidence for active farming in ambrosia beetles and strong heredity of garden microbiomes. Proc. R. Soc. B Biol. Sci. 289. doi: 10.1098/rspb.2022.1458.

Döring, H., Clerc, P., Grube, M., and Wedin, M. (2000). Mycobiont-specific PCR primers for the amplification of nuclear its and LSU rDNA from lichenized Ascomycetes. Lichenol. 32, 200–204. doi: 10.1006/lich.1999.0250.

Dowd, P. F. (1992). Insect fungal symbionts - A promising source of detoxifying enzymes. J Ind Microbiol 9, 149–161.

Enfjäll, K., and Leimar, O. (2009). The evolution of dispersal - The importance of information about population density and habitat characteristics. Oikos 118, 291–299. doi: 10.1111/j.1600-0706.2008.16863.x.

Estrada, C., Rojas, E. I., Wcislo, W. T., and Van Bael, S. A. (2014). Fungal endophyte effects on leaf chemistry alter the in vitro growth rates of leaf-cutting ants' fungal mutualist, *Leucocoprinus gongylophorus*. Fungal Ecol. 8, 37–45. doi: 10.1016/J.FUNECO.2013.12.009.

Fabig, W. (2011). The microbial community associated with the ambrosia beetle *Xyleborinus saxesenii* (Coleoptera: Curculionidae) and its influence on the growth of the mutualistic fungus.

Farrell, B. D., Sequeira, A. S., O'Meara, B. C., Normark, B. B., Chung, J. H., and Jordal, B. H. (2001). The Evolution of Agriculture in Beetles (Curculionidae: Scolytinae and Platypodinae). Evolution (N. Y). 55, 2011–2027.

Fernández-Marín, H., Nash, D. R., Higginbotham, S., Estrada, C., Zweden, J. S. van, d'Ettorre, P., et al. (2015). Functional role of phenylacetic acid from metapleural gland secretions in controlling fungal pathogens in evolutionarily derived leaf-cutting ants. Proc. R. Soc. B Biol. Sci. 282. doi: 10.1098/RSPB.2015.0212.

Fischer, M. (1954). Untersuchungen über den kleinen Holzbohrer (Xyleborus saxeseni). Pflanzenschutzberichte 12, 137–180.

Flórez, L. V., Biedermann, P. H. W., Engl, T., and Kaltenpoth, M. (2015). Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. Nat. Prod. Rep. 32, 904–936. doi: 10.1039/C5NP00010F.

Fox, J., and Weisberg, S. (2019). An R companion to applied regression. Thousand Oaks, CA: Sage Publications Inc.

Francke-Grosmann, H. (1956). Hautdrüsen als Träger der Pilzsymbiose bei Ambrosiakäfern. Zeitschrift für Morphol. und Ökologie der Tiere 45, 275–308. doi: 10.1007/BF00430256.

Francke-Grosmann, H. (1967). “Ectosymbiosis in wood-inhabiting insects,” in Symbiosis, ed. S. M. Henry (New York: Academic Press), 141–205. Available at: https://scholar.google.com/scholar?hl=en&q=ambrosia+fungus+storage+in+two+species&btnG=&as_sdt=1%2C16&as_sdtp=#3.

Francke-Grosmann, H. (1975). Zur epizoischen und endozoischen Übertragung der symbiotischen Pilze des Ambrosiakäfers *Xyleborus saxeseni* (Coleoptera: Scolytidae). Entomol. Ger. 1, 279–292.

French, J. R. J., and Roeper, R. A. (1972). Interactions of the ambrosia beetle, *Xyleborus dispar* (Coleoptera: Scolytidae), with its symbiotic fungus *Ambroszella hartzgii* (Fungi Imperfecti). Can. Entomol. 104, 1635–1641. doi: 10.4039/Ent1041635-10.

Friesen, M. L., Porter, S. S., Stark, S. C., von Wettberg, E. J., Sachs, J. L., and Martinez-Romero, E. (2011). Microbially Mediated Plant Functional Traits. Annu. Rev. Ecol. Evol. Syst. 42, 23–46. doi: 10.1146/annurev-ecolsys-102710-145039.

Fuller, D. Q., and Stevens, C. J. (2017). Open for competition: Domesticates, parasitic domesticoids and the agricultural niche. Archaeol. Int., 20 pp. 110-121. 20. doi: 10.5334/AI.359.

Gerardo, N. M., and Parker, B. J. (2014). Mechanisms of symbiont-conferred protection against natural enemies: An ecological and evolutionary framework. Curr. Opin. Insect Sci. 4, 8–14. doi: 10.1016/j.cois.2014.08.002.

Gloor, G. B., Macklaim, J. M., Pawlowsky-Glahn, V., and Egozcue, J. J. (2017). Microbiome datasets are compositional: And this is not optional. Front. Microbiol. 8, 2224. doi: 10.3389/FMICB.2017.02224/BIBTEX.

Goes, A. C., Barcoto, M. O., Kooij, P. W., Bueno, O. C., and Rodrigues, A. (2020). How Do Leaf-Cutting Ants Recognize Antagonistic Microbes in Their Fungal Crops? Front. Ecol. Evol. 8, 1–12. doi: 10.3389/fevo.2020.00095.

Goh, C. H., Veliz Vallejos, D. F., Nicotra, A. B., and Mathesius, U. (2013). The Impact of Beneficial Plant-Associated Microbes on Plant Phenotypic Plasticity. J. Chem. Ecol. 39, 826–839. doi: 10.1007/s10886-013-0326-8.

Green, P. W. C., and Kooij, P. W. (2018). The role of chemical signalling in maintenance of the fungus garden by leaf-cutting ants. Chemoecology 28, 101–107. doi: 10.1007/S00049-018-0260-X/METRICS.

Griffiths, H. M., and Hughes, W. O. H. (2010). Hitchhiking and the removal of microbial contaminants by the leaf-cutting ant *Atta colombica*. Ecol. Entomol. 35, 529–537. doi: 10.1111/J.1365-2311.2010.01212.X.

Groth, I., Schumann, P., Rainey, F. A., Martin, K., Schuetze, B., and Augsten, K. (1997). *Demetria terragena* gen. nov., sp. nov., a new genus of actinomycetes isolated from compost soil. Int. J. Syst. Bacteriol. 47, 1129–1133. doi: 10.1099/00207713-47-4-1129.

Grubbs, K. J., Surup, F., Biedermann, P. H. W., McDonald, B. R., Klassen, J. L., Carlson, C. M., et al. (2020). Cycloheximide-producing Streptomyces associated with *Xyleborinus saxesenii* and *Xyleborus affinis* fungus-farming ambrosia beetles. Front. Microbiol. 11, 1–12. doi: 10.3389/fmicb.2020.562140.

Hamilton, W. D. (1964). The genetical evolution of social behaviour. I. J. Theor. Biol. 7, 1–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/5875341.

Hanula, J. L., Mayfield, A. E., Fraedrich, S. W., and Rabaglia, R. J. (2008). Biology and Host Associations of Redbay Ambrosia Beetle (Coleoptera: Curculionidae: Scolytinae), Exotic Vector of Laurel Wilt Killing Redbay Trees in the Southeastern United States. J. Econ. Entomol. 101, 1276–1286. doi: 10.1093/JEE/101.4.1276.

Harrington, T. C. (2005). “Ecology and evolution of mycophagous bark beetles and their fungal partners,” in Ecological and evolutionary advances in insect-fungal associations, eds. F. E. Vega and M. Blackwell (Oxford: Oxford University Press), 257–291.

Hartig, F. (2021). DHARMa: Residual diagnostics for hierarchical (multi-level / mixed) regression models. Available at: https://cran.r-project.org/package=DHARMa.

Haughland, D. L., and Larsen, K. W. (2004). Exploration correlates with settlement: Red squirrel dispersal in contrasting habitats. Source J. Anim. Ecol. 73, 1024–1034. Available at: https://about.jstor.org/terms.

Heg, D., Bachar, Z., Brouwer, L., and Taborsky, M. (2004). Predation risk is an ecological constraint for helper dispersal in a cooperatively breeding cichlid. Proc. R. Soc. B Biol. Sci. 271, 2367–2374. doi: 10.1098/rspb.2004.2855.

Herz, H., Hölldobler, B., and Roces, F. (2008). Delayed rejection in a leaf-cutting ant after foraging on plants unsuitable for the symbiotic fungus. Behav. Ecol. 19, 575–582. doi: 10.1093/BEHECO/ARN016.

Hibbing, M. E., Fuqua, C., Parsek, M. R., and Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. Nat. Rev. Microbiol. 8, 15. doi: 10.1038/NRMICRO2259.

Hoppe, B., Krüger, D., Kahl, T., Arnstadt, T., Buscot, F., Bauhus, J., et al. (2015). A pyrosequencing insight into sprawling bacterial diversity and community dynamics in decaying deadwood logs of *Fagus sylvatica* and *Picea abies*. Sci. Reports 2015 51 5, 1–9. doi: 10.1038/srep09456.

Hosking, G. P. (1972). *Xyleborus saxeseni*, its life-history and flight behaviour in New Zealand. New Zeal. J. For. Sci. 3, 37–53.

Hubbard, H. G. (1897). The ambrosia beetles of the United States., ed. L. O. Howard Washington, DC: US department of agriculture bureau of entomology.

Hughes, C. L., Dytham, C., and Hill, J. K. (2007). Modelling and analysing evolution of dispersal in populations at expanding range boundaries. Ecol. Entomol. 32, 437–445. doi: 10.1111/J.1365-2311.2007.00890.X.

Hughes, D. P., Pierce, N. E., and Boomsma, J. J. (2008). Social insect symbionts: evolution in homeostatic fortresses. Trends Ecol. Evol. 23, 672–677. doi: 10.1016/j.tree.2008.07.011.

Hulcr, J., Mann, R., and Stelinski, L. L. (2011). The scent of a partner: ambrosia beetles are attracted to volatiles from their fungal symbionts. J. Chem. Ecol. doi: 10.1007/s10886-011-0046-x.

Hulcr, J., and Stelinski, L. L. (2017). The ambrosia symbiosis: From evolutionary ecology to practical management. Annu. Rev. Entomol. 62, 285–303. doi: 10.1146/annurev-ento-031616-035105.

Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J. H., Chinwalla, A. T., et al. (2012). Structure, function and diversity of the healthy human microbiome. Nat. 2012 4867402 486, 207–214. doi: 10.1038/nature11234.

Ibarra-Juarez, L. A., Burton, M. A. J., Biedermann, P. H. W., Cruz, L., Desgarennes, D., Ibarra-Laclette, E., et al. (2020). Evidence for succession and putative metabolic roles of fungi and bacteria in the farming mutualism of the ambrosia beetle *Xyleborus affinis*. mSystems 5, e00541-00520. doi: 10.1128/MSYSTEMS.00541-20/ASSET/527FF6DB-D4E5-4920-8F5B-3208E1531817/ASSETS/GRAPHIC/MSYSTEMS.00541-20-F0010.JPEG.

Johnston, S. R., Hiscox, J., Savoury, M., Boddy, L., and Weightman, A. J. (2019). Highly competitive fungi manipulate bacterial communities in decomposing beech wood (*Fagus sylvatica*). FEMS Microbiol. Ecol. 95, 225. doi: 10.1093/FEMSEC/FIY225.

Jong, Z. W., Kassim, N. F. A., Naziri, M. A., and Webb, C. E. (2017). The effect of inbreeding and larval feeding regime on immature development of *Aedes albopictus*. J. Vector Ecol. 42, 105–112. doi: 10.1111/JVEC.12244.

Jordal, B. H. (2002). Elongation Factor 1 α resolves the monophyly of the haplodiploid ambrosia beetles Xyleborini (Coleoptera: Curculionidae). Insect Mol. Biol. 11, 453–465. doi: 10.1046/j.1365-2583.2002.00354.x.

Joseph, R., and Keyhani, N. O. (2021). Fungal mutualisms and pathosystems: life and death in the ambrosia beetle mycangia.

Joy, J. B. (2013). Symbiosis catalyses niche expansion and diversification. Proc. R. Soc. B Biol. Sci. 280. doi: 10.1098/RSPB.2012.2820.

Käärik, A. A. (1974). “Decomposition of wood,” in Biology of Plant Litter Decomposition, eds. C. H. Dickinson and G. J. F. Pugh (Academic Press, London), 129–174.

Kajimura, H., and Hijii, N. (1992). Dynamics of the fungal symbionts in the gallery system and the mycangia of the ambrosia beetle, *Xylosandrus mutilatus* (Blandford) (Coleoptera: Scolytidae) in relation to its life history. Ecol. Res. 7, 107–117. doi: 10.1007/BF02348489.

Kandasamy, D., Gershenzon, J., Andersson, M. N., and Hammerbacher, A. (2019). Volatile organic compounds influence the interaction of the Eurasian spruce bark beetle *(Ips typographus*) with its fungal symbionts. ISME J. 13, 1788–1800. doi: 10.1038/s41396-019-0390-3.

Kandasamy, D., Gershenzon, J., and Hammerbacher, A. (2016). Volatile organic compounds emitted by fungal associates of conifer bark beetles and their potential in bark beetle control. J. Chem. Ecol. 42, 952–969. doi: 10.1007/s10886-016-0768-x.

Kassambara, A. (2020). ggpubr: “ggplot2” based publication ready plots. Available at: https://cran.r-project.org/package=ggpubr.

Kasson, M. T., Wickert, K. L., Stauder, C. M., Macias, A. M., Berger, M. C., Simmons, D. R., et al. (2016). Mutualism with aggressive wood-degrading *Flavodon ambrosius* (Polyporales) facilitates niche expansion and communal social structure in Ambrosiophilus ambrosia beetles. Fungal Ecol. 23, 86–96. doi: 10.1016/J.FUNECO.2016.07.002.

Katariya, L., Ramesh, P. B., Gopalappa, T., Desireddy, S., Bessière, J. M., and Borges, R. M. (2017). Fungus-farming termites selectively bury weedy fungi that smell different from crop fungi. J. Chem. Ecol. 43, 986–995. doi: 10.1007/s10886-017-0902-4.

Kent, D. S., and Simpson, J. A. (1992). Eusociality in the beetle *Austroplatypus incompertus* (Coleoptera: Curculionidae). Naturwissenschaften 79, 86–87.

Kingsolver, J. G., and Norris, D. M. (1977). The interaction of *Xyleborus ferrugineus* (Coleoptera: Scolytidae) behavior and initial reproduction in relation to its symbiotic fungi. Ann. Entomol. Soc. Am. 70, 1–4.

Kirkendall, L. R., Biedermann, P. H. W., and Jordal, B. H. (2015). “Evolution and diversity of bark and ambrosia beetles,” in Bark beetles: Biology and ecology of native and invasive species (Elsevier Academic Press), 85–156. doi: 10.1016/B978-0-12-417156-5.00003-4.

Kirkendall, L. R., Kent, D. S., and Raffa, K. F. (1997). “Interactions among males, females and offspring in bark and ambrosia beetles: the significance of

living in tunnels for the evolution of social behavior," in The evolution of social behavior in insects and arachnids, 181–215.

Knapp, J. J., Howse, P. E., and Kermarrec, A. (1990). "Factors controlling foraging patterns in the leaf-cutting ant *Acromyrmex octospinosus* (Reich)," in Applied Myrmecology: A World Perspective, eds. R. K. Vander Meer, K. Jaffe, and A. Cedeno (Westview, Boulder, CO), 382–409. doi: 10.1201/9780429043109-45.

Koenig, W. D., Pitelka, F. A., Carmen, W. J., Mumme, R. L., and Stanback, M. T. (1992). The Evolution of Delayed Dispersal in Cooperative Breeders. https://doi.org/10.1086/417552 67, 111–150. doi: 10.1086/417552.

Kok, L. T., Norris, D. M., and Chu, H. M. (1970). Sterol metabolism as a basis for a mutualistic symbiosis. Nature 225, 661–662. doi: 10.1038/225661b0.

Kolařík, M., Hulcr, J., and Kirkendall, L. R. (2015). New species of Geosmithia and Graphium associated with ambrosia beetles in Costa Rica. Czech Mycol. 67, 29–35. Available at: http://www.researchgate.net/profile/Miroslav%7B_%7DKolarik/publication/276293398%7B_%7DNew%7B_%7Dspecies%7B_%7Dof%7B_%7DGeosmithia%7B_%7Dand%7B_%7DGraphium%7B_%7Dassociated%7B_%7Dwith%7B_%7Dambrosia%7B_%7Dbeetles%7B_%7Din%7B_%7DCosta%7B_%7DRica/links/5556.

Kölliker-Ott, U. M., Bigler, F., and Hoffmann, A. A. (2003). Does mass rearing of field collected *Trichogramma brassicae* wasps influence acceptance of European corn borer eggs? Entomol. Exp. Appl. 109, 197–203. doi: 10.1046/J.0013-8703.2003.00104.X.

Konrad, M., Vyleta, M. L., Theis, F. J., Stock, M., Tragust, S., Klatt, M., et al. (2012). Social transfer of pathogenic fungus promotes active immunisation in ant colonies. PLoS Biol. 10, e1001300. doi: 10.1371/journal.pbio.1001300.

Kostovcik, M., Bateman, C. C., Kolarik, M., Stelinski, L. L., Jordal, B. H., and Hulcr, J. (2014). The ambrosia symbiosis is specific in some species and promiscuous in others: evidence from community pyrosequencing. ISME J. 9, 126–138. doi: 10.1038/ismej.2014.115.

Kostovcik, M., Bateman, C. C., Kolarik, M., Stelinski, L. L., Jordal, B. H., and Hulcr, J. (2015). The ambrosia symbiosis is specific in some species and promiscuous in others: Evidence from community pyrosequencing. ISME J. 9, 126–138. doi: 10.1038/ismej.2014.115.

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., and Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl. Environ. Microbiol. 79, 5112–5120. doi: 10.1128/AEM.01043-13.

Krokene, P. (2015). Conifer defense and resistance to bark beetles. Bark Beetles Biol. Ecol. Nativ. Invasive Species, 177–207. doi: 10.1016/B978-0-12-417156-5.00005-8.

Kukor, J. J., and Martin, M. M. (1983). Acquisition of digestive enzymes by siricid woodwasps from their fungal symbiont. Science. 220, 1161–1163. doi: 10.1126/SCIENCE.220.4602.1161.

Lahti, L., and Shetty, S. (2019). microbiome R package. Available at: http://microbiome.github.io.

Lamberty, M., Zachary, D., Lanot, R., Bordereau, C., Robert, A., Hoffmann, J. A., et al. (2001). Insect immunity: Constitutive expression of a cysteine-rich antifungal and a linear antibacterial peptide in a termite insect. J. Biol. Chem. 276, 4085–4092. doi: 10.1074/JBC.M002998200.

Lenth, R. V. (2021). emmeans: Estimated marginal means, aka least-squares means. Available at: https://cran.r-project.org/package=emmeans.

Leppla, N. C., Huettel, M. D., Chambers, D. L., and Turner, W. K. (1976). Comparative life history and respiratory activity of “wild” and colonized Caribbean fruit flies [Dipt.: Tephritidae]. Entomophaga 21, 353–357. doi: 10.1007/BF02371633.

Liedo, P., Salgado, S., Oropeza, A., and Toledo, J. (2007). Improving mating performance of mass-reared sterile mediterranean fruit flies (Diptera: Tephritidae) through changes in adult holding conditions: Demography and mating competitiveness. Florida Entomol. 90, 33–40. doi: 10.1653/0015-4040(2007)90[33:IMPOMS]2.0.CO;2.

Lin, Y. K., Keane, B., Isenhour, A., and Solomon, N. G. (2006). Effects of patch quality on dispersal and social organization of prairie voles: An experimental approach. J. Mammal. 87, 446–453. doi: 10.1644/05-MAMM-A-201R1.1.

Lladó, S., López-Mondéjar, R., and Baldrian, P. (2017). Forest Soil Bacteria: Diversity, Involvement in Ecosystem Processes, and Response to Global Change. Microbiol. Mol. Biol. Rev. 81. doi: 10.1128/mmbr.00063-16.

Lucas, J. A., García-Cristobal, J., Bonilla, A., Ramos, B., and Gutierrez-Mañero, J. (2014). Beneficial rhizobacteria from rice rhizosphere confers high protection against biotic and abiotic stress inducing systemic resistance in rice seedlings. Plant Physiol. Biochem. 82, 44–53. doi: 10.1016/j.plaphy.2014.05.007.

Lüdecke, D., Ben-Shachar, M. S., Patil, I., Waggoner, P., and Makowski, D. (2021). performance: An R package for assessment, comparison and testing of statistical models. J. Open Source Softw. 6, 3139. doi: 10.21105/joss.03139.

Luna, E., Cranshaw, W., and Tisserat, N. (2014). Attraction of Walnut Twig Beetle *Pityophthorus juglandis* (Coleoptera: Curculionidae) to the Fungus *Geosmithia morbida*. Plant Heal. Prog. 15, 135–140. doi: 10.1094/php-rs-14-0001.

Lutzoni, F., and Pagel, M. (1997). Accelerated evolution as a consequence of transitions to mutualism. Proc. Natl. Acad. Sci. 94, 11422–11427. doi: 10.1073/pnas.94.21.11422.

Malacrinò, A., Rassati, D., Schena, L., Mehzabin, R., Battisti, A., and Palmeri, V. (2017). Fungal communities associated with bark and ambrosia beetles trapped at international harbours. Fungal Ecol. 28, 44–52. doi: 10.1016/j.funeco.2017.04.007.

Mangiafico, S. (2021). rcompanion: Functions to support extension education program evaluation. Available at: https://cran.r-project.org/package=rcompanion.

Martin, M. M. (1979). Biochemical implications of insect mycophagy. Biol. Rew 54, 1–21.

Martin, M. M. (1992). The evolution of insect-fungus associations: From contact to stable symbiosis. Integr. Comp. Biol. 32, 593–605. doi: 10.1093/ICB/32.4.593.

Martinez Arbizu, P. (2020). pairwiseAdonis: Pairwise multilevel comparison using adonis.

Matos, M., Rose, M. R., Rocha Pité, M. T., Rego, C., and Avelar, T. (2000). Adaptation to the laboratory environment in *Drosophila subobscura*. J. Evol. Biol. 13, 9–19. doi: 10.1046/J.1420-9101.2000.00116.X.

Mayers, C. G., Harrington, T. C., and Biedermann, P. H. W. (2018). Mycangia Define the Diverse Ambrosia Beetle–-Fungus Symbioses. 105–142.

Mayers, C. G., Harrington, T. C., and Biedermann, P. H. W. (2022). “Mycangia define the diverse ambrosia beetle–fungus symbioses,” in The convergent evolution of agriculture in humans and insects (The MIT Press). doi: 10.7551/mitpress/13600.003.0013.

Mayers, C. G., Harrington, T. C., Mcnew, D. L., Roeper, R. A., Biedermann, P. H. W., Masuya, H., et al. (2020). Four mycangium types and four genera of ambrosia fungi suggest a complex history of fungus farming in the ambrosia beetle tribe Xyloterini. https://doi.org/10.1080/00275514.2020.1755209 112, 1104–1137. doi: 10.1080/00275514.2020.1755209.

Mayers, C. G., McNew, D. L., Harrington, T. C., Roeper, R. A., Fraedrich, S. W., Biedermann, P. H. W., et al. (2015). Three genera in the Ceratocystidaceae are the respective symbionts of three independent lineages of ambrosia beetles with large, complex mycangia. Fungal Biol. 119, 1075–1092. doi: 10.1016/j.funbio.2015.08.002.

Mburu, D. M., Maniania, N. K., and Hassanali, a (2013). Comparison of Volatile Blends and Nucleotide Sequences of Two *Beauveria bassiana* Isolates of Different Virulence and Repellency Towards the Termite *Macrotermes michealseni*. J. Chem. Ecol. 39, 101–108. doi: 10.1007/s10886-012-0207-6.

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217. doi: 10.1371/journal.pone.0061217.

Meats, A., Holmes, H. M., and Kelly, G. L. (2004). Laboratory adaptation of *Bactrocera tryoni* (Diptera: Tephritidae) decreases mating age and increases protein consumption and number of eggs produced per milligram of protein. Bull. Entomol. Res. 94, 517–524. doi: 10.1079/BER2004332.

Mehdiabadi, N. J., Hughes, B., and Mueller, U. G. (2006). Cooperation, conflict, and coevolution in the attine ant-fungus symbiosis. Behav. Ecol. 17, 291–296. doi: 10.1093/BEHECO/ARJ028.

Merckx, T., and Van Dyck, H. (2007). Habitat fragmentation affects habitat-finding ability of the speckled wood butterfly, *Pararge aegeria* L. Anim. Behav. 74, 1029–1037. doi: 10.1016/J.ANBEHAV.2006.12.020.

Meunier, J. (2015). Social immunity and the evolution of group living in insects. Philos. Trans. R. Soc. B Biol. Sci. 370, 20140102–20140102. doi: 10.1098/rstb.2014.0102.

Miadlikowska, J., Kauff, F., Högnabba, F., Oliver, J. C., Molnár, K., Fraker, E., et al. (2014). A multigene phylogenetic synthesis for the class Lecanoromycetes (Ascomycota): 1307 fungi representing 1139 infrageneric taxa, 317 genera and 66 families. Mol. Phylogenet. Evol. 79, 132–168. doi: 10.1016/j.ympev.2014.04.003.

Mighell, K., and Van Bael, S. A. (2016). Selective elimination of microfungi in leaf-cutting ant gardens. Fungal Ecol. 24, 15–20. doi: 10.1016/J.FUNECO.2016.08.009.

Milne, D. H., and Giese, R. L. (1970). The columbian Timber Beetle, *Corthylus columbianus* (Coleoptera: Scolytidae). X. Comparison of Yearly Mortality and Dispersal Losses with Population Densities. Entomol. News, 12–24.

Milner, R. J., Staples, J. A., and Lutton, G. G. (1998). The Selection of an Isolate of the Hyphomycete Fungus, *Metarhizium anisopliae*, for Control of Termites in Australia. Biol. Control 11, 240–247. doi: 10.1006/BCON.1997.0574.

Morales-Rodríguez, C., Sferrazza, I., Aleandri, M. P., Dalla Valle, M., Speranza, S., Contarini, M., et al. (2021). The fungal community associated with the ambrosia beetle *Xylosandrus compactus* invading the mediterranean maquis in central Italy reveals high biodiversity and suggests environmental acquisitions. Fungal Biol. 125, 12–24. doi: 10.1016/j.funbio.2020.09.008.

Morowitz, M. J., Carlisle, E. M., and Alverdy, J. C. (2011). Contributions of Intestinal Bacteria to Nutrition and Metabolism in the Critically Ill. Surg. Clin. North Am. 91, 771–785. doi: 10.1016/j.suc.2011.05.001.

Mueller, U. G., Gerardo, N. M., Aanen, D. K., Six, D. L., and Schultz, T. R. (2005). The evolution of agriculture in insects. Annu. Rev. Ecol. Evol. Syst. 36, 563–595. doi: 10.1146/annurev.ecolsys.36.102003.152626.

Mueller, U. G., Poulin, J., and Adams, R. M. M. (2004). Symbiont choice in a fungus-growing ant (Attini, Formicidae). Behav. Ecol. 15, 357–364. doi: 10.1093/BEHECO/ARH020.

Mueller, U. G., and Sachs, J. L. (2015). Engineering microbiomes to improve plant and animal health. Trends Microbiol. 23, 606–617. doi: 10.1016/J.TIM.2015.07.009.

Mueller, U. G., Scott, J. J., Ishak, H. D., Cooper, M., and Rodrigues, A. (2010). Monoculture of leafcutter ant gardens. PLoS One 5, e12668. doi: 10.1371/JOURNAL.PONE.0012668.

Mullon, C., Keller, L., and Lehmann, L. (2018). Social polymorphism is favoured by the co-evolution of dispersal with social behaviour. Nat. Ecol. Evol. 2, 132–140. doi: 10.1038/s41559-017-0397-y.

Myles, T. G. (2002). Alarm, aggregation, and defense by *Reticulitermes flavipes* in response to a naturally occurring isolate of *Metarhizium anisopliae*. Sociobiology 40, 243–255.

Nakashima, T., Iizuka, T., Ogura, K., Maeda, M., and Tanaka, T. (1982). Isolation of some microorganisms associated with five species of ambrosia beetles and two kinds of antibiotics produced by Xv-3 strain in these isolates. J. Fac. Agric. Hokkaido Univ. 61, 60–72. Available at: http://hdl.handle.net/2115/12972.

Neger, F. W. (1908). Die Pilzkulturen der Nutzholzborkenkäfer. Zentralblatt für Bakteriol. Jena Abt II 20, 279–282.

Nones, S., Fernandes, C., Duarte, L., Cruz, L., and Sousa, E. (2022). Bacterial community associated with the ambrosia beetle *Platypus cylindrus* on declining Quercus suber trees in the Alentejo region of Portugal. Plant Pathol. 71, 966–979. doi: 10.1111/ppa.13536.

Norberg, U., Enfjäll, K., and Leimar, O. (2002). Habitat exploration in butterflies - An outdoor cage experiment. Evol. Ecol. 16, 1–14. doi: 10.1023/A:1016007521178/METRICS.

Norris, D. M. (1979). “The Mutualistic Fungi of xyleborini Beetles.,” in Insect-Fungus Symbiosis, 53–63.

North, R. D., Jackson, C. W., and Howse, P. E. (1999). Communication between the fungus garden and workers of the leaf-cutting ant, *Atta sexdens rubropilosa*, regarding choice of substrate for the fungus. Physiol. Entomol. 24, 127–133. doi: 10.1046/J.1365-3032.1999.00122.X.

Nuotclà, J. A., Biedermann, P. H. W., and Taborsky, M. (2019). Pathogen defence is a potential driver of social evolution in ambrosia beetles. Proc. R. Soc. B Biol. Sci. 286, 1–9. doi: 10.1098/rspb.2019.2332.

Nuotclà, J. A., Diehl, J. M. C., and Taborsky, M. (2021). Habitat quality determines dispersal decisions and fitness in a beetle-fungus mutualism. Front. Ecol. Evol. 9, 1–15. doi: 10.3389/fevo.2021.602672.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2020). vegan: Community ecology package.

Pagnocca, F. C., Masiulionis, V. E., and Rodrigues, A. (2012). Specialized fungal parasites and opportunistic fungi in gardens of attine ants. Psyche (London). doi: 10.1155/2012/905109.

Papen, H., and Werner, D. (1979). N2-fixation in *Erwinia herbicola*. Arch. Microbiol. 120, 25–30. doi: 10.1007/BF00413267.

Peer, K., and Taborsky, M. (2004). Female ambrosia beetles adjust their offspring sex ratio according to outbreeding opportunities for their sons. J. Evol. Biol. 17, 257–264. doi: 10.1111/j.1420-9101.2003.00687.x.

Peer, K., and Taborsky, M. (2007). Delayed dispersal as a potential route to cooperative breeding in ambrosia beetles. Behav. Ecol. Sociobiol. 61, 729–739. doi: 10.1007/s00265-006-0303-0.

Pie, M. R., Rosengaus, R. B., and Traniello, J. F. A. (2004). Nest architecture, activity pattern, worker density and the dynamics of disease transmission in social insects. J. Theor. Biol. 226, 45–51. doi: 10.1016/j.jtbi.2003.08.002.

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C. M., and Bakker, P. A. H. M. (2014). Induced systemic resistance by beneficial microbes. Annu. Rev. Phytopathol. 52, 347–375. doi: 10.1146/annurev-phyto-082712-102340.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., and R Core Team (2021). _nlme: Linear and nonlinear mixed effects models_. Available at: https://cran.r-project.org/package=nlme.

Piñol, J., Senar, M. A., and Symondson, W. O. C. (2019). The choice of universal primers and the characteristics of the species mixture determine when DNA metabarcoding can be quantitative. Mol. Ecol. 28, 407–419. doi: 10.1111/MEC.14776.

Poulsen, M., and Boomsma, J. J. (2005). Mutualistic fungi control crop diversity in fungus-growing ants. Science 307, 741–744. doi: 10.1126/SCIENCE.1106688.

Poulsen, M., Bot, A. N. M., Currie, C. R., and Boomsma, J. J. (2002). Mutualistic bacteria and a possible trade-off between alternative defence mechanisms in Acromyrmex leaf-cutting ants. Insectes Soc. 49, 15–19. doi: 10.1007/S00040-002-8271-5.

R Core Team (2021). R: A language and environment for statistical computing.

Rassati, D., Marini, L., and Malacrinò, A. (2019). Acquisition of fungi from the environment modifies ambrosia beetle mycobiome during invasion. PeerJ 2019, e8103. doi: 10.7717/PEERJ.8103/SUPP-5.

Redman, R. S., Sheehan, K. B., Stout, R. G., Rodriguez, R. J., and Henson, J. M. (2002). Thermotolerance generated by plant/fungal symbiosis. Science. 298, 1581. doi: 10.1126/science.1072191.

Richard, F. J., Poulsen, M., Drijfhout, F., Jones, G., and Boomsma, J. J. (2007). Specificity in chemical profiles of workers, brood and mutualistic fungi in Atta, Acromyrmex, and Sericomyrmex fungus-growing ants. J. Chem. Ecol. 33, 2281–2292. doi: 10.1007/S10886-007-9385-Z/FIGURES/2.

Ridley, P., Howse, P. E., and Jackson, C. W. (1996). Control of the behaviour of leaf-cutting ants by their “symbiotic” fungus. Experientia 52, 631–635. doi: 10.1007/BF01969745/METRICS.

Rocha, S. L., Evans, H. C., Jorge, V. L., Cardoso, L. A. O., Pereira, F. S. T., Rocha, F. B., et al. (2017). Recognition of endophytic Trichoderma species by leaf-cutting ants and their potential in a Trojan-horse management strategy. R. Soc. Open Sci. 4. doi: 10.1098/RSOS.160628.

Roeper, R. A., Treeful, L. M., Foote, R. A., and Bunce, M. A. (1980a). In Vitro Culture of the Ambrosia Beetle *Xyleborus affinis* (Coleoptera: Scolytidae). Gt. Lakes Entomol. 13, 33–35.

Roeper, R. A., Treeful, L. M., O’brien, K. M., Foote, R. A., and Bunce, M. A. (1980b). Life history of the ambrosia beetle *Xyleborus affinis* (Coleoptera: Scolytidae) from in vitro culture. Gt. Lakes Entomol. 13, 141–143.

Römer, D., Aguilar, G. P., Meyer, A., and Roces, F. (2022). Symbiont demand guides resource supply: leaf-cutting ants preferentially deliver their harvested fragments to undernourished fungus gardens. Sci. Nat. 109, 1–5. doi: 10.1007/S00114-022-01797-7/TABLES/1.

Rosengaus, R. B., Guldin, M. R., and Traniello, J. F. A. (1998). Inhibitory effect of termite fecal pellets on fungal spore germination. J. Chem. Ecol. 1998 2410 24, 1697–1706. doi: 10.1023/A:1020872729671.

Rosengaus, R. B., Jordan, C., Lefebvre, M. L., and Traniello, J. F. A. (1999). Pathogen alarm behavior in a termite: A new form of communication in social insects. Naturwissenschaften 1999 8611 86, 544–548. doi: 10.1007/S001140050672.

Rosengaus, R. B., and Traniello, J. F. A. (2001). Disease susceptibility and the adaptive nature of colony demography in the dampwood termite *Zootermopsis angusticollis*. Behav. Ecol. Sociobiol. 50, 546–556. doi: 10.1007/s002650100394.

Roy, B. A., and Kirchner, J. W. (2000). Evolutionary dynamics of pathogen resistance and tolerance. Evolution (N. Y). 54, 51–63. doi: 10.1111/J.0014-3820.2000.TB00007.X.

Royce, L. A., Rossignol, P. A., Burgett, D. M., and Stringer, B. A. (1991). Reduction of tracheal mite parasitism of honey bees by swarming. Philos. Trans. R. Soc. London. Ser. B Biol. Sci. 331, 123–129. doi: 10.1098/RSTB.1991.0003.

Sarkar, D. (2008). Lattice: Multivariate data visualization with R. New York: Springer.

Saucedo-Carabez, J. R., Ploetz, R. C., Konkol, J. L., Carrillo, D., and Gazis, R. (2018). Partnerships between ambrosia beetles and fungi: Lineage-specific promiscuity among vectors of the laurel wilt pathogen, *Raffaelea lauricola*. Microb. Ecol. doi: 10.1007/s00248-018-1188-y.

Saunders, J. L., and Knoke, J. K. (1967). Diets for rearing the ambrosia beetle *Xyleborus ferrugineus* (Fabricius) in vitro. Science. 157, 460–463. doi: 10.1126/science.157.3787.460.

Saverschek, N., Herz, H., Wagner, M., and Roces, F. (2010). Avoiding plants unsuitable for the symbiotic fungus: learning and long-term memory in leaf-cutting ants. Anim. Behav. 79, 689–698. doi: 10.1016/J.ANBEHAV.2009.12.021.

Saverschek, N., and Roces, F. (2011). Foraging leafcutter ants: olfactory memory underlies delayed avoidance of plants unsuitable for the symbiotic fungus. Anim. Behav. 82, 453–458. doi: 10.1016/J.ANBEHAV.2011.05.015.

Schmidt, S., Kildgaard, S., Guo, H., Beemelmanns, C., and Poulsen, M. (2022). The chemical ecology of the fungus-farming termite symbiosis. Nat. Prod. Rep. 39, 231–248. doi: 10.1039/D1NP00022E.

Schooley, R. L., Wiens Schooley, J. A., Schooley, R. L., and Wiens, J. A. (2003). Finding habitat patches and directional connectivity. Oikos 102, 559–570. doi: 10.1034/J.1600-0706.2003.12490.X.

Schultz, T. R. (2022). “The convergent evolution of agriculture in humans and fungus-farming ants,” in The Convergent Evolution of Agriculture in Humans and Insects, 281–313. doi: 10.7551/mitpress/13600.003.0021.

Scott, J. J., Oh, D.-C., Yuceer, M. C., Klepzig, K. D., Clardy, J., and Currie, C. R. (2008). Bacterial protection of beetle-fungus mutualism. Science. 322, 63. doi: 10.1126/science.1160423.Bacterial.

Seibold, S., Müller, J., Baldrian, P., Cadotte, M. W., Štursová, M., Biedermann, P. H. W., et al. (2019). Fungi associated with beetles dispersing from dead wood – Let’s take the beetle bus! Fungal Ecol. 39, 100–108. doi: 10.1016/J.FUNECO.2018.11.016.

Shigo, A. L. (1967). Successions of organisms in discoloration and decay of wood. Int. Rev. For. 2, 237–299. doi: 10.1016/B978-1-4831-9976-4.50012-1.

Shinzato, N., Muramatsu, M., Watanabe, Y., and Matsui, T. (2005). Termite-regulated fungal monoculture in fungus combs of a macrotermitine termite *Odontotermes formosanus*. Zoolog. Sci. 22, 917–922. doi: 10.2108/ZSJ.22.917.

Six, D. L., and Bentz, B. J. (2007). Temperature determines symbiont abundance in a multipartite bark beetle-fungus ectosymbiosis. Microb. Ecol. 54, 112–118. doi: 10.1007/S00248-006-9178-X/FIGURES/2.

Skelton, J., Johnson, A. J., Jusino, M. A., Bateman, C. C., Li, Y., and Hulcr, J. (2019a). A selective fungal transport organ (mycangium) maintains coarse

phylogenetic congruence between fungus-farming ambrosia beetles and their symbionts. Proc. R. Soc. B Biol. Sci. 286, 1–9. doi: 10.1098/rspb.2018.2127.

Skelton, J., Jusino, M. A., Carlson, P. S., Smith, K., Banik, M. T., Lindner, D. L., et al. (2019b). Relationships among wood-boring beetles, fungi, and the decomposition of forest biomass. Mol. Ecol. 28, 4971–4986. doi: 10.1111/mec.15263.

Slowikowski, K. (2021). ggrepel: Automatically position non-overlapping text labels with “ggplot2”. Available at: https://cran.r-project.org/package=ggrepel.

Stacey, P. B. (1979). Habitat saturation and communal breeding in the acorn woodpecker. Anim. Behav. 27, 1153–1166. doi: 10.1016/0003-3472(79)90063-0.

Sun, X., Wang, J., Fang, C., Li, J., Han, M., Wei, X., et al. (2020). Efficient and stable metabarcoding sequencing from DNBSEQ-G400 sequencer examined by large fungal community analysis. bioRxiv, 2020.07.02.185710. doi: 10.1101/2020.07.02.185710.

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., and Willerslev, E. (2012). Towards next-generation biodiversity assessment using DNA metabarcoding. Mol. Ecol. 21, 2045–2050. doi: 10.1111/j.1365-294X.2012.05470.x.

Taborsky, M., Frommen, J. G., and Riehl, C. (2016). Correlated pay-offs are key to cooperation. Philos. Trans. R. Soc. B Biol. Sci. 371. doi: 10.1098/RSTB.2015.0084.

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N. S., Wijesundera, R., et al. (2014). Global diversity and geography of soil fungi. Science. 346, 1256688. doi: 10.1126/SCIENCE.1256688/SUPPL_FILE/TEDERSOO-SM.PDF.

Tedersoo, L., Bahram, M., Zinger, L., Nilsson, R. H., Kennedy, P. G., Yang, T., et al. (2022). Best practices in metabarcoding of fungi: From experimental design to results. Mol. Ecol. 31, 2769–2795. doi: 10.1111/MEC.16460.

Tedersoo, L., and Lindahl, B. (2016). Fungal identification biases in microbiome projects. Environ. Microbiol. Rep. 8, 774–779. doi: 10.1111/1758-2229.12438.

Thompson, B. M., Bodart, J., McEwen, C., and Gruner, D. S. (2014). Adaptations for symbiont-mediated external digestion in *Sirex noctilio* (Hymenoptera: Siricidae). Ann. Entomol. Soc. Am. 107, 453–460. doi: 10.1603/AN13128.

Tranter, C., Lefevre, L., Evison, S. E. F., and Hughes, W. O. H. (2015). Threat detection: contextual recognition and response to parasites by ants. Behav. Ecol. 26, 396–405. doi: 10.1093/BEHECO/ARU203.

Ulyshen, M. D. (2016). Wood decomposition as influenced by invertebrates. Biol. Rev. 91, 70–85. doi: 10.1111/brv.12158.

Undabarrena, A., Beltrametti, F., Claverías, F. P., González, M., Moore, E. R. B., Seeger, M., et al. (2016). Exploring the diversity and antimicrobial potential of marine actinobacteria from the Comau Fjord in Northern Patagonia, Chile. Front. Microbiol. 7. doi: 10.3389/FMICB.2016.01135.

Van Arnam, E. B., Currie, C. R., and Clardy, J. (2018). Defense contracts: molecular protection in insect-microbe symbioses. Chem. Soc. Rev. 47, 1638–1651. doi: 10.1039/C7CS00340D.

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. New Phytol. 206, 1196–1206. doi: 10.1111/nph.13312.

Viana, A. M. M., Frézard, A., Malosse, C., Della Lucia, T. M. C., Errard, C., and Lenoir, A. (2001). Colonial recognition of fungus in the fungus-growing ant *Acromyrmex subterraneus subterraneus* (Hymenoptera: Formicidae). Chemoecology 11, 29–36. doi: 10.1007/PL00001829.

von Arx, J. A., and Hennebert, G. L. (1965). Deux champignons ambrosia. Mycopathol. Mycol. Appl. 25, 309–315. doi: 10.1007/BF02049918.

Weikl, F., Ghirardo, A., Schnitzler, J.-P., and Pritsch, K. (2016). Sesquiterpene emissions from *Alternaria alternata* and *Fusarium oxysporum*: Effects of age, nutrient availability, and co-cultivation OPEN. Nat. Publ. Gr. doi: 10.1038/srep22152.

White, T. J., Bruns, S., Lee, S., and Taylor, J. (1990). “Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics,” in PCR Protocols: A Guide to Methods and Applications, eds. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (San Diego: Academic Press), 315–322. doi: citeulike-article-id:671166.

Wickham, H. (2016). ggplot2: Elegant graphics for data analysis. New York: Springer-Verlag New York.

Wickham, H., François, R., Henry, L., and Müller, K. (2021). dplyr: A grammar of data manipulation. Available at: https://cran.r-project.org/package=dplyr.

Wickham, H., and Seidel, D. (2020). scales: scale functions for visualization. Available at: https://cran.r-project.org/package=scales.

Wiggins, W. D., Bounds, S., and Wilder, S. M. (2018). Laboratory-reared and field-collected predators respond differently to same experimental treatments. Behav. Ecol. Sociobiol. 72, 1–8. doi: 10.1007/S00265-017-2437-7.

Wilke, C. O. (2020). cowplot: Streamlined plot theme and plot annotations for “ggplot2”. Available at: https://cran.r-project.org/package=cowplot.

Wilson, E. O. (1971). The Insect Societies. Cambridge: Belknap Press of Harvard University Press.

Wisselink, M., Aanen, D. K., and van ’t Padje, A. (2020). The longevity of colonies of fungus-growing termites and the stability of the symbiosis. Insects 11, 1–15. doi: 10.3390/INSECTS11080527.

Wood, T. G., and Thomas, R. J. (1989). “The Mutualistic Association between Macrotermitinae and Termitomyces,” in Insect-Fungus Interactions, eds. N. Wilding, N. M. Collins, P. M. Hammond, and J. F. Webber (New York: Academic Press), 69–92.

Woodwell, G. M., Whittaker, R. H., and Houghton, R. A. (1975). Nutrient concentrations in plants in the Brookhaven oak-pine forest. Ecology 56, 318–332. doi: 10.2307/1934963.

Yanagawa, A., Fujiwara-Tsujii, N., Akino, T., Yoshimura, T., Yanagawa, T., and Shimizu, S. (2012). Odor aversion and pathogen-removal efficiency in grooming Behavior of the termite *Coptotermes formosanus*. PLoS One 7, e47412. doi: 10.1371/journal.pone.0047412.

Yanagawa, A., and Shimizu, S. (2007). Resistance of the termite, *Coptotermes formosanus* Shiraki to *Metarhizium anisopliae* due to grooming. BioControl 52, 75–85. doi: 10.1007/s10526-006-9020-x.

Yang, A. S. (2001). Modularity, evolvability, and adaptive radiations: a comparison of the hemi- and holometabolous insects. Evol. Dev. 3, 59–72. doi: 10.1046/J.1525-142X.2001.003002059.X.

Zhang, Y., Chen, M., Guo, J., Liu, N., Yi, W., Yuan, Z., et al. (2022). Study on dynamic changes of microbial community and lignocellulose transformation mechanism during green waste composting. Eng. Life Sci. 22, 376–390. doi: 10.1002/elsc.202100102.

Zhou, J., Huang, H., Meng, K., Shi, P., Wang, Y., Luo, H., et al. (2009). Molecular and biochemical characterization of a novel xylanase from the symbiotic Sphingobacterium sp. TN19. Appl. Microbiol. Biotechnol. 85, 323–333. doi: 10.1007/S00253-009-2081-X.

Zilber-Rosenberg, I., and Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: The hologenome theory of evolution. FEMS Microbiol. Rev. 32, 723–735. doi: 10.1111/j.1574-6976.2008.00123.x.

Zimmermann, G., and Butin, H. (1973). Untersuchungen über die Hitze- und Trockenresistenz holzbewohnender Pilze. Flora 162, 393–419. doi: 10.1016/S0367-2530(17)31722-X.

Zuur, A. F., Ieno, E. N., Walker, N., Saveliev, A. A., and Smith, G. M. (2009). Mixed effects models and extensions in ecology with R. New York, NY: Springer New York doi: 10.1007/978-0-387-87458-6.

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First of all, I would like to express my gratitude to my supervisor, Prof. Dr. Peter Biedermann. He gave me the opportunity to work on this fascinating system and supported me throughout the process. I have always appreciated our team atmosphere and the social events that accompanied this time. Thank you, Peter, for believing in me and letting me go my own way. I think I can say that this thesis showed me my limits, but also taught me to grow and become stronger.

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Good premises and infrastructure were needed to carry out my research. Many thanks to the Julius-Maximilians-University of Würzburg and the Albert-Ludwigs-University of Freiburg for these excellent working places. It was great to be a part of this community there and as an alumni I will keep my subsequent colleagues in mind in the future.

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And now comes my Zoo3. What a great department, what amazing people and scientists. I loved being a part of this group and I made so many amazing memories. Thank you, to Prof. Dr. Ingolf Steffan-Dewenter for this amazing department and work atmosphere. Thank you, to Prof. Dr. Thomas Schmitt, who is such a cool dude. I loved our time together at conferences in Vienna and Helsinki and appreciate your support and help during my whole PhD. You took care of me and integrated me into your group, when Peter got his call to Freiburg. Your door was always open to me when I needed help or had questions. I am happy you have agreed to chair my defence. Thank you, Thomas.

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If I would continue with each one of the department there would be no end, but still I want to thank all of you guys, may it be professors, doctors, Michaela, Beate, Gudrun & Suse or the PhD students. Thank you for having me these three years in Würzburg.

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About half a year after Peter moved to Freiburg with the lab, he asked me to follow and join the Forest Entomology and Protection. In the beginning I was a bit sad, about leaving my friends and Würzburg, but Freiburg welcomed me with open arms and I am really happy that I got the chance to get to know all these amazing people here. Angela, Anita, Andrea, Katrin and Hannes you are amazing. It is so much fun working with you and you really made me feel like part of your families. Thank you for your technical, organizational or mental help, whenever I needed it. Otti and Tim, thank you for introducing me to new research topics and your expertises. I am grateful for all the new things I learned from you.

And now my crazy fellows and partners in crime. Sifat, Antoine, Helge, Tobi, Lucile, Antonio, Lennart, Lena, Julio (my sunshine 😊), Deja, Vienna and Ralu

you are amazing. I loved our hikes and adventures, may it be Feldsee, Ravennaschlucht, Saint Peter, Zweibachwasserfälle, Opfinger Lake, 90s parties or driving to the Alsace. It was these moments, that kept me motivated and which I value very much. Thank you, for your friendship, long discussions on science, crazy one's on vegetables, whiskey tastings, and all your support now in the final phase. You kept me standing and continuing. No one understands better what we are going through and it is all the nicer that we can stand by each other and overcome all the work, short nights and stress. Especially in the last days, my dear Ralu, you were a great help and support. I don't take it for granted and it means the world to me. Thank you, honeybunny!!!! *hugs*

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In the past five years, my family in particular has had to do without me a lot. I didn't always have time to come home, and even when I did, I was usually busy or had work to do. Thank you for your understanding, your support, the good food at home, doing the laundry when I couldn't get to it and putting up with my moods. I hope I can make you proud and give it all back.

Once again, THANK YOU everyone, this work is not my merit alone, but a product of teamwork, in which everyone has contributed in his or her own way to the success!

AUTHOR CONTRIBUTIONS

Statement of individual author contributions and of legal second publication rights to manuscripts included in the dissertation

1. Publication: Diehl JMC, Kowallik V, Keller A & Biedermann PHW. 2022. First experimental evidence for active farming in ambrosia beetles and strong heredity of garden microbiomes. *Proc. R. Soc. B* 289: 20221458. https://doi.org/10.1098/rspb.2022.1458

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	JMCD, PHWB	AK			
Data Collection	JMCD				
Data Analysis and Interpretation	JMCD	PHWB, AK	VK		
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	JMCD	PHWB, VK	AK		

Explanations (if applicable):

1. Manuscript: Diehl JMC, Kassie D & Biedermann PHW. submitted to *Symbiosis* (December 2022, in review). Friend or Foe: Ambrosia Beetle response to volatiles of common threats in their fungus gardens.

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	JMCD, DK	PHWB, ML, GC			
Data Collection	DK	LQ, MP, FF			
Data Analysis and Interpretation	DK, JMCD				
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	JMCD	PHWB, DK			

Explanations (if applicable): ML (Max Lehenberger) and GC (Giulio Criscione) designed the set-up of the modified petri dish for the adult beetles. We thank them for this valuable design in the acknowledgments. LQ (Lara Quaas), MP (Max Philippi) and FF (Franziska Fass) assisted with data collection as part of a student's course project. We thank them for their support in the acknowledgements.

2. Manuscript: Working title: Diehl JMCD & Biedermann PHW. In preparation. Ambrosia beetle response to pathogen pressure – A glance at life-history, behaviour and symbiont community over multiple generations.

Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	JMCD, PHWB				
Data Collection	JD	SB, TW, MEG	LK, SK		
Data Analysis and Interpretation	JMCD	PHWB			
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	JMCD	PHWB	RH		

Explanations (if applicable): SB collected the data for the side study on pathogen lethality; TW (Tom Wolf) & MEG (Mona El Gaa) supported as student assistants collecting a part of the behavioural data during the one-year observation period. During a practical course, LK (Luise Kühlborn) and SK (Stephan Kreidl) assisted SB in the data collection. We thanked the students for their help in the acknowledgements. RH (Raluca Hedes) commented on the first draft of the manuscript. We thanked her in the acknowledgements.

2. Publication: Nuotclà JA, Diehl JMCD & Taborsky M. 2021. Habitat quality determines dispersal decisions and fitness in a beetle – fungus mutualism. *Front. Ecol. Evol.* 9:602672. doi: 10.3389/fevo.2021.602672					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	JAN, MT	JMCD			
Data Collection	JAN	JMCD			
Data Analysis and Interpretation	JAN	JMCD			
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	JAN, MT	JMCD			

Explanations (if applicable): JMCD developed and carried out the molecular analysis and analysed the data in this study.

3. Manuscript: Diehl JMC, Keller A & Biedermann PHW. submitted to *Front. Microbiol.* (January 2023). Succession of ambrosia beetle microbial community structure throughout development in field and laboratory galleries.					
Participated in	**Author Initials,** Responsibility decreasing from left to right				
Study Design Methods Development	JMCD, PHWB				
Data Collection	JMCD				
Data Analysis and Interpretation	JMCD	AK, PHWB			
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	JMCD	PHWB	AK, VK		

Explanations (if applicable): VK (Vienna Kowallik) commented on the manuscript. We thanked her in the acknowledments.

If applicable, the doctoral researcher confirms that she/he has obtained permission from both the publishers (copyright) and the co-authors for legal second publication.

The doctoral researcher and the primary supervisor confirm the correctness of the above mentioned assessment.

Janina Marie Christin Diehl	27.02.2023	Freiburg	
Doctoral Researcher's Name	Date	Place	Signature

Prof. Dr. Peter Biedermann	27.02.2023	Freiburg	
Primary Supervisor's Name	Date	Place	Signature

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Statement of individual author contributions to figures/tables of publications or manuscripts included in the dissertation

1. Publication: Diehl JMC, Kowallik V, Keller A & Biedermann PHW. 2022. First experimental evidence for active farming in ambrosia beetles and strong heredity of garden microbiomes. *Proc. R. Soc. B* 289: 20221458. https://doi.org/10.1098/rspb.2022.1458

Figure	**Author Initials,** Responsibility decreasing from left to right				
1	JMCD				
2	JMCD	PHWB	VK		
3	JMCD	PHWB	VK		
Suppl. 1	JMCD				
Suppl. 2	JMCD	PHWB	VK		
Suppl. 3	JMCD	PHWB	VK		
Suppl. 4	JMCD	PHWB	VK		
Suppl. 5	JMCD	PHWB	VK		
Suppl. 6	JMCD	PHWB	VK		
Suppl. 7	JMCD	VK			
Suppl. 8	JMCD	VK			
Suppl. 9	JMCD	VK			
Suppl. 10	JMCD	VK			
Table	**Author Initials,** Responsibility decreasing from left to right				
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Suppl. 1	JMCD				
Suppl. 2	JMCD				
Suppl. 3	JMCD				

Explanations (if applicable): PHWB and VK commented and helped improving the figures.

1. Manuscript: Diehl JMC, Kassie D & Biedermann PHW. submitted to *Symbiosis* (December 2022). Friend or Foe: Ambrosia Beetle response to volatiles of common threats in their fungus gardens.

Figure	**Author Initials,** Responsibility decreasing from left to right				
1	DK	JMCD			
2	JMCD	PHWB	DK		
Table	**Author Initials,** Responsibility decreasing from left to right				
1	JMCD				

Explanations (if applicable): PHWB commented on the figure, design based on the original figure by DK.

2. Manuscript: Working title: Diehl JMCD & Biedermann PHW. In preparation. Ambrosia beetle response to pathogen pressure – A glance at life-history, behaviour and symbiont community over multiple generations.					
Figure	**Author Initials,** Responsibility decreasing from left to right				
1	JMCD				
2	JMCD				
3	JMCD				
4	JMCD				
5	JMCD				
6	JMCD	DV			
7	JMCD	DV			
8	JMCD	DV			
9	JMCD				
Suppl. 1	JMCD				
Suppl. 2	JMCD				
Suppl. 3	LK, SK				
Suppl. 4	JMCD				
Suppl. 5	JMCD				
Suppl. 6	JMCD				
Suppl. 7	JMCD				
Table	**Author Initials,** Responsibility decreasing from left to right				
1	JMCD				
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Suppl. 1	JMCD				
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Suppl. 3	JMCD				
Suppl. 4	JMCD				
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Suppl. 6	JMCD				
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2. Publication: Nuotclà JA, Diehl JMCD & Taborsky M. 2021. Habitat quality determines dispersal decisions and fitness in a beetle – fungus mutualism. *Front. Ecol. Evol.* 9:602672. doi: 10.3389/fevo.2021.602672					
Figure	**Author Initials,** Responsibility decreasing from left to right				
1	JAN				
2	JAN				
3	JMCD				
4	JAN				
Suppl. 1	JAN				
Suppl. 2	JMCD				
Suppl. 3	JMCD				
Suppl. 4	JMCD				
Suppl. 5	JMCD				
Suppl. 6	JMCD				
Table	**Author Initials,** Responsibility decreasing from left to right				
Suppl. 1	JMCD				
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Suppl. 3	JMCD				
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Suppl. 5	JAN				

Explanations (if applicable):

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3. Manuscript 5: Diehl JMC, Keller A & Biedermann PHW. submitted to *Front. Microbiol.* (January 2023). Succession of ambrosia beetle microbial community structure throughout development in field and laboratory galleries.					
Figure	**Author Initials,** Responsibility decreasing from left to right				
1	JMCD	PHWB	DV		
2	JMCD	PHWB			
3	JMCD	PHWB			
4	JMCD	PHWB			
5	JMCD	PHBW			
Suppl. 1	JMCD				
Suppl. 2	JMCD				
Suppl. 3	JMCD				
Suppl. 4	JMCD				
Suppl. 5	JMCD	PHWB			
Suppl. 6	JMCD				
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Suppl. 8	JMCD	PHWB			
Suppl. 9	JMCD				
Suppl. 10	JMCD				
Suppl. 11	JMCD	PHWB			
Suppl. 12	JMCD				
Suppl. 13	JMCD				
Table	**Author Initials,** Responsibility decreasing from left to right				
1	JMCD				
Suppl. 1	JMCD				
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Explanations (if applicable): The picture of the artificial rearing tube was provided by DV (Davide Vallotto). We thank him in the acknowledgements.

I also confirm my primary supervisor's acceptance.

Janina Marie Christin Diehl 27.02.2023 Freiburg

Doctoral Researcher's Name Date Place Signature

AFFIDAVIT

Affidavit

I hereby confirm that my thesis entitled "Ecology and evolution of symbiont management in ambrosia beetles" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Freiburg, 27.02.2023
Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation "Ökologie und Evolution des Symbiontenmanagements bei Ambrosiakäfern" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Freiburg, 27.02.2023
Ort, Datum Unterschrift