# MICROBIAL DRIVERS OF SOCIALITY – FROM MULTICELLULARITY TO ANIMAL SOCIETIES

EDITED BY: Dino McMahon, Peter H. W. Biedermann, Marko Rohlfs and Joël Meunier

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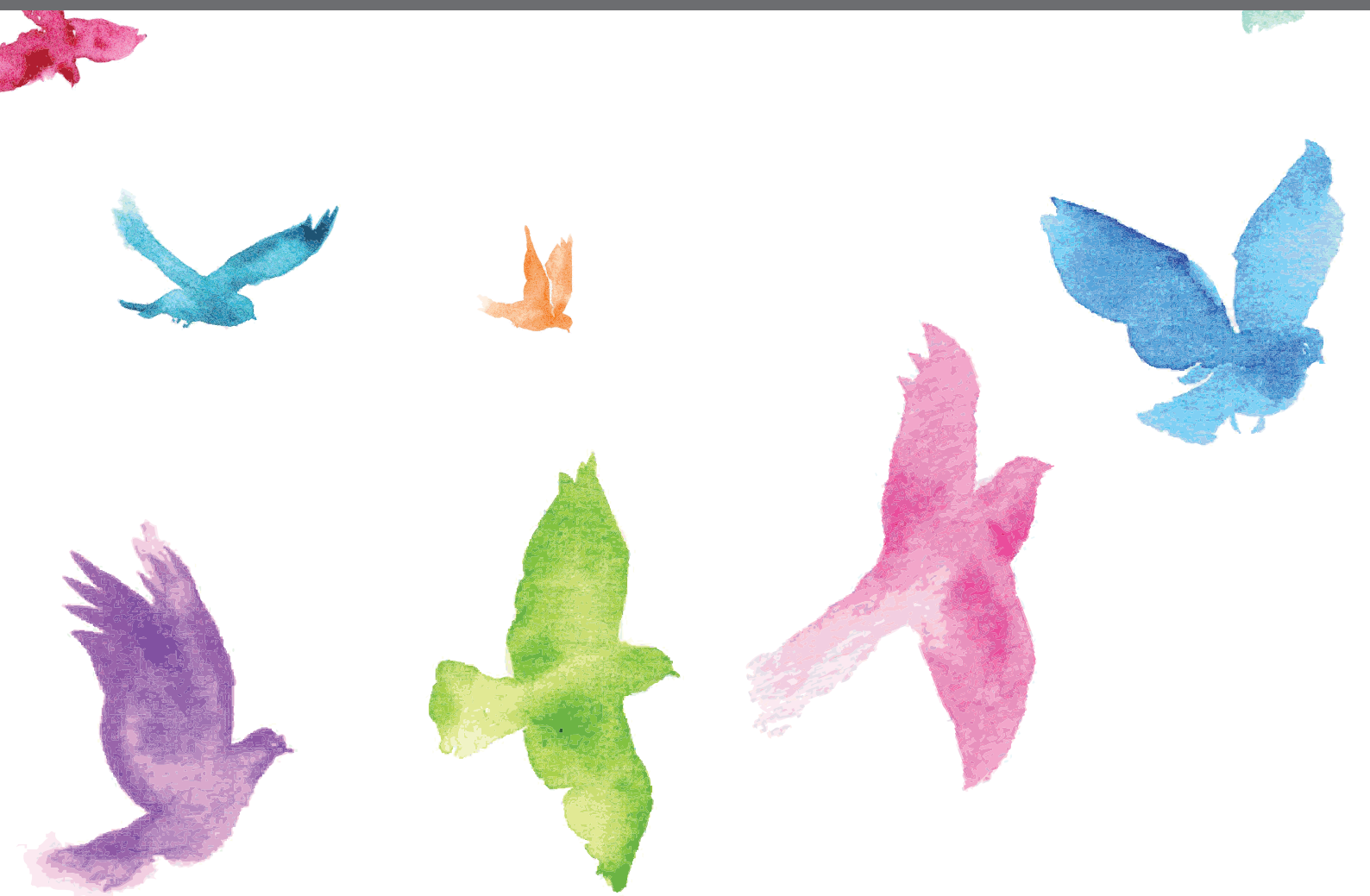

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# MICROBIAL DRIVERS OF SOCIALITY – FROM MULTICELLULARITY TO ANIMAL SOCIETIES

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# Editorial: Microbial Drivers of Sociality – From Multicellularity to Animal Societies

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**Editorial on the Research Topic**

**Microbial Drivers of Sociality – From Multicellularity to Animal Societies**

## MICROBES—AN ECOLOGICAL DRIVER OF SOCIAL EVOLUTION

While sociality is present in a taxonomically diverse number of species, most animals remain solitary (Bourke, 2011). Over the last centuries, this apparent imbalance in social and non-social animals has led to a great deal of research aimed at shedding light on the biotic and abiotic factors explaining the emergence and maintenance of sociality in nature (West et al., 2015). Among them, microbes were quickly identified as a major problem for the evolution of social life, because frequent contact between group members typically facilitates the transmission of pathogens, high nest fidelity favours the establishment of microbial pathogens close to their social hosts and, finally, because social groups often exhibit limited genetic diversity and thus limited genetic resistance against certain pathogen strains (Schmid-Hempel, 1998; Cremer et al., 2007). However, this long-standing view has changed considerably over the last few years. Recent research indeed revealed that group living may be more effective than solitary living to limit the risk of infection by pathogenic microbes because group living also allows the development of an additional layer of defence against pathogens in the form of social immunity (Cremer et al., 2007; Cotter and Kilner, 2010). Under strong pressure from pathogens, microbes could therefore promote, rather than hinder, the evolutionary transition from solitary to group living (Meunier, 2015; Biedermann and Rohlfs, 2017). Moreover, we are increasingly aware that many microbes provide essential benefits to their hosts by performing critical digestive, physiological, and reproductive functions (Engel and Moran, 2013; McFall-Ngai et al., 2013). The need to access beneficial microbes may thus have played a role in the expression of frequent and tight interactions between conspecifics and ultimately promoted social evolution (Wilson, 1971; Onchuru et al., 2018). Finally, a growing number of studies suggest that microbes could enforce the aggregation and expression of cooperative behaviours of the hosts to increase their chance of reaching new hosts and may therefore be involved in the evolution of host sociality (Lewin-Epstein et al., 2017) (but see Johnson and Foster, 2018).

In this Research Topic, we aimed to provide an overview of these recent advances and the potential limitations of our understanding of the roles of microbes in the social evolution of hosts. The collection of articles presented here responds to these objectives by focusing on five major points: (1) a potential limit in our understanding of the roles of microbes in social evolution comes from the multiple definitions of sociality and the persistent boundaries between research communities, (2) the access to social immunity does not necessarily modify investment into

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individual immunity, (3) further researches are needed to explore the wide diversity of social immunity in non-eusocial species and to shed light on the mechanisms of recognition of microbial pathogens in social groups, (4) habitat quality can be a prime candidate to explain the association between microbes and social evolution, and (5) the necessity to acquire symbionts from conspecifics could be a key evolutionary driver of sociality. In the following parts, we will briefly summarise how the studies contained in this Research Topic address these five points and then conclude on potential future directions of research.

## On the Multiple Definitions of Sociality and the Necessity to Break Boundaries Between Research Communities

Social life is not only present in various organisms but also in all kinds of forms: simple aggregations of individuals, facultative or obligatory parental care, cooperatively breeding groups and super-organismal eusocial societies (Bourke, 2011). However, research on animal sociality has long focused exclusively on the fascinating eusocial organisation of certain hymenopteran and isopteran insects, hampering our general understanding of the evolution of all forms of social life in nature (Elgar, 2015; Meunier and Steiger, 2018). Somewhat surprisingly, the results of our call for this Research Topic suggest that this bias is being corrected: 7 of the 16 published articles are based on non-eusocial systems (**Table 1**). All else being equal, this indicates that the research community studying the link between microbes and social evolution has moved away from exclusive eusocial model systems. However, the results of our call also point out existing partitions between communities, as we only managed to receive two non-insect articles and unfortunately none on a non-eukaryotic system (**Table 1**). This bias may come from the fact that the editors of this special issue are mostly working on insect models, and have failed to reach out and/or to attract members of the other communities. This bias may also stem from the multiple definitions of sociality present in the literature (Rubenstein and Abbot, 2017), which are often very specific to each community, and which may have—for instance—excluded vast communities of researchers studying family life and aggregation behaviours much more frequent in non-insects species.

## On the Links Between Individual and Social Immunities

A long-standing question about the link between microbial pathogens and social evolution is whether access to additional defences against microbial pathogens (i.e., social immunity) necessarily comes with reduced investment in individual immunity. Five manuscripts of this Research topic illustrate the complexity of this question and the diversity of answers that can be given. First, Meusemann et al. used genomic and transcriptomic data from eight termite species, representing wood-dwelling and foraging species, plus 14 other winged insects (Pterygota) and found that differences in the types of sociality do not reflect differences in the intensity of natural selection on immune genes. Instead, they found evidence for a genome-wide pattern of relaxed selection on these genes in termites. Second, Baeuerle et al. used experimental approaches on 14 bumblebee species to show that the investment in an external defence against pathogens (in the venom) does not trade-off against the investment in internal immune defence present in the hemolymph. Third, Esparza-Mora et al. revealed in a termite that inhibiting an external enzyme capable of degrading entomopathogenic fungi does not trigger collective defences such as allogrooming, but instead reduce defensive cannibalistic behaviours. This suggests that the individual immune system is linked to certain collective immune behaviours in this termite. Fourth, Cole and Rosengaus emphasised the importance of social environment on pathogen resistance, as they showed that the presence of a king may help to mitigate the negative effects of a queen's infection during colony foundation in a dampwood termite. Finally, Pull and McMahon propose a comprehensive review of social immunity and emphasise that "superorganism immunity" may fulfil an analogous function to the immune system of Metazoa.

## On the Diversity of Social Immunity and Mechanisms of Recognition of Microbial Pathogens

The recent proposal that social immunity is not specific to eusocial systems (Cotter and Kilner, 2010; Van Meyel et al., 2018) opened numerous questions about the diversity of its forms, the type of immune benefits provided by the social environment and the mechanisms mediating the recognition of pathogenic microbes in social species. Two manuscripts of this Research Topic offer an overview of these questions. First, Trienens and Rohlfs investigated forms of social immunity in groups of fruit fly larvae. They showed that larvae suppress the invasion of a harmful fungus by the summative effect of individuals at high densities and that larger groups of larvae at the same density can control fungal growth more efficiently. This indicates a potential collective defence against habitat invasion by pathogenic fungi in insects that exhibit mere aggregation behaviour. Second, Goes et al. reviewed the literature on leaf-cutting ants' social immunity to investigate how workers protect their fungal garden against harmful microbes. They reveal that workers discriminate against harmful microbes *via* chemical cues originating from the antagonistic microbe and/or semiochemicals released by the fungus-garden during harmful interactions, as well as *via* associative learning when workers connect the microbe cues with damage in the fungus garden.

## On the Key Role of Habitat Quality in the Association Between Microbes and Social Evolution

Several articles dealt with the issue that habitat can shape interactions of animals with microbes, which in turn affects the animal's social behaviour. Three of these articles specifically looked at wood as a substrate for insects, which is acknowledged to be very favourable for the evolution of sociality because of its structural resistance and longevity compared to the life of an insect (Hamilton, 1978; Kirkendall et al., 2015). In a review article, Dillard and Benbow argue that two additional factors may

**TABLE 1 |** Articles included in our Research Topic.

| References | Type | Study organism(s) | Level of sociality |
|---|---|---|---|
| Cole and Rosengaus | Research article | *Zootermopsis angusticollis* (Blattodea) | Eusociality |
| Meusemann et al. | Research article | Various Blattodea | Various levels of sociality |
| Tragust et al. | Research article | *Lasius niger* (Hymenoptera) | Eusociality |
| Baeuerle et al. | Research article | 14 species of bumblebees (Hymenoptera) | Eusociality |
| Sinotte et al. | Review | Eusocial insects in general | Eusociality |
| Esparza-Mora et al. | Research article | *Reticulitermes flavipes* (Blattodea) | Eusociality |
| Figueiredo and Kramer | Review | Animals in general | All levels of sociality |
| Nalepa | Review | Lower termites and *Cryptocercus* spp. (Blattodea) | Eusociality and subsociality |
| Dunn et al. | Review | Hominins | Different levels of sociality |
| Bratburd et al. | Review | Social insects | Different levels of sociality |
| Trienens and Rohlfs | Research article | *Drosophila melanogaster* (Diptera) | Aggregation |
| Goes et al. | Review | Attini (Hymenoptera) | Eusociality |
| Biedermann | Research article | *Xyleborus affinis* (Coleoptera) | Cooperatively breeding |
| Pull and McMahon | Review | Eusocial insects in general | Eusociality |
| Dillard and Benbow | Review | Various wood-dwelling insects | Different levels of sociality |
| Nuotclà et al. | Research article | *Xyleborinus saxesenii* (Coleoptera) | Cooperatively breeding |

facilitate prolonged parent-offspring contact and cooperative behaviours in insects. First, the low nutritional value of wood selects for associations with nutrient-enriching microbes that need to be transferred to offspring, which is often through social contact. Furthermore, insects compete with many antagonistic microbes in this habitat and collective defence is often better to keep them in cheque. A research study by Biedermann found evidence for collective pathogen defence in a cooperatively breeding, fungus-farming ambrosia beetle. Delayed dispersing female offspring showed more social hygienic behaviour at places within the nest with higher abundances of antagonistic fungi. Nuotclà et al. showed in a closely related ambrosia beetle species with a similar social system that the two nutritionally important fungal mutualists of this species vary in their relative abundance depending on the dryness of the wood substrate. Interestingly this fed back on the social behaviour and the delayed dispersal periods of daughters in this facultatively eusocial beetle. Finally, a study by Tragust et al. on founding *Lasius niger* ant queens showed that queens exposed to pathogens invest simultaneously in formic acid defence and higher worker production. Surprisingly there was no measurable trade-off between this individual immune defence and reproduction at an early nest stage, but this may have effects on later fitness.

## On the Role of Symbionts as Promoters of Hosts' Social Evolution

The growing awareness that microbes residing on and in a host can provide it with major benefits has recently stimulated a great number of experimental and theoretical research on the impacts of symbionts in the social evolution of hosts. This is illustrated by five articles in this Research Topic. First, Bratburd et al. examined the general role of defensive microbial symbionts in host protection against pathogens in terms of behavioural and immune responses and discussed why insects are good models to study issues relating to human health and agriculture. Second, Dunn et al. used a comparative approach to study how microbiomes of hominins have changed over evolutionary time, questioning their impact on the evolution of several host functions and discussing the possibility that prosocial microbes promoted hominin social behaviour. Third, Nalepa discussed why symbiont transmission *via* proctodeal trophallaxis and sociality are likely to have entangled evolutionary histories and conclude that the vertical transmission of gut microbes (flagellates) and the origin of host subsociality are two sides of the same coin in termites. Fourth, Sinotte et al. explored the link between the division of labour and symbiosis in social insects. Their review suggests that structured microbiomes have evolved in parallel to social complexity, and predicts that mature social insect colonies with the most extreme division of labour shows the strongest distinction between caste microbiomes. This suggests that caste-specific microbiomes may enhance symbiotic benefits and the efficiency of division of labour. Finally, Figueiredo and Kramer took another perspective and focused on the microbes themselves. Their review describes cooperation and conflict within the microbiota. They discuss how these parameters can affect animal hosts and conclude that an explicit consideration of social dynamics within symbiont communities is crucial to advance our understanding of how microbes shape animal function and evolution.

## CONCLUSION

Overall, this Research Topic emphasises the multiple roles of microbes in the social evolution of their hosts, which range from obstruction to promotion. It also illustrates why group-living animals specifically face an intense tug-of-war between the necessity to limit the inherently high risk of pathogen infection and transmission within the nest as well as the necessity to protect and efficiently transmit essential symbionts within the nest and to dispersing sexuals. The outcome of this war can

have profound impacts on the life-history traits of a given species. Finally, it also points out that although our current understanding of the link between microbes and social evolution is based on a wider range of social systems and is thus becoming more comprehensive, it still needs to bring together the results of all research communities studying different organisms. We believe that this Research Topic is a first step toward achieving this goal.

## AUTHOR CONTRIBUTIONS

All authors wrote the manuscript.

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# Pathogenic Dynamics During Colony Ontogeny Reinforce Potential Drivers of Termite Eusociality: Mate Assistance and Biparental Care

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As an ecologically dominant taxon, termites appear to be resilient to environmental stressors. However, swarming alates (winged-individuals) encounter a myriad of environmental pressures that drastically reduce the probability of colony foundation. Dispersing alates face high rates of predation, desiccation, nitrogen limitation, and risks of infection, among others. We propose that alates benefit from mate assistance and biparental care to overcome some of these challenges. We assessed whether the bacteria, *Serratia marcescens* (an ecologically relevant, gram-negative, facultative termite pathogen), negatively affected the growth of newly founded termite colonies. Additionally, we revealed the significance of the king's presence in improving successful establishment of incipient colonies. Virgin queens of the dampwood termite, *Zootermopsis angusticolli*s, were subjected to one of four treatments: naïve (untreated), or injections with either sterile saline, heat-killed *S. marcescens,* or a sublethal does of live *S. marcescens*. Each queen was then paired with a naïve, virgin king. The incipient colonies underwent censuses every 4 days for 80 days. We estimated survival rates and compared the onset of oviposition and hatching, overall egg production and larval hatching success, all as a function of queen treatment and the presence of a mate. We identified factors that, under pathogenic stress, influenced these fitness-related milestones. Queen infection significantly reduced the number of successfully established colonies. Moreover, both the presence of a king and his mass significantly influenced the queen's survival, her onset of oviposition, overall egg production, and hatching success. We conclude that termite colonies incur significant fitness costs after a queen suffers an acute infection and that the presence of a king (and his stored resources) may help mitigate the negative effects of a queen's infection. Pathogenic pressures, combined with the significant role of kings in colony success, appear to reinforce two-parent colony foundation, mate assistance, biparental care, and ultimately the overlap of generations, all of which have been considered pre-adaptations for eusociality. By studying the fitness consequences of pathogenic stress during the ontogeny of a termite colony, we can infer some of the conditions and pressures under which termite sociality likely emerged.

**Keywords: social evolution, social insect, mate assistance, pathogens, colony foundation, fitness, biparental care, isoptera**

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## INTRODUCTION

Termites, and other eusocial insects (social Hymenoptera), have long garnered the interest of evolutionary biologists (e.g., Boomsma and Gawne, 2018, and references therein). In spite of the convergent social organization across all eusocial insects, termites represent an especially interesting taxon, differing from their social Hymenopteran counterparts in striking ways. Termites are hemimetabolous, diploid insects, their worker and/or soldier castes are typically composed of both males and females, they feed on a nitrogen-limited cellulose-based diet, and their colonies are mostly established by a monogamous reproductive pair that exhibits biparental care (Krishna and Wesner, 1970; Wilson, 1971; Shellman-Reeve, 1990, 1997a; Rosengaus and Traniello, 1991; Bignell et al., 2011). Unfortunately, studies on their evolutionary trajectory toward eusociality are hampered, in part, by the fact that this taxon lacks graded levels of sociality (Thorne, 1997; Korb and Throne, 2017). All termites are considered eusocial, whether facultatively (i.e., the worker and soldier castes retain the potential for reproduction) or obligatorily (workers and soldiers are sterile; Boomsma, 2013; Boomsma and Gawne, 2018). Hence, contrary to the common approach used in bees and wasps which exhibit a full spectrum of sociality (Hunt and Toth, 2017; Wcislo and Fewell, 2017), comparative approaches across levels of sociality within the single termite clade are not possible. Instead, we can make inferences about the origins and maintenance of their eusociality by using a combination of molecular phylogenetics, and ecological, physiological, nutritional, and behavioral comparisons with its related sister taxa, the subsocial wood roach, *Cryptocercus* (e.g., Wheeler, 1904; Shellman-Reeve, 1990; Nalepa, 1991, 2010, 2011; Inward et al., 2007; Klass et al., 2008; Todaka et al., 2010; Bourguignon et al., 2014, 2017; Tai et al., 2015; Korb and Heinze, 2016, Maekawa et al., 2008; Nalepa and Arellano, 2016; Harrison et al., 2018).

Here we use an alternative approach that may prove helpful in elucidating the selective pressures and intermediate steps that culminated in the evolution of termite eusociality. Studying the current selective pressures faced by incipient colonies in basal termite species allows us to make inferences about the transitions in social complexity based on proposed evolutionary scenarios (**Figure 1**). We therefore examined multiple fitness parameters of newly founded colonies while under pathogenic stress. We identified factors that constrained or promoted the successful establishment of a colony. These conditions may have been similar to the conditions and pressures under which termite sociality originated ∼150 million years ago (Thorne, 1997; Bourguignon et al., 2014; Harrison et al., 2018).

As termite colonies develop, they progress through graded levels of sociality. First, winged reproductives swarm away from their natal colony without displaying any apparent social cohesion (∼solitary stage). After attracting a mate, the de-winged alates establish a monogamous pair that engages in frequent social interactions (Nutting, 1969; Shellman-Reeve, 1990, 1999; Rosengaus and Traniello, 1991; Brent et al., 2007). Such monogamy often correlates with biparental care and mate assistance in termites (Shellman-Reeve, 1997a,b; Klug, 2018). These three traits are considered prerequisites for the evolution of termite eusociality, and eusocial evolution more broadly (Boomsma, 2009, 2013). Subsequently, the royal pair enters a subsocial phase, where the king and queen provide biparental care, functioning as a family unit while exploiting their nitrogen-poor wooden resources (Nalepa and Jones, 1991; Rosengaus and Traniello, 1991, 1993a). These family units then proceed toward more complex levels of sociality. The formerly altricial larvae start contributing labor in the colony (nest expansion, hygiene, royal, and brood care; Rosengaus and Traniello, 1993a; Brent and Traniello, 2001; Chouvenc and Su, 2017) while remaining within their natal nest (i.e., non-dispersing brood) and forgoing their own reproduction (either temporarily or permanently; Thorne, 1997; Boomsma, 2009, 2013; Boomsma and Gawne, 2018). It is at this developmental stage, that a termite colony attains the required traits of any eusocial species: reproductive division of labor, overlap of generations and cooperative brood care; as defined by Wilson (1971). By focusing on the incipient stages of colony foundation in the basal termite, *Zootermopsis angusticollis*, we identified some of the selective pressures affecting colony establishment. We also quantified their fitness-related milestones and made inferences about how mate assistance and biparental care help overcome the myriad stressors encountered by the founding pair.

In spite their reputation for hardiness, termite colonies have extremely low probabilities of becoming established, even under ideal laboratory conditions (Rosengaus and Traniello, 1993b; Fei and Henderson, 2003; Calleri et al., 2006; Hartke and Rosengaus, 2013; Cole et al., 2018). In nature, swarming alates fall prey to diverse aerial and terrestrial predators (Sheppe, 1970; Delighne et al., 1981; Dial and Vaughan, 1987; Lepage, 1991; Matsuura and Nishida, 2002). The few that survive have to quickly locate a mate, shed their wings, and search for an adequate nesting site before desiccation takes a toll (Nutting, 1969). The search for a nest involves scurrying above ground, under the leaf-litter and/or subterraneously, environments known for their high microbial, potentially pathogenic, loads (Cruse, 1998; Schmid-Hempel, 1998; Rosengaus et al., 2003, 2011; Tunaz and Stanley, 2009; Chouvenc et al., 2011). Once the nesting site is located, the future king and queen sequester themselves within the copularium where they likely encounter additional bacterial, fungal, viral pathogens as well as entomopathogenic nematodes (Schmid-Hempel, 1998; Rosengaus et al., 2000, 2003, 2011; Wilson-Rich et al., 2007).

Beyond the above-mentioned environmental challenges, the royal pair has to cope with intrinsic factors that further reduce their probability of colony establishment (Cole et al., 2018). These include their own genetic background, behavioral incompatibility with their mate, nutritional stress due to their cellulose-based diets, and limited stored resources (Cowling and Merrill, 1966; Nalepa, 1988; Hunt and Nalepa, 1994; Higashi et al., 2000; Bauerfeind and Fischer, 2005; Shellman-Reeve, 2013; Cole et al., 2018; Nottingham et al., 2018). The latter two are particularly important, as restricted energy must be allocated to several competing demands (Cole et al., 2018). These include nest construction, nest sanitation (via the deposition of antimicrobial

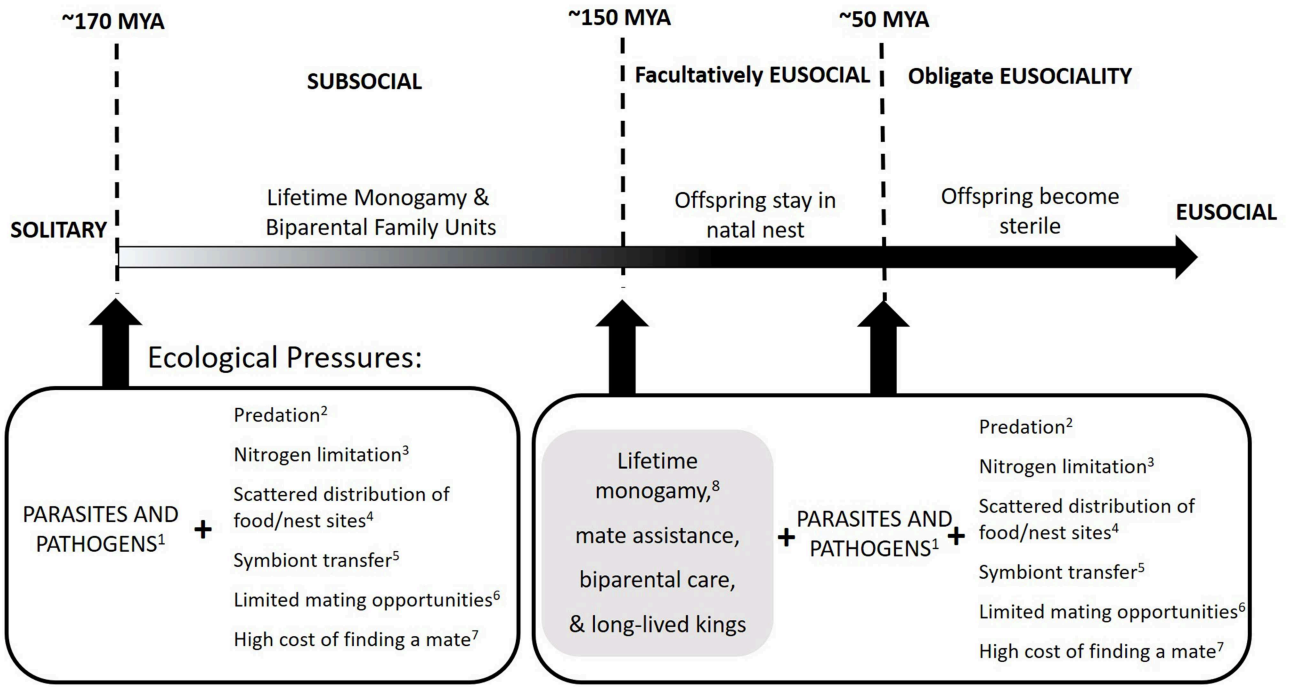


**FIGURE 1 |** Proposed scenario of the evolution of termite sociality. Based on basic termite biology, previous empirical studies and theoretical frameworks, we propose an evolutionary scenario in which the transitions between levels of social complexity occurred along a continuum, spanning ∼120 million years (MYA). Throughout this continuum, multiple, and sustained ecological pressures (including pathogenic stress) likely promoted the transition from a solitary to a subsocial lifestyle, which in the putative termite ancestor, likely consisted of monogamy, longer life expectancies of parents, mate assistance, and biparental care. These subsocial traits served as preadaptations (in gray box) which, together with continued strong ecological pressures, might have selected for non-dispersing progeny who retained reproductive potential (facultative eusociality; Boomsma, 2009, 2013; Boomsma and Gawne, 2018) by around ∼150 MYA (Bourguignon et al., 2014). By ∼50 MYA (Bourguignon et al., 2014, 2017), the evolution of sterility in the offspring took place (obligatory eusociality; Boomsma, 2009, 2013; Boomsma and Gawne, 2018). Given the risks posed by multiple environmental stressors, the fitness benefits accrued by progeny while rearing siblings likely outweighed any benefits from nest dispersal and personal reproduction. The dashed lines represent the timing of these major transitions in social complexity (Bourguignon et al., 2014, 2017; Harrison et al., 2018). Possible environmental factors influencing sociality throughout this continuum: [1]Cruse (1998), Schmid-Hempel (1998), Rosengaus et al. (2000, 2003, 2011), Tunaz and Stanley (2009), Chouvenc et al. (2011) and Wilson-Rich et al. (2007). [2]Delighne et al. (1981), Dial and Vaughan (1987), Lepage (1991), Matsuura and Nishida (2002) and Sheppe (1970). [3]Nalepa (1991, 2010, 2015), Hunt and Nalepa (1994), Higashi et al. (2000) and Shellman-Reeve (2013). [4]Hamilton (1978), Korb and Heinze (2008) and Nutting (1969). [5]Nalepa (1991, 2015). [6]Hamilton (1978) and Nutting (1969). [7]Hamilton (1978) and Nutting (1969). [8]Boomsma (2009, 2013).

compounds on their nesting substrate), courtship, copulation, gametogenesis, parental care (at least until the first altricial larvae become independent), and the generation of costly immune responses if exposed to pathogens (Armitage et al., 2003; Schwenke et al., 2016; Brace et al., 2017).

Recently, while investigating the short-term pathogen-induced fitness costs at the colony level, Cole et al. (2018) identified several extrinsic and intrinsic factors that affect the successful establishment of a termite colony within the first 30 days post-establishment. Here, through a series of new experiments, we expand on these results and assessed the longer-term, colony-wide fitness costs associated with queen's bacterial infection. We asked whether the initial pathogen-induced fitness costs are temporary or sustained for up to 80 days post-pairing. We compared queen and king survival, onset of oviposition, overall egg production, onset of hatching as well as hatching success during colony foundation, all as a function of queen bacterial exposure. By framing our results around basic termite biology, previous empirical and theoretical studies (Wilson, 1971, 1975; Hamilton, 1978; Nalepa, 1991, 2010, 2011; Thorne, 1997; Higashi et al., 2000; Korb, 2008a; Boomsma, 2009, 2013; Korb and Heinze, 2016; Nalepa and Arellano, 2016; Boomsma and Gawne, 2018), we reveal some of the underlying factors and dynamics fostering termite biparental care, mate assistance, and king longevity—all putative pre-adaptations for sociality while under a scenario of pathogenic stress.

# METHODS

## Collection, Maintenance, and Establishment of Incipient Colonies

Male and female *Z. angusticollis* alates were retrieved from 10 different mature stock colonies, all collected from the Redwood East Bay Regional Parks, Oakland California. These colonies were transported to our USDA approved containment room (Northeastern University, Boston, MA; USDA Permit P526P-17-03814) and maintained at 25°C. Upon molting, the alates were removed, sexed, de-winged, weighed, queens were experimentally treated (see below) and then, paired inside a

Petri dish (60 × 15 mm; lined with moist Whatman # 1 filter paper) with a naïve king ($n = 271$ pairs in total). We only used heavily pigmented alates who were physiologically and motivationally ready to mate (Cole et al., 2018). To each incipient colony, we added approximately ∼2.5 mg of white birch (*Betula*) to provide a nesting/feeding substrate in which to build their initial mating chamber (copularium; Nutting, 1969). The incipient colonies were stacked inside covered plastic boxes lined with wet paper towel to keep high humidity. The colonies were watered and additional wood chips added as needed. To ensure robust sample sizes of surviving incipient colonies across all treatments (see below), we purposefully established a surplus of incipient colonies headed by queens infected with a sublethal dose of live *Serratia marcescens* (see below). Our original bias explains the unbalanced sample sizes across treatments. Based on the non-synchronized swarming under our laboratory conditions, we establish incipient colonies headed by either, kings and queens collected from the same (nestmates, $n = 178$) or different parental colonies (non-nestmates, $n = 93$).

## Experimental Design and Colony Census

We quantified the colony-wide fitness consequences of a queen's bacterial infection by establishing colonies headed by naïve queen + naïve king: $n = 76$; Burnes-Tracey Saline (BTS) - injected queen + naïve king: $n = 49$; $10^7$ CFU/mL heat-killed *S. marcescens* (HK-*Sm*) queen + naïve king: $n = 50$; $2 \times 10^5$ CFU/mL sublethal dose of live *S. marcescens* (live-*Sm*) queen + naïve king: $n = 96$ (bacterial culturing protocol in **Supplementary Methods 1**). *S. marcescens* is a common Gram-negative soil bacterium known to naturally infect termites upon entering the hemocoel, either through the gut lining or via cuticular wounding (Chouvenc et al., 2011; Mirabito and Rosengaus, 2016). Previous work has shown that more than 50% of individuals survive a dose 1 µl injection of $2 \times 10^5$ CFU/mL of live *S. marcescens*, and hence it is considered sublethal (Cole et al., 2018). Such a sub-lethal dose allows us to study the effects of pathogenic stress in incipient colonies during the production of the first brood while maintaining a reasonable sample size. Periodic census were performed every 4 days for 80 days in which we recorded queen and king survival, number of days elapsed till the onset of oviposition, total number of eggs at 80 days (a measure of overall fecundity), onset of hatching and hatching success.

## Microinjections

To mimic the natural infectious process, and to administer precise known pathogen loads, we cultured (**Supplementary Methods 1**) and injected 1 µL suspensions of *S. marcescens* suspended in sterile BTS or sterile BTS alone. All injections were performed as described previously in Cole et al. (2018). Briefly, after 10–20 min of cold immobilization, the queens were injected with a picospritzer III (Parker Hannifin) and a pulled borosilicate capillary tube with a 2 µm diameter at its point. Queens were allowed to recover for 1 h prior to pairing with their naïve mates.

## Statistical Analysis

All statistics were run using IBM SPSS version 24.

### Survival of Queens and Kings

We first ran separate Cox proportional hazards regression models (henceforth, Cox model) for each sex across the entire 80-day census period (**Supplemental Tables 1, 2**). These models included queen treatment and whether the mate was present or not (i.e., mate death), as extrinsic covariates. Mate death in these (and subsequent) models allowed us to estimate fitness-related benefits attained from mate assistance and biparental care. Queen mass, king mass, and "nestmate vs. non-nestmate pairs" were also included as intrinsic (inherent to the individual) covariates. All survival curves across 80 days post-pairing showed a clear inflection point at ∼day 20 post-pairing (**Supplemental Figure 1**). Given these inflection points, the survival data violated the assumptions of proportional hazards (Kleinbaum and Klein, 2012). For this reason, we ran two additional separate Cox models for each sex, one targeting days 0–20 and the other focusing on days 21–80 post-pairing. Both models included the same extrinsic and intrinsic variables described for the 80–day analysis. Where appropriate, *post-hoc* pairwise comparisons were performed using a Bonferroni correction, setting a more conservative threshold $p$-value of 0.008 (Rice, 1989).

### Fitness Consequences of Queen's Treatment

#### *Likelihood and onset of oviposition*

We first ran a single Cox model for the entire 0–80 day period to estimate the likelihood of oviposition. The model included all established incipient colonies—those in which the parents survived even if they yielded no eggs as well as those in which one or both of the parents died after oviposition. The following extrinsic covariates were included: queen treatment, mate death and whether or not they were paired with a nestmate or non-nestmate. The intrinsic covariates included queen mass and king mass. From these Cox models, we can draw information on both the onset (time course of egg-laying on the x-axis) as well as the likelihood (proportion of egg-laying colonies on the y-axis) of oviposition. We also ran an identical second Cox model, in which, only colonies that had produced at least one egg were included. Together, both models provide a more complete picture of the effects that each intrinsic and extrinsic variable has on the production of the first brood of eggs.

#### *Total egg and larvae counts by day 80*

To assess the effect of queen treatment on the overall number of eggs and larvae produced by day 80, regardless of the viability of those eggs, we ran two mixed effect models. The first model was generated for the total number of eggs produced within each colony, and the second on total number of larvae produced by day 80, both as a function of queen treatment. These models were identical except in the dependent variable, and included all of the originally established colonies. The fixed variables included queen treatment, nestmate vs. non-nestmate pairs, and mate death. Queen and king mass, and

onset of oviposition were included as continuous covariates. Accounting for onset of oviposition was important as a later onset of oviposition was bound to result in lower overall egg count due to the fixed endpoint of the 80-day experiment. The models also included the following interactions: queen treatment × queen mass, queen treatment × king mass, queen treatment × king death, and queen treatment × queen death.

*Likelihood and onset of hatching*

Two Cox models were generated, one to estimate the likelihood of hatching and the second to determine the onset of hatching. The former model included all colonies regardless of queen and king survival, oviposition, and larval hatching status. The second model included only those colonies that had at least one larvae by day 80. Both models encompassed the entire 80-day period, and included both extrinsic categorical covariates (queen treatment, mate death, nestmate vs. non-nestmate pairs) and intrinsic continuous covariates (queen and king mass, onset of oviposition).

*Hatching success*

We defined hatching success as the percent of oviposited eggs that actually yielded larvae. Given that hatching success is constrained by the number of eggs present in the incipient colony, this analysis only included colonies that produced eggs regardless of whether king and/or queen died or not. This GLMM included queen treatment, nestmate vs. non-nestmate pairs, mate death, as fixed factors; queen mass, king mass and onset of oviposition as continuous covariates and the following interactions: queen treatment × queen mass, queen treatment × king mass, queen treatment × king death, and queen treatment × queen death.

*Proportion of intact colonies 80 days post-pairing*

We assumed that hatched larvae had the potential for maturing into functional workers past the 80-day experimental period. Thus, to estimate the probability that an incipient termite colony, while under pathogenic pressures, reached the initial colony growth phase (Cole et al., 2018), we defined "intact colonies" as those with both a surviving king, queen and at least one hatched larvae. To test whether the number of intact incipient colonies was disproportionately lower when queens experienced pathogenic stress, we compared the absolute number of intact vs. non-intact incipient colonies as a function of queen treatment with a 2 × 4 chi-square test.

# RESULTS

## Queen Survival

Queen survival between days 0–20 was significantly influenced by both queen treatment (Wald = 19.1, df = 3, $p < 0.001$; **Figure 2A**) and the king's survival (Wald = 12.5, df = 1, $p < 0.001$; **Figure 2B**). Live-*Sm* injected queens were 2.9 times more susceptible than naïve queens (**Figure 2A**) and after controlling for the effect of all other variables in the model, including queen treatment, queens without a mate were 1.9 times more likely to die than queens with a living partner were (**Figure 2B**). No other factor influenced queen survival (**Supplemental Table 3**).

In contrast to the 0–20 day period, queen survival between days 21–80 post-pairing was not influenced by her treatment (Wald = 5.3, df = 3, $p = 0.2$; **Figure 3A**). Instead, her survival was significantly influenced by whether she was accompanied by her mate or not (Wald = 20.9, df = 1, $p < 0.001$, **Figure 3B**) and his mass (Wald = 3.7, df = 1, $p = 0.05$). After controlling for the effects of all other variables in the model, queens without a living partner were 10.6 times more likely to die than their paired counterparts (Wald = 20.9, df = 1, $p < 0.001$; **Figure 3B**; **Supplemental Table 4**).

## King Survival

Between days 0–20 post-pairing, survival of the naïve kings was significantly influenced only by the survival of his queen (Wald = 8.7, df = 1, $p = 0.003$; **Figures 2C,D**). Kings who lost their queen were 2.1 times more likely to die than kings accompanied by a living partner (**Figure 2D**). No other variable, including queen treatment (Wald = 0.8, df = 3, $p = 0.9$), influenced king survival at this early stage of colony life (**Supplemental Table 5**).

King survival between days 21–80 post-pairing was also not influenced by queen treatment (Wald = 0.9, df = 3, $p = 0.8$; **Figure 3C**; **Supplemental Table 6**). His survival however, was significantly dependent on the queen's survival (Wald = 20.9, df = 1, $p < 0.001$; **Figure 3D**), with kings whose queens died being 11.5 times more likely to die than those with surviving queens. Interestingly, between days 21–80, whether or not a king was paired with a nestmate or non-nestmate queen was also a significant factor influencing his survival (Wald = 4.8, df = 1, $p = 0.03$, **Supplemental Table 6**).

## Likelihood and Onset of Oviposition

Queen treatment was a marginally significant predictor of her likelihood to oviposit (Wald Statistic = 7.3, df = 3, $p = 0.06$, **Figure 4**, **Supplemental Table 7**). In pairwise comparisons and after adjusting the *p*-value with a Bonferroni correction, the live-*Sm* treatment had a tendency for lower likelihood of oviposition relative to the other three treatments. Compared to naïve queens, live-*Sm* queens were 1.8 times less likely to oviposit (Wald = 5.9, df = 1, $p = 0.015$). Live-*Sm* queens were also 1.7 and 1.9 times less likely to oviposit than saline and HK-*Sm* queens (Wald = 3.4, df = 1, $p = 0.065$; Wald = 5.0, df = 1, $p = 0.025$, respectively; **Figure 4A**). Both queen death and king mass were significant and independent predictors of likelihood of oviposition (Wald = 83.8, df = 1, $p < 0.001$, **Figure 4B**; Wald = 14.2, df = 1, $p < 0.001$, respectively). Colonies whose queens died within the 80-day period were 9.7 times less likely to produce at least one egg (**Figure 4B**). King mass was also a significant predictor of the onset of oviposition (Wald = 17.4, df = 1, $p < 0.001$). No other factors were a significant predictor of either the likelihood or the onset of oviposition (**Supplemental Tables 7, 8**; **Supplemental Figure 2**).

## Overall Egg Count

Onset of oviposition was predictive of overall egg count ($F = 123.0$, df = 1, 247, $p < 0.001$). After accounting

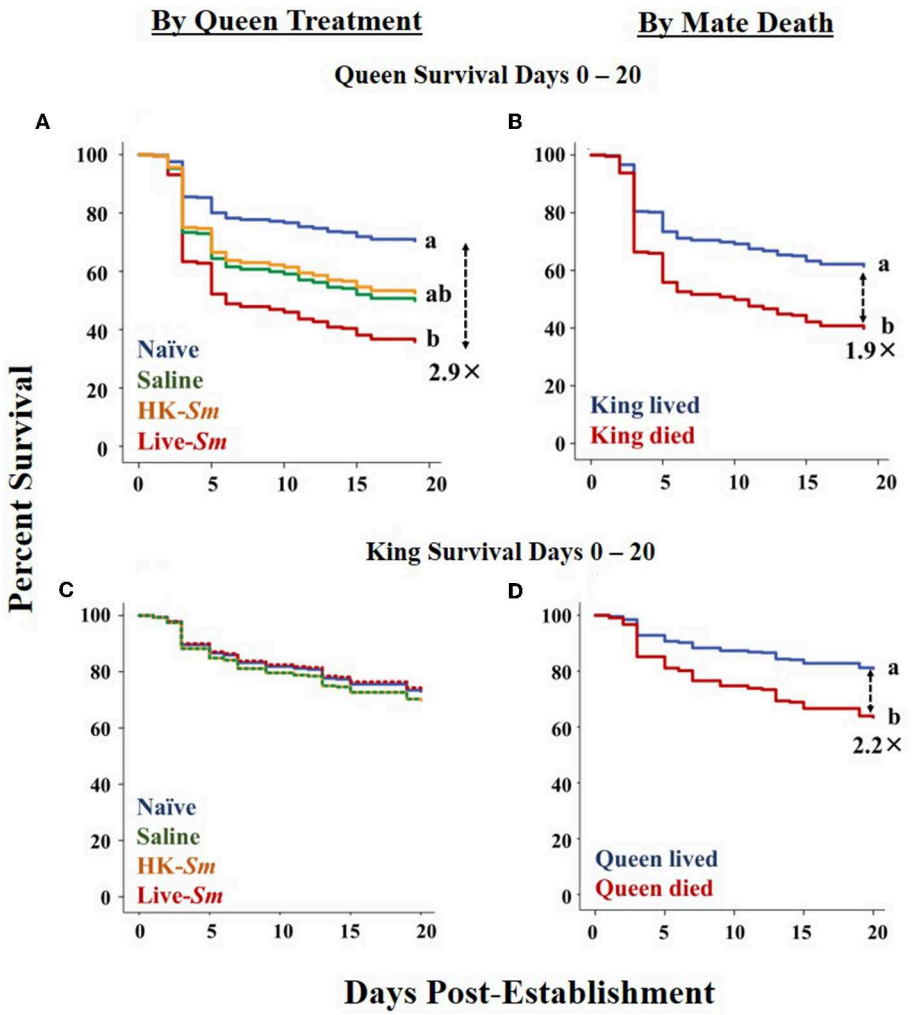


**FIGURE 2 |** Survival curves across the first 20 days post-establishment (Cox model). **(A)** Queen survival as a function of queen treatment. **(B)** Queen survival as a function of king death. **(C)** King survival as a function of queen treatment. The survival curves for kings mated to saline queens overlaps with those mated to HK-*Sm* queens. Similarly, there is overlap between the curves of kings mated to saline and live-*Sm* queens. **(D)** King survival as a function of the death of his queen. Dashed arrows with corresponding numbers indicate hazard ratios of death between the significant pairwise comparisons.

for the effects of onset of oviposition, queen and king mass had both significant main effects ($F = 8.6$, df = 1, 247, $p = 0.004$, **Supplemental Table 10**; $F = 5.2$, 1, 247, $p = 0.02$, **Supplemental Table 11**, respectively), and significant interactions with queen treatment ($F = 5.1$, df = 3, 247, $p = 0.002$; $F = 4.4$, 3, 247, $p = 0.005$, respectively). When queens were injected with saline, their mass positively correlated with egg count, producing 0.34 ± 0.0.08 SE eggs/mg. Given that queen mass ranged from 24.2 to 82.2 mg, the heaviest queens produced ∼19 more eggs on average than the lighter queens. Likewise, when queens were naïve, king mass positively correlated with egg count: for every mg of his mass, his queens produced 0.35 ± 0.03 SE more eggs. King mass also positively correlated with egg count when queens received the HK-*Sm* treatment (0.31 ± 0.19 SE eggs/mg). King mass did not appear to impact egg production when queens were infected with live *S. marcescens* (**Supplemental Table 11**). No other variable in the model was significant (**Supplemental Table 10**).

## Likelihood and Onset of Hatching

After controlling for the effect of onset of oviposition (Wald = 35.5, df = 1, $p < 0.001$), queen treatment was not a significant predictor of larval hatching (Wald = 1.8, df = 3, $p = 0.6$; **Supplemental Figure 3**; **Supplemental Table 12**). The death of the king, however, was a significant an independent predictor of how likely eggs were to hatch by the end of the 80-day period (Wald = 9.1, df = 1, $p = 0.003$). If the king lived, a colony was 3.3 times more likely to produce larvae than their counterparts with no kings. The onset of hatching was similar for colonies with and without surviving kings (**Supplemental Table 13**). The death of the queen also predicted if a colony produced larvae (Wald = 3.8, df = 1, $p = 0.05$). Colonies with surviving queens were 2.6 times more likely to produce larvae. As with the kings,

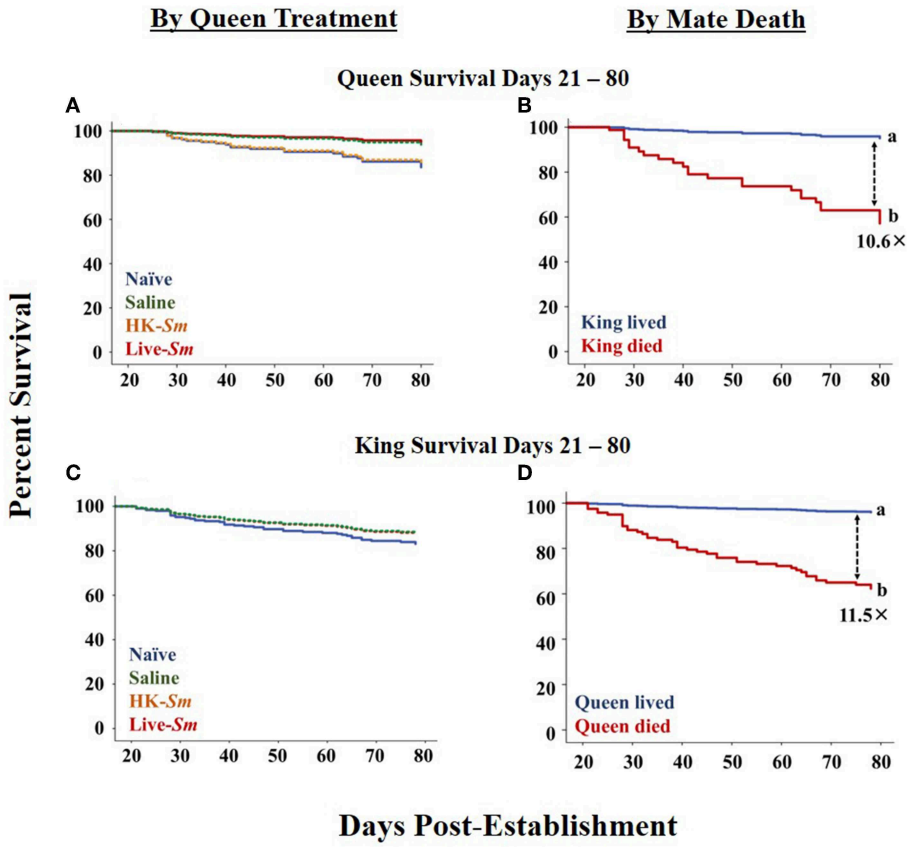


**FIGURE 3 |** Survival curves across days 21–80 post-establishment (Cox model). **(A)** Queen survival as a function of queen treatment. The survival curves for live-*Sm* and saline, and the naive and HK-*Sm,* treatments overlap. **(B)** Queen survival as a function of king death. **(C)** King survival as a function of queen treatment. The survival curves for kings mated to saline, HK-*Sm* and live-*Sm* queens overlap, and resemble a single line. **(D)** King survival as a function of queen death. Dashed arrows with corresponding numbers indicate hazard ratios of death between significant pairwise comparisons. Note that the magnitude of the hazard ratios of death when an individual's mate dies (B and D) are an order of magnitude higher on days 21–80 post-establishment than on days 0–20 (**Figures 2B,D**).

queen death did not impact the onset of hatching, only its likelihood. No other variables significantly predicted likelihood of hatching or the onset of hatching (**Supplemental Figure 4**; **Supplemental Tables 12, 13**).

## Hatching Success

After controlling for onset of oviposition ($F = 8.8$, df $= 1, 97$, $p = 0.004$), the presence or absence of the king was a significant and independent predictor of the percentage of eggs that hatched. On average, colonies with surviving kings hatched three times the proportion of eggs compared to those in which kings died (**Figure 5**). Queen treatment and queen death had no impact on percent hatching ($F = 0.1$, df $= 3, 97$, $p = 0.9$; $F = 0.5$, df $= 1, 97$, $p = 0.5$, respectively). No other factor was significant (**Supplemental Table 14**).

## Total Number of Larvae

After controlling for the significant effect of onset of oviposition ($F = 37.4$, df $= 1, 246$, $p < 0.001$), neither queen treatment nor king death were significant predictors of total larval count ($F = 0.6$, df $= 3, 246$, $p = 0.6$; $F = 1.9$, df $= 1, 246$, $p = 0.2$). The interaction "queen treatment × queen mass" approached significance ($F = 1.9$, df $= 3, 246$, $p = 0.06$; **Supplemental Table 15**). No other factor was significant (**Supplemental Table 15**).

## Proportion of Intact Colonies by Day 80 Post-establishment

Only 14 and 14.6% of the originally established colonies headed by HK-*Sm* and live-*Sm* injected queens were scored as intact colonies relative to 20 to 25% of the colonies founded by naïve and saline-injected queens, respectively. These differences, however, were not statistically significant ($\chi^2 = 3.9$, df $= 3$, $p = 0.3$).

# DISCUSSION

To understand the drivers and dynamics that may have led to the evolution of eusociality as a viable strategy in termites, we can use present-day incipient colonies to identify current selection pressures and extrapolate how pathogens may have promoted their social evolution. Moreover, because newly established

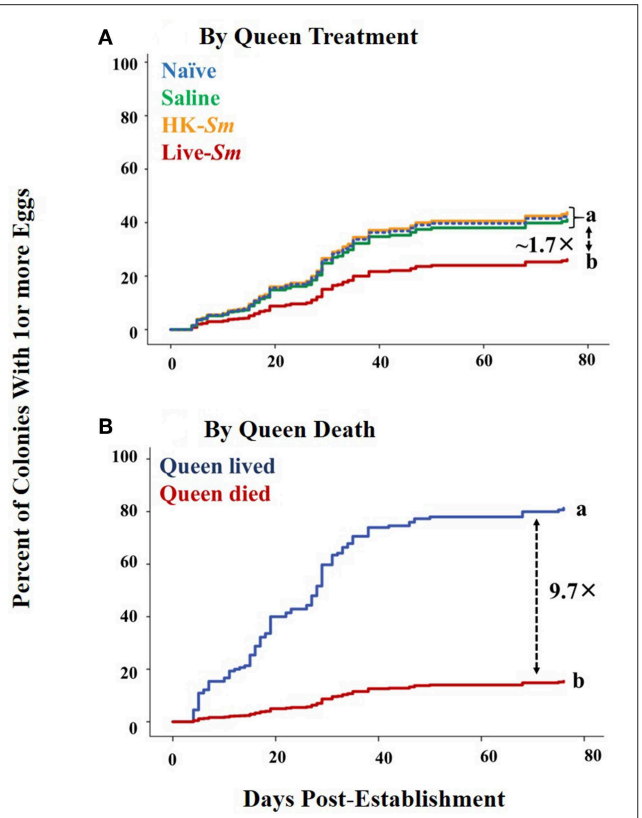


**FIGURE 4 |** Likelihood of oviposition across the first 80 days post-establishment (Cox models). **(A)** Likelihood of oviposition as a function of queen treatment. Of the established 271 incipient colonies, only 120 (44.3%) oviposited at least one egg within the first 80 days post-pairing ($n = 45$ colonies headed by naïve queens + naïve kings; $n = 22$ by saline queens + naïve kings; $n = 26$ by HK-*Sm* queens + naïve kings; $n = 27$ by live-*Sm* queens + naïve kings). **(B)** Likelihood of oviposition as a function of queen death. Differing letters denote statistical significance. Dashed arrow with corresponding number indicate how many times greater the likelihood of oviposition is between significant pairwise comparisons.

colonies transition during their ontogeny from solitary → subsocial → eusocial (whether facultatively or obligatorily; Boomsma and Gawne, 2018), we can tease apart the role and significance of each of these selection pressures against the backdrop of increased social complexity.

There are multiple factors influencing insect social evolution (**Figure 1**, and references therein). Here we focus on pathogenic pressures, which are particularly stringent in termites (Rosengaus and Traniello, 1993b; Schmid-Hempel, 1998; Rosengaus et al., 2000, 2003, 2011; Calleri et al., 2006). In the present study, we explored how colony foundation under pathogenic stress results in a bottleneck that is comprised of the two "fitness checkpoints" (**Supplemental Figure 5**). Our analyses also indicate that mate assistance mitigates some of the pathogen-induced fitness-related costs. Given that kings play such a significant role in present-day colonies, we propose that a king's presence and participation in tasks associated with colony establishment were crucial during the origins and maintenance of termite sociality. Below, we first introduce the amended checkpoint model of colony foundation, followed by a description of the role of pathogens during each checkpoint. We then discuss how mate assistance and biparental care may help mitigate fitness costs during these stages. Finally, we place our findings within the broader context of current theory in the evolution of insect sociality.

## The "Checkpoints" Model of Colony Foundation

Cole et al. (2018) identified factors that influenced the successful establishment of a colony during the first 30-days. Based on these data, a colony passes through two "all-or-nothing" fitness checkpoints: initial survival (checkpoint 1) and oviposition (checkpoint 2). By expanding our census to encompass 80 days of colony life (**Supplemental Figure 5**), which now includes the subsocial phase, and using a new set of alates from different natal nests from those used by Cole et al. (2018), we not only confirm and replicate our initial observations, but also refine this model.

Based on the current data, checkpoint 1, characterized by a steep decline in the survival of reproductives, extends through the first 20 days post-establishment rather than the first 10 days as reported previously (Cole et al., 2018; **Figures 2**, **3**). Checkpoint 2, which encompasses the onset of oviposition, now extends to day 40 (**Figure 4**). Irrespective of bacterial infection of one or both members of the royal pair, these two checkpoints result in a significant bottleneck with a massive loss of colonies (present study; Cole et al., 2018). These results, in combination with previous work (Rosengaus and Traniello, 1993b; Shellman-Reeve, 1997a; Hartke and Rosengaus, 2013; Chouvenc et al., 2014; Cole et al., 2018), supports the assertion that the incipient stage of colony foundation in termites is a vulnerable time, influenced by multiple intrinsic and extrinsic factors, including pathogens. Once oviposition begins, the colony enters the "initial growth phase" (Cole et al., 2018), characterized by the presence of eggs and the first dependent hatchlings (**Supplemental Figure 5**). We did not follow colonies past this phase, yet, we assume that once the hatchlings become independent, functional workers (∼3rd instar; Rosengaus and Traniello, 1993b; Crosland et al., 1998), the colony reaches the ergonomic growth phase (Oster and Wilson, 1978).

## Pathogenic Stress Negatively Influences the First Two Checkpoints

### Checkpoint 1: Survival

Our initial 80-day model of survival showed a clear inflection point at day ∼20 (**Supplemental Figure 1**), suggesting that different selection pressures influence the survival of reproductives at different times during colony ontogeny. Separating our survival analyses within each sex into the periods before and after day 20 post-establishment, we identified relatively short-term effects of pathogenic stress on the survival of treated queens (**Figure 2A**). There was no evidence that pathogenic stress affected queen survival from days 21–80 (**Figure 3A**), or that queens infected their kings at any time throughout the census period (**Figures 2C**, **3C**). Likewise, Cole et al. (2018) found that direct exposure of kings to live *Serratia*

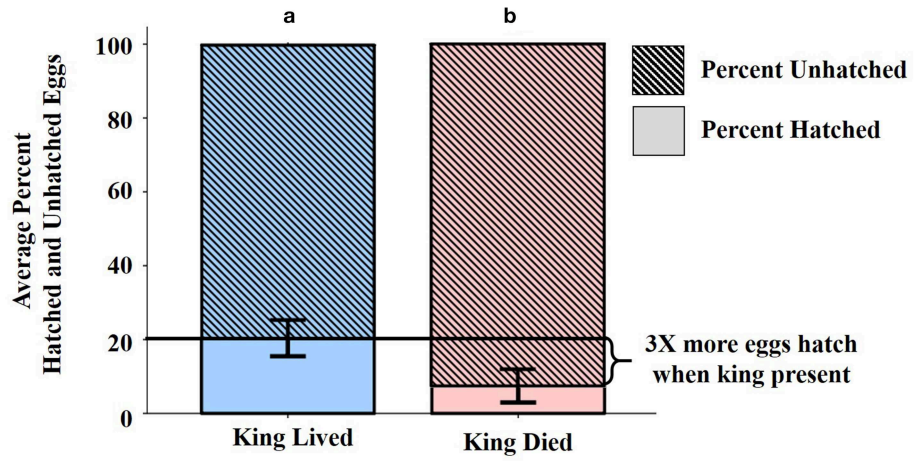


**FIGURE 5 |** Average percent hatched and unhatched eggs as a function of king death. When kings lived, an average of 20% of a colony's eggs hatched compared to an average of only 7% when kings died. Differing letters denote statistical significance. Error bars represent ± 2 SD around the average.

also reduced their survival. These two studies strongly suggest that direct pathogenic exposure reduces survival and thus, decreases the number of colonies that pass through the first checkpoint (**Supplemental Figure 5**).

### Checkpoint 2: Oviposition

We compared both the likelihood and onset of oviposition as a function of queen treatment. Live-*Sm* queens were the least likely to produce eggs, with only 26.1% of queens laying at least one egg compared to the ∼42% of any of the other three treatments (**Figure 4A**). The likelihood of oviposition by live-*Sm* injected queens was the lowest relative to all other three treatments, hinting at possible physiological and fitness-related costs associated with bacterial exposure throughout checkpoint 2. When focusing only on those colonies that produced at least one egg, the majority began oviposition between days 15–35 (**Supplemental Figure 2**). Only 4% of these colonies initiated oviposition after day 40 (**Supplemental Figure 2**). In agreement with Cole et al. (2018), the present data indicate that queen treatment did not significantly influence the onset of egg laying even after expanding the colony census by an additional 50 days from Cole et al. (2018; **Supplemental Figure 2**). This lends support to the idea that the timing of oviposition is a fixed trait, impervious to pathogenic stress.

### Initial Growth Phase

Although queen treatment was not a significant predictor of hatching success, only ∼14% of colonies exposed to *S. marcescens* (live or heat-killed) were intact, having two surviving parents and larvae by day 80, compared to the 25 and 20% of intact naïve and saline colonies, respectively. These proportions did not differ significantly from each other, however, we suspect this is due to the low sample size of intact colonies after the bottleneck of checkpoints 1 and 2. The lack of statistical significance do not rule out the possibility of longer-term, cascading effects of pathogenic stress beyond colony foundation.

Our present data, together with that of Cole et al. (2018), demonstrate that *S. marcescens* has relatively short-term effects, reducing the survival of the royal pair during checkpoint 1 and the likelihood of oviposition during checkpoint 2 (**Supplemental Figure 5**). Bacterial exposure did not affect the onset of oviposition, nor egg quality (Cole et al., 2018). We argue that any strategy that ameliorates the fitness costs during these checkpoints would provide a significant fitness advantage and therefore, should be favored by natural selection. The current data provide evidence that the presence of the king (i.e., his mate assistance and biparental care) help realize this advantage.

## Mate Assistance Increases Colony Success

### Checkpoint 1: Mate Presence Enhanced Survival

In agreement with previous work (Shellman-Reeve, 1997a; Rosengaus et al., 2000), both queens and kings benefited from the presence of a living mate throughout the first 80 days of colony life (**Figures 2**, **3**). Interestingly, the magnitude of these benefits differed when comparing days 0–20 vs. 21–80. Within the first 20 days post-establishment, queens without a mate were almost twice as likely to die, but, during days 21–80, these queens were 10.6 times more likely to die. Kings without a mate exhibited similar patterns, showing 2.2 and 11.5 times higher likelihood of death, relative to accompanied kings on days 0–20 and 21–80, respectively. Thus, in terms of survival, the persistence of a royal pair helps to shield the new colony against the many stressors of colony foundation, including pathogenic stress.

Mechanisms underlying these advantages could include mutual grooming to remove cuticular pathogens (Cruse, 1998; Rosengaus et al., 2000), proctodeal exchanges resulting in the transfer of protein-rich secretions (Shellman-Reeve, 1990) and/or the exchange immune-factors via trophallaxis (e.g., Traniello et al., 2002; Hamilton et al., 2011; but see Mirabito and Rosengaus, 2016), all of which can mitigate the threat

of pathogens. Evolutionarily, such co-dependency between queens and kings could have reinforced the reproductive caste's longer-lifespans and two-parent colony foundation, presumed steppingstones in the evolution of eusociality (Boomsma, 2009; Davies and Gardner, 2018; Klug, 2018).

### Checkpoint 2: Kings Enhance Oviposition and Overall Egg Production

Surviving queens mated to heavier kings (i.e., assumed to have greater energetic/metabolic resources) were more likely to oviposit and initiated oviposition sooner than queens mated to lighter kings (**Supplemental Table 8**). With respect to the overall number of eggs laid by day 80, naïve, saline-, and HK-*Sm*-treated queens mated with well-resourced kings produced a greater number of eggs than those mated to lighter kings (**Supplemental Tables 9, 11**). Interestingly, this effect was absent in live-*Sm*-injected queens, suggesting that the contributions made by a king are insufficient to boost the number of eggs produced by an infected queen. Thus, total egg production appeared to suffer due to pathogenic stress during the first 80-days post-establishment.

The fact that king mass consistently influenced the queen's reproductive output (current study; Cole et al., 2018; Chouvenc, 2019), suggests that well-resourced kings provide superior mate assistance, which in turn, increases colony fitness. Such assistance could be in the form of transfer of nutritious secretions via proctodeal trophallaxis (Shellman-Reeve, 1990), aid in housekeeping tasks such as nest construction and sanitation (Rosengaus and Traniello, 1991), and the promotion of ovarian maturation (Shellman-Reeve, 1999; Brent and Traniello, 2001; Brent et al., 2005). Behavioral observation are needed to pinpoint which of these forms of assistance play a role, if any.

### The Initial Growth Phase: Kings Enhance Hatching Success

The likelihood of a colony producing larvae, and the proportion of hatched eggs, were most influenced by the presence of a surviving king (**Supplemental Figure 3**, **Supplemental Tables 12, 14**). On average, by day 80 post-establishment, colonies with surviving kings had three times the hatching success of those without kings. Hence, the king's presence, but not the queen's, significantly enhances hatching success. These data are in agreement with Shellman-Reeve (1990) who suggested that kings specialize in brood care, although Rosengaus and Traniello (1991) found no sex-based bias in brood care.

In spite the significant role that kings have on colony fitness between days 21–80, we were surprised that ∼80% of eggs from colonies with a living king never hatched (**Figure 5**), indicating that colonies still face many stressors. There are at least three possible explanations for the low hatching success recorded in all colonies. We observed some eggs across all treatments that appeared to be infected with various bacterial or fungal pathogens, or were desiccated and misshapen (**Supplemental Figure 6**). There is also a high probability that kings and queens consumed some of the eggs. Cannibalism in termites is common (Sun et al., 2018) and has been considered to be a mechanism by which termites cope with nitrogen limitation (e.g., Hunt and Nalepa, 1994). By consuming eggs, the royal pair can recoup some of the nitrogen contained within those eggs. Additionally, culling a proportion of their eggs would reduce the number of dependent larvae needing future attention (Nalepa, 1988, 2010). By tailoring the number of larvae in a context-dependent fashion, the royal pair could optimize the energy allotted to all of the demands of colony foundation. Examples of culling are seen in a variety of taxa (e.g., Mehlis et al., 2009; Miller and Zink, 2012; Takata et al., 2013).

## Monogamy, Mate Assistance, and Biparental Care: Preadaptations in the Evolution of Termite Eusociality

The hostile environment faced by dispersing alates may have promoted the evolution of monogamy, mate assistance, king longevity and biparental care in the termite ancestor (Hamilton, 1978; Nalepa, 1991, 2011; Hunt and Nalepa, 1994; Shellman-Reeve, 1997a,b; Thorne, 1997; Korb, 2008a; Korb and Heinze, 2016). We argue that, in addition to the many ecological pressures faced by new kings and queens (see **Figure 1** and references therein), pathogenic pressures also selected for these same life-history traits that ultimately, served as pre-adaptations for the evolution of termite sociality.

In the present study, we used the "subsocial" phase of colony development in a basal termite species that is consistent with the proposed ancestral life style of one-piece nesting (Abe, 1987; Nalepa, 1988, 2010, 2011; Thorne, 1997; Inward et al., 2007; Klass et al., 2008; Korb, 2008a,b; Korb and Heinze, 2008; Bourguignon et al., 2014; Nalepa and Arellano, 2016; Harrison et al., 2018) to speculate on the possible role of pathogens in termite evolution. Our data show that in the face of disease, the presence of kings enhance colony fitness in several important ways (**Figure 6**; **Supplemental Figure 5**). The enhanced queen survival (**Figures 2**, **3**) and enhanced hatching success (**Figure 5**) due to the king's presence may explain, in part, the longer lifespan of termite kings relative to that of drones of the social Hymenoptera, who die shortly after mating (Wilson, 1971). In the latter, any potential contributions a drone makes toward his queen or progeny can only occur during copulation. In termites, his continued contributions appear to have a sustained impact on colony fitness. If these results accurately reflect the circumstances under which termites evolved, then it is reasonable to hypothesize that long-lived ancestral males who provided mate assistance for longer periods would have reaped enhanced fitness benefits, reinforcing a monogamous mating strategy. This in turn, would have set the stage for prolonged family life.

Boomsma (2009), for example, suggested that under conditions of lifetime monogamy by the diploid parental generation, genetic gains would have been equivalent whether progeny opted for personal reproduction or for becoming helpers at the nest. Workers would have still had on average, half of their genetic makeup represented. However, Korb and Schneider (2007) found that average relatedness in colonies of *Cryptotermes secundus* did not predict whether termite

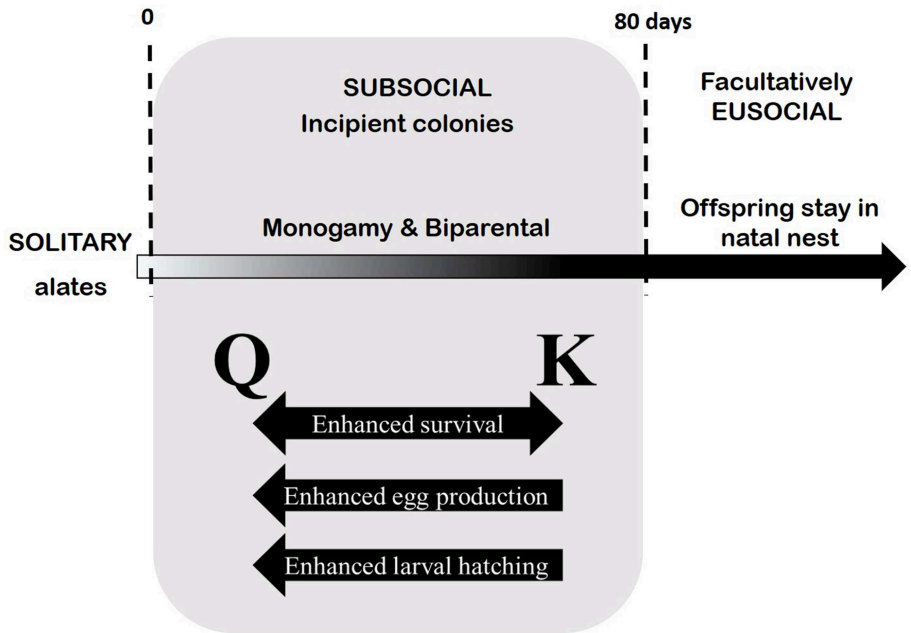


**FIGURE 6 |** Ontogeny of present-day *Z. angusticollis* colonies informed by our current data. Gray box summarizes the results of our experiment manipulations, which inform our understanding of the dynamics between the queens and kings during the subsocial phase of colony life (~ first 80 days post-establishment). Specifically, the continued presence of the king and/or his stored resources (i.e., mass) result in enhanced fitness, as measured by queen survival, egg production and larval hatching success.

workers remained in the nest or developed into dispersing adults. Korb (2008a,b) and Korb and Heinze (2016) suggest that the harsh environment faced by dispersing individuals selects for progeny to remain in the nest. Such false-workers in one-piece nesting species reap direct fitness by inheriting the nest and the reproductive position. Although no consensus exists yet, it is likely that both the equivalent fitness gains derived from monogamy in the diploid king and queens, together with the harsh ecological pressures faced by dispersing alates, played significant roles in the origins of termite eusociality. Additional benefits of colony life could have further promoted social cohesion of early termites, including improved nitrogen recycling (Potrikus and Breznak, 1981; Machida et al., 2001), the reproductive specialization of queens (Oster and Wilson, 1978), and notably, colony-wide social immunity (Traniello et al., 2002; Cremer et al., 2018). We argue that pathogens, along with other environmental stressors, could have posed significant selection pressures that reinforced monogamy, king longevity, mate assistance, and brood care. The data we present here support the maintenance of two-parent colony foundation in termites which may have promoted monogamy thereby paving the way for inclusive fitness benefits for non-reproducing termites as predicted by Boomsma (2009, 2013). Our data also support the costs associated with dispersal, as alates that attempt to found new colonies face a reduced risk of survival, even in the absence of predators. Such costs of dispersal, in combination with the probability of becoming a reproducing individual likely promoted staying in the natal nest (Korb, 2008a; Korb and Heinze, 2016).

## CONCLUSION

Dispersing termite alates face a hostile environment, including pathogens. Even under ideal conditions, few incipient colonies pass through the bottleneck (checkpoints 1 and 2). Yet, the presence of pathogens further exacerbates colony failure. An acute sublethal bacterial infection reduces alate survival. For those that survive, pathogenic stress has cascading consequences on several fitness-related parameters. Our results indicate that mate assistance and biparental care can mitigate some of the negative effects of infection. By identifying some of the factors that currently influence colony establishment, we can infer some of the conditions and pressures faced by the termite ancestor that may have led to the origins and maintenance of termite eusociality.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## AUTHOR CONTRIBUTIONS

EC and RR collected termite colonies in the field, set up incipient colonies in the lab and prepared the manuscript. EC treated queens and performed periodic census and ran statistical analyses. RR financially supported collection trips.

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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.2019.00473/full#supplementary-material

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# Synergies Between Division of Labor and Gut Microbiomes of Social Insects

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Social insects maximize resource acquisition and allocation through division of labor and associations with microbial symbionts. Colonies divide labor among castes and subcastes, where the plasticity of caste roles decreases in clades with higher social grades. Recent studies indicate that specific castes may also foster distinct gut microbiomes, suggesting synergies between division of labor and symbiosis. The social organization of a colony potentially partitions evolutionary persistent microbial partners to optimize symbioses and complement division of labor. However, research in this area has received limited attention. To elucidate if a structured microbiota is adaptive, we present three testable predictions to address consistent community structure, beneficial functions, and selection for microbiota that support caste roles. First, we posit that social insect groups spanning lower to higher social grades exhibit increasingly distinct caste microbiomes, suggesting that structured microbiomes may have evolved in parallel to social complexity. Second, we contend that the development of these microbiomes during colony maturation may clarify the extent to which they support division of labor. Third, we predict that mature social insect colonies with the most extreme division of labor demonstrate the strongest distinctions between caste microbiomes, carrying the greatest promise of insight into microbiome composition and function. Ultimately, we hypothesize that caste-specific microbiomes may enhance symbiotic benefits and the efficiency of division of labor, consequently maximizing fitness.

**Keywords: symbiosis, division of labor, gut microbiome, major evolutionary transition, superorganism**

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## INTRODUCTION

Organisms are selected to optimize resource use through their own actions and interactions, in turn maximizing reproductive success. Ants, termites, and the social bees and wasps compose more than half of the biomass of land-dwelling insects (Hölldobler and Wilson, 2009) and have comprehensive ecological impacts as pollinators, predators, herbivores, and decomposers (Bignell and Eggleton, 2000; Richter, 2000; Aizen et al., 2008; Del Toro et al., 2012). Their success has, in

part, been attributed to their division of labor (Oster and Wilson, 1978), where castes within the colony optimally divide tasks and thus improve the efficiency by which resources are amassed, distributed, and utilized (Anderson and Ratnieks, 1999; Duarte et al., 2011).

Microbial gut symbionts augment social insect metabolism and defense, further allowing the colonies to monopolize various resources. Termites digest recalcitrant plant substrates with obligate gut symbionts that degrade lignocellulose and upgrade dietary nitrogen (Cleveland, 1923; Brune, 2014). Social corbiculate bees host bacterial communities that aid in nutrient acquisition from pollen (Kwong and Moran, 2016; Kesnerova et al., 2017; Zheng et al., 2017). Herbivorous ants maintain gut bacteria that offset host dietary and metabolic limitations through amino acid supplementation, nitrogen recycling, and catabolism of glucose and citrate (Russell et al., 2009; Hu et al., 2018; Sapountzis et al., 2018). Gut microbes also contribute to disease defense in bees and termites by imparting pathogen colonization resistance (Koch and Schmid-Hempel, 2011; Peterson and Scharf, 2016; Raymann et al., 2017; Inagaki and Matsuura, 2018), further enabling host persistence and resource acquisition across environments.

The colony gut microbiota may be organized and optimized to complement division of labor, with castes partitioning the gut microbiota to support specialized roles. This could potentially enhance symbiont productivity to meet host needs and increase the efficiency of division of labor. A partitioned microbiota may incur a selective benefit if it improves resource acquisition and allocation. To begin to assess this hypothesis, we review division of labor in the social insects, known gut microbiota compositional differences between castes and subcastes, and the role of social interactions in influencing persistent gut microbiota. We then discuss three future avenues of research that may allow insights into putative interfaces between social organization and microbiomes: (1) structure and function of colony microbiomes across social insects that vary in social complexity, (2) development of caste microbiomes during colony maturation, and (3) caste-specific gut microbiomes within colonies with the most extreme division of labor.

## DIVISION OF LABOR IS A CORNERSTONE IN SOCIAL INSECT BIOLOGY

Colony-level social complexity of social insect species is dictated by division of labor between worker and reproductive castes. Workers perform non-reproductive tasks such as brood care, defense, and foraging while the reproductive caste comprises one or a few individuals that secure colony fecundity. Variation in the degree of this division of labor can be interpreted along an evolutionary gradient of increasing social complexity and decreasing individual-level reproductive plasticity (Taylor et al., 2019) (**Figure 1**). In the most derived social insects, workers are morphologically precluded from attaining a reproductive role, resulting in strong suppression of inter-caste reproductive conflict (Boomsma and Gawne, 2018).

Morphological reproductive-worker differentiation and repression of conflict between interdependent castes represent a major evolutionary transition to higher complexity (Maynard Smith and Szathmáry, 1995; West et al., 2015). This transition aligns the fitness interests of all colony members, with selection acting more on the colony than on individual colony members. The developmentally irreversible distinction between worker and reproductive castes makes their division of labor comparable to that of the germline and soma, and they consequently have come to be regarded as superorganisms (Wheeler, 1911; Boomsma and Gawne, 2018).

The division of non-reproductive labor among workers also becomes more specialized along the sociality gradient (**Figure 1**). A variety of temporal, physical, and spatial factors may direct division of non-reproductive labor (Duarte et al., 2011). Temporal subcastes, with workers transitioning from tasks within to outside the nest as they age (e.g., nurse bees transitioning to foragers), and physical subcastes with morphological distinctions exhibiting task specialization (e.g., minor and major workers and soldiers), are commonly identified across social insects (Oster and Wilson, 1978) (**Figure 1**). Superorganismal clades exhibit the highest degree of specialization among workers, where their cohesive actions constitute colony-level adaptations for resource acquisition, comparable to the specialized functions of somatic tissues in a multicellular organism (Wheeler, 1911; Boomsma and Gawne, 2018).

## CURRENT KNOWLEDGE ON CASTE-DISTINCT GUT MICROBIOMES

Microbial symbioses and division of labor are well-known to enhance resource acquisition, but few studies have compared gut microbiomes of castes and subcastes (**Figure 1**). In some social insect clades, the reproductive caste microbiome is distinct and drastically simplified compared to that of the worker caste. Foraging lower termite reproductives generally lack symbiotic protists that dominate the worker guts (Shimada et al., 2013; Inagaki and Matsuura, 2016), and the reproductive caste of Termitidae termites shows reduced diversity and disparate bacterial community composition (Otani et al., 2019) compared to the worker caste (Dietrich et al., 2014; Otani et al., 2014, 2016, 2019). Honey bee queens also host a simplified bacterial community that is significantly reduced compared to workers (Kapheim et al., 2015; Tarpy et al., 2015; Anderson et al., 2018). Albeit limited, data on the gut microbiomes of ants suggest some differences between reproductive and workers (Johansson et al., 2013; Brown and Wernegreen, 2016). The gut microbiomes of social wasps remain understudied, although some clades appear to retain consistent gut microbes (Stefanini et al., 2012, 2016; Gruber et al., 2019; Suenami et al., 2019).

Differences between worker subcastes have almost exclusively been explored in termites and bees. Studies suggest that the gut microbiome composition of worker bees varies with age and could be task-dependent, although patterns inferred are

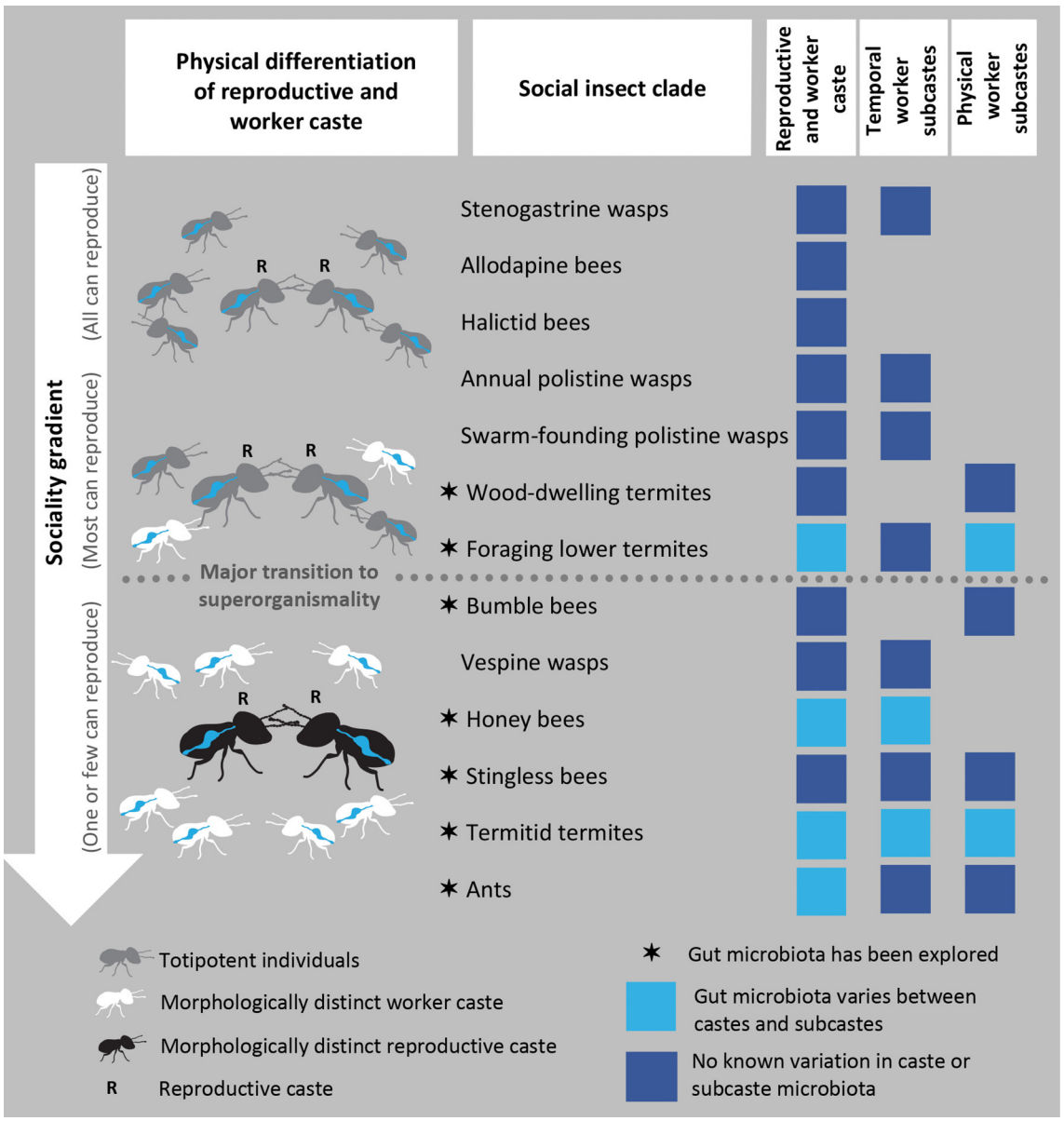


**FIGURE 1 |** The sociality gradient and gut microbial symbionts of social insects. The sociality gradient is illustrated by the greyscale changes in the insects, indicative of reductions in individual reproductive plasticity. The gradient begins with colonies where totipotent workers help reproductives through adulthood, to which subsocial family life is a critical evolutionary precursor and culminates in superorganismal colonies with morphologically distinct castes. The position of clades along the gradient is adapted from Boomsma and Gawne (2018). Clades for which the gut microbiota has been explored are marked with a star, while evidence or lack thereof on distinct (sub)caste microbiomes is represented by light and dark blue squares, respectively. The first column of boxes illustrates that all clades divide reproductive labor between the reproductive and worker caste. The second and third columns signify if members of the clade have specialized division of non-reproductive labor among temporal and physical worker subcastes (temporal: bees, Seeley, 1982; Wille, 1983; termites, Watson and Sewell, 1985; ants, Hölldobler and Wilson, 1990; physical: bees, Goulson et al., 2002; Spaethe and Weidenmüller, 2002; Evans, 2006; wasps, Jandt and Toth, 2015; Li et al., 2015; Du et al., 2017; Grüter et al., 2017; termites, Korb and Thorne, 2017).

subtle (Guo et al., 2015; Anderson et al., 2018; Jones et al., 2018). Similarly, the fungus-farming subfamily of the Termitidae exhibits differences in gut bacterial composition related to food-processing by temporal subcastes (Hinze et al., 2002; Hongoh et al., 2006; Li et al., 2015, 2016). Termites also present differences among physical subcastes, where soldier microbiomes can be distinct from other subcastes (Hongoh et al., 2006; Shimada et al., 2013; Inagaki and Matsuura, 2016; Otani et al., 2019, but see Berlanga et al., 2011), and in highly polymorphic termites, such as the genus *Macrotermes*, minor and major workers and soldiers display variable microbiomes (Hongoh et al., 2006; Schnorr et al., 2019).

## SOCIALITY SUPPORTS PERSISTENT SYMBIOTIC GUT MICROBIOTA

For an individual insect, the repeated remodeling of the gut and shedding of the microbiota throughout development can impede extracellular gut symbionts from persisting within individuals and over generations (Engel and Moran, 2013). Group living overcomes this challenge by providing access to microbiota through social interactions (Troyer, 1984; Nalepa et al., 2001; Lombardo, 2008; Engel and Moran, 2013).

Microbiota characteristic of natal social insect colonies have been suggested to be transmitted by reproductives in colonies founded independently (Benjamino and Graf, 2016; Meirelles et al., 2016; Stefanini et al., 2016; Diouf et al., 2018), or by workers and reproductives (Kwong et al., 2014) in colonies that are founded dependently, such as honey bees and army ants. Workers within colonies then stabilize the gut microbiota through intracolonial transfer of symbionts; newly-eclosed microbe-free workers typically receive inocula from mature workers through fecal-oral transmission (Wheeler, 1984; Ohkuma and Brune, 2010; Powell et al., 2014; Lanan et al., 2016). Additional oral exchanges, interactions, and the shared nest environment and resources facilitate continuous microbiota transmission and homogenization between colony members (Martinson et al., 2012; Stefanini et al., 2012; Powell et al., 2014; Zhukova et al., 2017). The reliable transmission of gut microbes between and within colonies thus allows transgenerational persistence of microbial communities.

Host transmission of heritable extracellular symbionts has been hypothesized to result in long-term associations between some social insects and specialized microbiota. Termites, social corbiculate bees, and clades of ants consistently host microbial phylotypes over evolutionary timescales, as indicated by patterns of phylogenetic congruence of microbial communities with hosts (Russell et al., 2009; Dietrich et al., 2014; Sanders et al., 2014; Kwong et al., 2017; Lukasik et al., 2017; Bourguignon et al., 2018; Sapountzis et al., 2019). Although this indicates vertical transmission across generations, most symbioses are characterized by the presence of at least some degree of host switching, suggesting that horizontal transmission persists across host clades (Koch et al., 2013; Sanders et al., 2014; Kwong et al., 2017; Bourguignon et al., 2018). Nevertheless, the extensive evolutionary histories of hosts and symbionts are presumably facilitated by social interactions and insect physiological or morphological characteristics that promote specific microbial partners (c.f. Kwong and Moran, 2015; Lanan et al., 2016; Sapountzis et al., 2019).

## DISCUSSION

### Is the Colony Gut Microbiota Optimized Through Social Organization?

We hypothesize that hosts may organize and optimize the gut microbiota among colony members to increase the benefits of symbioses and social organization. Consistent caste-specific differences in honey bee and termite microbiomes suggest that microbial community compositions have been selected over evolutionary time to align with caste roles. If these caste-specific microbiomes enhance productivity of symbioses and efficacy of division of labor, they may be selected for as an emergent property of the combined effects of division of caste and microbiome labor.

Hosts are under strong selection to promote a beneficial microbiota; particularly in semi-closed systems like the gut (Foster et al., 2017). While microbes may compete within the gut ecosystem, potentially reducing symbiont expression of cooperative traits, hosts wrangle microbiota into stable and productive communities (Frank, 1996; Coyte et al., 2015). Hosts may shape microbiomes through mechanisms that regulate the immigration of microbes, support specific microbial community members through immune, physiological or dietary responses, and compartmentalize microbial communities (Frank, 1996; Foster et al., 2017), which in the social insects can occur both at the level of individuals and the colony. This could increase the net benefit of symbioses because optimization of microbiomes within specific castes may promote a simplified microbiota to meet distinct needs, enhancing the productivity of individual communities, while the colony-level conglomerate remains diverse.

Conclusions about optimization of symbiotic communities require elucidation of their compositions, functions, and impacts on host reproductive success. Since some microbes within a community could be driven by diet, gut morphology and physiochemistry, social interactions, or environmental exposure without further implications to the host, we propose three necessary levels of evidence to elucidate symbiotic benefit: (a) identification of consistent microbial communities within clades and castes, (b) characterization of conserved functions and benefits to hosts, and (c) determination of how hosts select for microbial community compositions and functions. With these needs in mind, we propose three focal areas that we believe can provide insight into the implications of structured colony microbiomes.

### Caste Microbiomes Along the Sociality Gradient

The distinctiveness of caste microbiomes should predictably increase along the sociality gradient. In lower social grades, all colony members remain totipotent and typically undergo physical remodeling when transitioning to a different caste (Roisin, 1990; Sumner et al., 2010), which may prevent strong specialization of their gut microbiota. In contrast, early developmental determination of committed castes may fine-tune the composition of microbial communities over their lifetime to meet divergent needs, resulting in distinct microbiota structure, and function. Termites and wasps are promising models to test this prediction as they have representative clades across the sociality gradient (**Figure 1**).

Social structure constrains the level of adaptation and thus the potential disparity in caste and subcaste microbiota. In social grades that have not undergone the major transition to superorganismality, the distinct microbiomes may represent

individual-level adaptations, and host interests in direct fitness benefits may limit specialization (Boomsma and Gawne, 2018; Cooper and West, 2018). After the major transition, however, workers can only improve their reproductive success through indirect fitness benefits gained at the colony level; thus, the colony acts as a fitness maximizing agent that can develop group-level adaptations (Gardner and Grafen, 2009), such as distinct caste and subcaste microbiota that optimize resource use and maximize colony fitness.

## Colony and Microbiota Development

The microbiomes of organisms are characterized by shifts associated with changing physiology and needs over the lifetime of the individual, and social insect colonies likely undergo comparable processes, from founding (possibly excluding taxa with dependent founding) through growth and development to maturity (left part of **Figure 2**). If caste specificities in microbiomes play important roles for colony reproductive success, the characterization of their development over colony maturation, during which caste roles are established and maintained, may allow us to corroborate functional predictions aligned with the division of labor.

As colonies grow, workers liberate reproductives from non-reproductive tasks, and both worker and reproductive castes become increasingly specialized (Wheeler, 1911; Oster and Wilson, 1978). Similarly, we predict that the structure and function of worker and reproductive gut microbiomes diverge during colony growth (**Figure 2**). The gut microbiomes of termite reproductives indicate a transition from diverse to simplified communities from founding to maturity (Shimada et al., 2013; Benjamino and Graf, 2016; Inagaki and Matsuura, 2016; Diouf et al., 2018; Otani et al., 2019) (**Figure 2**), which has been suggested to be a consequence of specialization on reproduction and dependence on workers (Chouvenc and Su, 2017). Similarly, the gut microbiome of honey bee queens exhibits slight changes with age (Tarpy et al., 2015; Anderson et al., 2018), suggesting that physiological and dietary changes related to reproductive specialization and duration may influence gradual shifts the reproductive microbiota. Additionally, as the number of workers increases, temporal and physical subcastes may develop unique microbiota, where age-dependent tasks, environments, and diets could influence microbial community composition. Exploration of changes in host characteristics and microbiota during colony development could thus clarify mechanisms of selection and caste complementarity, while examining changes in the functional microbiome and its influence on colony growth may elucidate how a divided microbiome supports colony maturation and the accretion of fitness benefits. Secondarily, the significance of independent or dependent colony founding should explored, as it independent founding may cause a microbial bottleneck that reduces both the stability and diversity

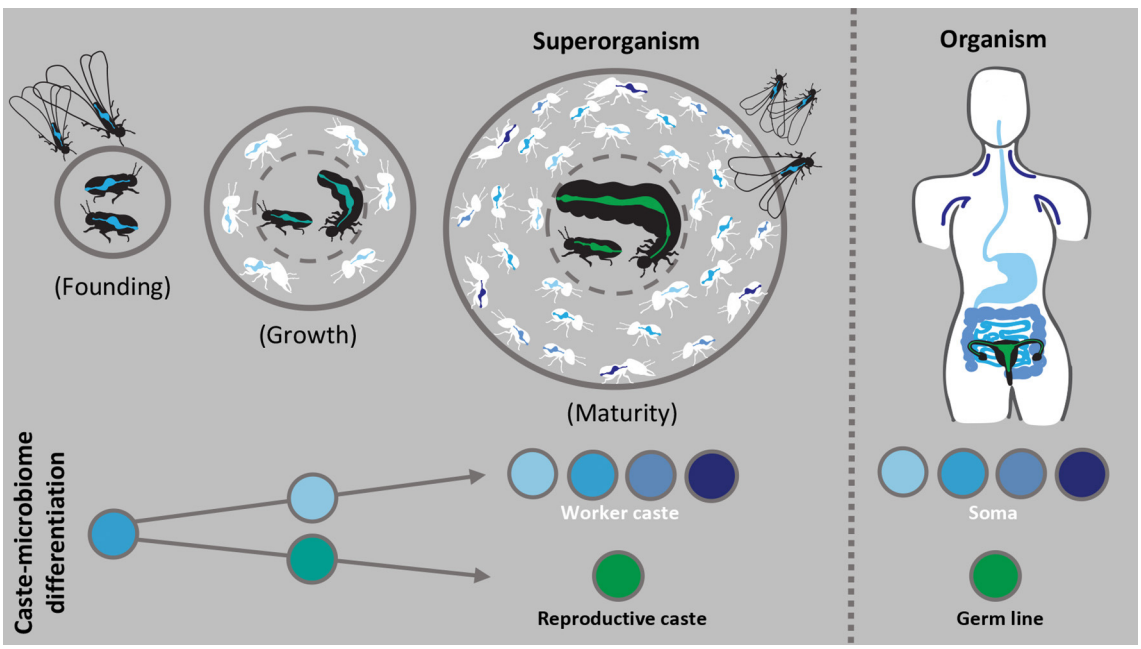


**FIGURE 2 |** Caste-specific microbiomes during growth and maturity of the superorganism. The left panel illustrates how gut microbiomes (spectrum of blue to green) of the reproductive (black) and worker caste (white) may structurally and functionally differentiate during the development of an independently founded colony. Such colonies undergo ergonomic growth before reaching maturity, during which caste roles establish and gut microbiota may similarly diverge. Reproductive gut microbiota (green) likely simplifies in structure and function while worker microbiota (blue) remains relatively diverse and may be partitioned among subcastes. The right panel proposes that the general structure and function of the superorganism microbiota could be analogous to that of multicellular organisms, in this case microbiota of human tissues. The major transition to superorganismality causes selection to act on the colony as a unit, similar to an individual organism, and the binary distinction between the worker and reproductive caste is comparable to the soma and germ line. Greater understanding of the cohesive actions carried out by the divisible units of superorganisms and their microbial partners may allow us to derive novel insights into higher-level emergent characteristics typically associated with and observed in organisms.

of the colony gut microbiome (Kwong et al., 2017; Lukasik et al., 2017). Consistent with our previous inferences, we expect that insights into these processes can be achieved through comparative analyses of social insects across the spectrum of social organization.

## The Structured Gut Microbiota of the Superorganism

We contend that the tightest associations and most distinct caste-specific microbiomes will be in clades with extreme and irreversible division of labor, the superorganisms. In particular, social insects such as honey bees, certain ants and the Termitidae may provide the greatest insights because the complex division of non-reproductive labor supports remarkable fecundity of reproductives (Winston, 1991; Kaib et al., 2001; Hölldobler and Wilson, 2009). A simple reproductive gut microbiota may be influenced by traits of the individual, such as their unique diet, typically consisting of buccal secretions from workers (Noirot, 1969; Haydak, 1979), and limited exposure to environmental microbes (Cremer et al., 2018; Stroeymeyt et al., 2018), thus, reducing microbial immigration. The reproductive microbiome then likely supports specific metabolic demands, critical disease defense, or increased longevity, while most other colony functions are outsourced to workers and their microbiomes (**Figure 2**).

Complexity in the worker gut microbiota is likely driven by variable host tasks, diets, and interactions. Immigration of microbiota into the worker gut is controlled during post-eclosion microbial inoculation (Ohkuma and Brune, 2010; Powell et al., 2014; Lanan et al., 2016) and remodeling of gut morphology (Zhukova et al., 2017). Changes in host diet and physiology, such as the differential diet and gut enzyme expression of honey bee workers with age (Crailsheim et al., 1992), may influence the gut microbiota. Subcaste microbiota could additionally be influenced by interaction and transmission networks, resulting in compartmentalization. Notably, since the majority of microbiota studies utilize 16S rRNA profiling, it is likely that compositional differences at the bacterial strain level have been overlooked (Engel et al., 2014; Ellegaard and Engel, 2016), as indicated by compartmentalization of strains between individual honey bees and variable functional gene content across age groups (Ellegaard and Engel, 2019). Compartmentalization among subcastes would conceivably allow microbes to cater to specific host roles, create adaptive heterogeneity (Masuda et al., 2015; Kennedy et al., 2017), and reduce the potential for competition between symbionts (Frank, 1996). Consequently, the various roles and interactions of worker (sub)caste(s) should promote functionally diverse microbiomes that hosts optimally divide to complement tasks.

A superorganism's highly structured colony microbiome, shaped by distinct (sub)caste communities, may be adaptive to the colony as a unit, similar to the composite of different microbiomes observed in tissues or body regions of multicellular organisms such as humans (The Human Microbiome Consotrium, 2012) (**Figure 2**). The major evolutionary transition to superorganismal social complexity shifts selection to act more strongly at the colony-level, similar to selection on multicellular organisms, and the coherent actions of morphologically committed reproductive and non-reproductive (sub)castes is comparable to the division of cells into germ and soma (Wheeler, 1911; Boomsma and Gawne, 2018). The analogous organization of the two systems allows comparisons and insights into higher-level characteristics typically associated with and observed in organisms (Helanterä, 2016; Kennedy et al., 2017), which could include the structure and function of the microbiome (**Figure 2**). For example, the significance of microbial partners in the framework of social immunity, another emergent property enabled by social structure (Cremer et al., 2007, 2018), may be compared to the interplay between microbiota and the human immune system (Hooper et al., 2012). We believe that considering this analogy in future research will help improve our fundamental understanding of the impact of symbioses on individuals, (sub)castes and superorganismal division of labor.

# CONCLUSION

Current research on social insect symbioses focuses on microbiome function and the importance of social life in maintaining a consistent microbiota. However, we have yet to fully integrate these paradigms. The division of labor, which directs social roles and interactions, may secondarily partition the microbiota, shaping caste-specific microbiomes that dually enhance productivity of symbioses and efficiency of castes. While individual and colony-level mechanisms may drive distinctions in gut microbiota, microbial interactions within these communities should be examined to fully understand structured microbiota as a potential adaptation. Overall, we contend that integration of gut symbionts into the framework of sociality defined by division of labor may elucidate its potential adaptive value to individual insects and the colony as a whole.

# AUTHOR CONTRIBUTIONS

VS, JR-H, and MP conceived the ideas. VS drafted the first version of text and figures. VS, JR-H, BT, KE, PS, MV-C, and MP contributed with expertise, input, and edits throughout the remainder of the text.

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REVIEW
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# Origin of Mutualism Between Termites and Flagellated Gut Protists: Transition From Horizontal to Vertical Transmission

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Lower termites, as well as their sister group, the subsocial wood-feeding cockroach *Cryptocercus*, rely on flagellated eukaryotic symbionts in the hindgut to cooperatively digest their wood diet. In *Cryptocercus* these flagellates undergo encystment cycles tightly coordinated with the molting cycle of their host, yet the resultant cysts play no demonstrated role in their transmission to neonates; the trophozoite stage of the flagellates is passed directly from parents to offspring via hindgut fluids (proctodeal trophallaxis). This pattern suggests that encystment is a vestige from a gregarious cockroach ancestor, when the flagellates had a functional, two-stage life cycle and the cysts were horizontally transmitted among hosts via coprophagy. The strong integration between flagellate encystment cycles and host developmental physiology in *Cryptocercus* indicates that the relationship of the flagellates with their proposed gregarious cockroach ancestor was not commensal, but parasitic, with flagellates likely obtaining benefits by taking advantage of host gut metabolites and ingested plant debris. When vertical transmission evolved the parasites were 'captured,' and their fitness became inescapably embedded in the fitness of their host. The vertical transmission of gut flagellates and the origin of host subsociality via proctodeal trophallaxis can be considered two sides of the same coin. From the host point of view proctodeal trophallaxis marks the origination of parental care by provisioning neonates with nourishment, metabolites and beneficial microbiota. From the flagellate point of view, proctodeal trophallaxis was a shift from horizontal to vertical transmission, pushing them from the parasitic to the mutualistic end of the symbiotic spectrum, arguably making this host behavioral change the most critical juncture in the evolutionary trajectory of the termite lineage.

**Keywords: parasitism, symbiosis, *Cryptocercus*, evolution, lignocellulose digestion, commensalism**

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> *"... given the complexity of host-microbe interactions, humility and nuance are probably wise stances when surveying the topic."*
>
> –Casadevall and Pirofski, 2019

## INTRODUCTION

Some of the best studied insect gut communities are those in termites (Robinson et al., 2010), yet as in most symbiotic systems (Huitzil et al., 2018), mechanisms explaining the role of microbiota in the social evolution of the host are poorly understood. The historic literature is dominated by the role of the flagellated protists in the termite gut and their influence on termite social behavior.

These protists die prior to the molt of their termite hosts (reviewed by Nalepa, 2017) and must be regained by feeding on the hindgut fluids of a donor nestmate, a behavior called proctodeal trophallaxis (McMahan, 1969). This mandatory interdependence between hosts led to the proposal that termites originated as feeding communities bound by the necessity of exchanging flagellates and only later evolved social care of the brood (Cleveland, 1926; Cleveland et al., 1934; Lin and Michener, 1972; Wilson, 1975). The bulk of evidence, however, indicates that protozoan death at host molt in termites did not precede eusociality. It was a downstream effect of the initial stages of the eusocial condition (alloparental care) and associated with the physiology of developmental arrest and caste control in the hosts (Nalepa, 1994, 2017). Nonetheless, the death of protists at host molt was an evolutionary tipping point, in that beyond that threshold, intrinsic processes in the system drove accelerating change (Nalepa, 2015).

Here I review evidence that a pivotal change in the host-flagellate relationship occurred very early in their shared evolutionary history. Specifically, I examine how the shift from horizontal transmission of protists in a distant gregarious ancestor (as typified by extant gregarious cockroaches), to vertical transmission in the immediate subsocial ancestor of termites (as typified by the wood-feeding cockroach *Cryptocercus*, sister group to termites) set the stage not only for the evolution of eusociality in descendants, but also for their ecological domination. A caveat is that reliance on conjectured historical associations makes the illumination of processes that led to current patterns challenging.

The term symbiosis used here is the currently accepted one: a symbiont lives in or on a living host, implying neither physiological dependence nor benefit or harm between the organisms involved; it is an interaction between species. Further categorization of symbiosis relates to the cost or benefits to each partner. In commensalism the smaller partner benefits from the relationship, with no fitness effect on the host. There are fitness gains for both partners in mutualism, and a parasite benefits by exploiting but not directly killing its host. The term symbiosis is not synonymous with mutualism (Thompson, 1994; Corliss, 2002; Goater et al., 2014; Tipton et al., 2019).

## THE HINDGUT MICROBIOME

The hindgut microbiome in *Cryptocercus* and lower termites is a diverse assemblage of bacteria, archaea and viruses, as well as two groups of protists from the "Excavata," a deep branching supergroup of Eukarya. Most protists are parabasalids, in the phylum Parabasalia, and oxymonads, in the Class Oxymonadea, Phylum Preaxostyla. Members of these two groups are of unclear phylogenetic origin and unique to the termite lineage (Brune, 2011; Ohkuma and Brune, 2011). The bacterial members of the hindgut community are affiliated with more than 15 phyla (Hongoh et al., 2005; Ohkuma, 2008; Dietrich et al., 2014), and many of those in *Cryptocercus*/lower termites are endo- or ectosymbionts, associated with the surface, cytoplasm or nucleus of the flagellates (Brune and Stingl, 2005; Noda et al., 2006, 2009, 2018; Ikeda-Ohtsubo and Brune, 2009; Sato et al., 2014; Mikaelyan et al., 2017b; Hongoh and Ohkuma, 2018).

Although the physiology of host-microbial interactions in the lineage is far from clear, it is unchallenged dogma (Bignell, 2011) that neither *Cryptocercus* nor termites can exist without their microbial partners (Cleveland et al., 1934; Breznak and Brune, 1994). The hosts, however, are not completely dependent on flagellates for lignocellulose digestion (Slaytor, 1992). All studied cockroach and termite species have endogenous cellulase genes (Genta et al., 2003; Lo et al., 2003b, 2011; Watanabe and Tokuda, 2010; Tokuda et al., 2014) suggesting a widespread ability to utilize cellulose-based materials as food in the clade. Only *Cryptocercus* and lower termites have a collaborative relationship with flagellates for lignocellulose digestion. The flagellates and their prokaryotic symbionts orchestrate a microbial feeding chain driven by the primary fermentations of carbohydrates to short-chain fatty acids, the major source of energy for the host. Each of the different flagellate populations appears to have a specific role in lignocellulose digestion (Brugerolle and Radek, 2006; Brune, 2011, 2014).

The hindguts of *Cryptocercus* and lower termites are flagellate dominated communities. These unicellular eukaryotes can reach population sizes of $10^5$ per host individual, representing 60% of total hindgut weight (Bignell, 2011; Brune, 2011). Nonetheless, the literature on host-microbiome interaction in insects in general and termites in particular is based primarily on their bacterial and fungal symbionts (e.g., Aanen et al., 2002; Engel and Moran, 2013; Mikaelyan et al., 2015a, 2017a; Graf, 2016; Diouf et al., 2018; Otani et al., 2019). This is partly because the mutualistic partnership with flagellates appears unique among insects, but also because of methodological difficulties. Analysis of genomic information in protists is more convoluted than in prokaryotes because of their cytological complexity: they have multiple chromosomes and carry large nuclear and extranuclear genomes (Caron et al., 2009). High variability in 18S rDNA copy number and discordance between eukaryotic genetics and morphology makes characterizing their microbiome more challenging (Chabé et al., 2017; Popovic and Parkinson, 2018); technology, however, is advancing quickly (Hongoh, 2010; Carpenter et al., 2013; Altermatt et al., 2015).

The flagellates of *Cryptocercus* and the lower termites are derived from parabasalid and oxymonadid lineages that were acquired before *Cryptocercus* and termites split in the late Jurassic (∼140–170 Mya, Lo et al., 2003a; Lo and Eggleton, 2011; Bourguignon et al., 2015); it is likely that *Cryptocercus* harbors descendants of the original set of symbiotic flagellates that gave rise to their current diversity (Carpenter et al., 2009; Ohkuma et al., 2009). Most are found nowhere else in nature (Honigberg, 1970), however, a few taxa exhibit a much wider occurrence and are present in cockroaches other than *Cryptocercus* as well as in a variety of metazoan groups (Wenrich, 1935; Nalepa, 1991).

Here the focus is on the flagellated protists in the termite lineage, with the understanding that they are just one link in a vast array of complex metabolic networks distributed among microbial populations (Hongoh, 2011). Attention is further narrowed to the large flagellates known to engulf wood particles extensively studied by L. R. Cleveland; I acknowledge a bias

against the small, often bacterivorous protists in using this approach. Although extraordinarily diverse, the large flagellates will here be treated as a group, as they have variable but similar responses to their environment during the molting cycle of their host (see Nalepa, 2017), a central topic here.

## FLAGELLATES

Flagellates are predominantly free-swimming organisms (Raven, 2000; Bogitsh et al., 2018), and require water for the trophic stage of their life cycle. Most cannot tolerate the viscid, moist rather than wet environments in which bacteria thrive (Bradbury, 1987). The oxymonad and parabasalid flagellates are furthermore amitochondriate and typically inhabit anoxic or hypoxic environments, either free-living in rich organic matter in the sediments of water bodies, or as symbionts in anaerobic sites within their animal hosts (Brugerolle and Müller, 2000; Treitli et al., 2018). Flagellates that live in hindguts originally derive from free-living anaerobic protozoa (Wenrich, 1935; Fenchel, 1987), an easy transition as hindguts satisfy both their major habitat requirements: the hindgut environment is both liquid and anoxic, with steep gradients at the oxic-anoxic interface (Brune, 1998; Brune and Friedrich, 2000).

If desiccation and oxygen levels are not a problem, the active, trophic phase of animal associated flagellates are able to travel through the outside environment to a new host (Foissner, 2006). Because protists are so fragile in the trophic stage of their life cycle, however, it is reasonable to assume that dispersal occurs primarily via cysts in terrestrial environments (Corliss and Esser, 1974; Foissner, 2007; Parfrey, 2015). Encystment is a complex, highly sophisticated, gene-regulated differentiating process in which the mobile trophozoite transforms into a dormant, resistant life stage. It typically involves drastic cytoplasmic dehydration, metabolic inactivation, autophagic activity, formation of a cyst wall, gene-silencing, and DNA repair (Gutiérrez et al., 1998, 2001; Schaap and Schilde, 2018). The 'biological goal' of encystation is differentiation into a form that can survive in unfavorable conditions. While encystment of free-living protists allows them to survive harsh ecological circumstances, cysts of animal associated protists are first and foremost a transmission strategy (Bradbury, 1987; Vickerman, 2000; Corliss, 2002; Foissner, 2006; Lauwaet et al., 2007).

## TRANSMISSION

The mechanism by which symbionts are transmitted is important because it influences the extent to which symbiont fitness interests are aligned with those of the host. There are two basic strategies: horizontal transmission, which occurs across positions in space and is assumed to be the basal condition, and vertical, which takes place across generations in time, from parent to offspring (Baquero, 2017). It is vertical transmission that favors mutualistic relationships, because it is the mechanism by which a lineage of symbionts becomes consistently associated with a host, allowing the relationship to become obligatorily codependent. Once 'captured' via vertical transmission, selection on the symbionts becomes inescapably embedded in selection on the host (Ewald, 1987; Alizon et al., 2009; Sachs et al., 2011; Mushegian and Ebert, 2016; Fisher et al., 2017; Brown and Akçay, 2019).

The evolution of transmission mode of hindgut flagellates in the termite ancestor was closely associated with changes in host social structure and feeding behaviors (Nalepa et al., 2001). Horizontal transmission of microbes is typified by extant gregarious cockroaches, where hatchlings are inoculated primarily via coprophagy, the ingestion of feces produced by conspecifics which contain protozoan cysts, bacterial cells and spores (Hoyte, 1961b; Cruden and Markovetz, 1984; Hackstein and Stumm, 1994). Vertical transmission is exemplified by *Cryptocercus*, a subsocial cockroach whose neonates obtain their gut symbionts by feeding directly on the gut fluids of parents (proctodeal trophallaxis) (Seelinger and Seelinger, 1983; Nalepa, 1984; Park et al., 2002). These two taxa bracket the horizontal versus vertical transmission dichotomy in cockroaches. The key question, then, is how was the transition from horizontal to vertical made, and what were the consequences for the flagellate-host relationship?

### Horizontal Transmission – *Lophomonas* Model

In termite ancestors, the step prior to subsociality as exemplified by *Cryptocercus* was likely a gregarious lifestyle, with groups of kin and non-kin hosts of various developmental stages living together in aggregations with a relatively fluid membership. Gregarious behavior is the most basic social adaptation for microbial transmission, and is exhibited to some degree by a number of unrelated detritivores (Nalepa et al., 2001). The ancestral host-flagellate relationship in the termite clade may have been similar to the one extant gregarious cockroaches have with *Lophomonas*, a flagellate found sporadically in several species of synanthropic cockroaches and closely related to the Trichonymphida found in the gut of *Cryptocercus* and lower termites (Kudo, 1926b; Gile and Slamovits, 2012). *Lophomonas* is typically found in 8–14% of examined hosts, but can reach levels of 48% (see Martinez-Girón et al., 2017). As in other protists found in non-*Cryptocercus* cockroaches (e.g., Lucas, 1927; Kidder, 1937; Hoyte, 1961a), *Lophomonas* is not known to engulf wood particles and is consistently described as a commensal (Kudo, 1926a,b; Gile and Slamovits, 2012; Martinez-Girón and Van Woerden, 2013; Martinez-Girón et al., 2017); there is, however, no empirical evidence to support that categorization (discussed further below).

*Lophomonas* has a basic two-stage life cycle (Hoyte, 1961a): an active trophozoite (vegetative, trophic stage) in the gut, and a cyst, the dormant, resistant stage excreted to the outside environment by the host. Cysts of *Lophomonas* reach new hosts via coprophagy; they then by-pass the gizzard and digestive enzymes of the coprophage before reaching the fluid environment of the gut (midgut, according to Lucas, 1927), where the trophozoite emerges from the cyst and establishes itself anew as part of the gut microbiome. Environmental conditions that

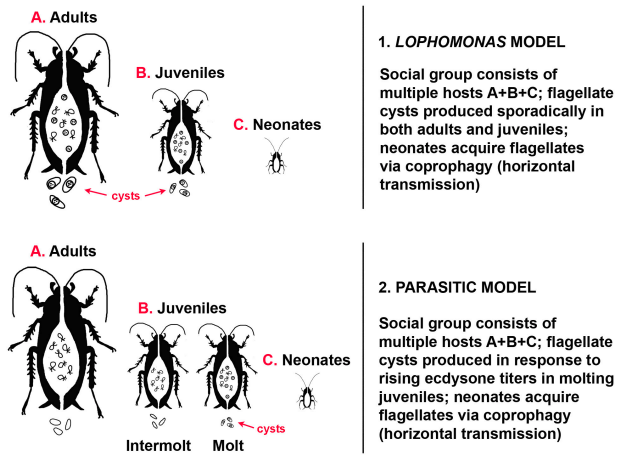


**FIGURE 1 |** Models of transmission dynamics in gregarious cockroaches. **(1)** *Lophomonas* model. Adults **(A)** do not molt, trophozoites and cysts are present in the gut, cysts are present in the feces. Trophozoites and cysts are present in the gut of molting and intermolt juveniles **(B)**, cysts are present in the feces. Neonates **(C)** hatch without protists in the gut. **(2)** Parasitic model. Adults **(A)** do not molt, trophozoites are present in the gut, no cysts are present in the feces. Trophozoites are present in the gut of intermolt juveniles **(B)**, no cysts in the feces; cysts are present in both the gut and feces of molting juveniles. Neonates **(C)** hatch without protists in the gut.

trigger encystation of protists are not precisely defined, but may occur in response to deficiency of nutrients, osmotic pressure, temperature changes, low pH, accumulation of waste products, and crowding (Bradbury, 1987; Bogitsh et al., 2018). Cysts of *Lophomonas* are produced sporadically in both juvenile and adult cockroaches, and can be found throughout the year (Kudo, 1926b). There is no evidence that encystation is coordinated with host reproduction or development; both cysts and trophozoites can be found in the gut during host ecdysis. Trophozoites survive the molting process and the size and shape of cysts does not change (Hoyte, 1961a) (**Figure 1**).

Horizontal transmission of *Lophomonas* is related to gregarious behavior in cockroaches because coprophagy requires some degree of site fidelity and host social contact (Nalepa et al., 2001; Bignell, 2011). Aggregation sites serve as infection banks, concentrations of fecal pellets containing encysted protists together with potential hosts in a limited space. Transmission of the flagellates relies on the excretory and feeding behavior of the cockroaches, and dispersal of the protists in space depends on the movement of the hosts among aggregation sites. It should be noted, however, that airborne cysts of *Lophomonas* occur and may cause a form of human bronchopulmonary disease (Martinez-Girón and Van Woerden, 2013; Fakhar et al., 2019).

## Vertical Transmission – *Cryptocercus*

In sharing a common ancestor with lower termites, *Cryptocercus* provides evidence that both vertical transmission of symbionts and metabolic interdependence between host and flagellates were present prior to the evolution of eusociality (Noirot, 1995). Furthermore, the unique life history of *Cryptocercus* can be assumed to reflect an early evolutionary stage of the termite clade (Klass et al., 2008). Characteristics of *Cryptocercus* most relevant to the evolution of symbiont transmission are that they are subsocial, living in biparental family groups, and that they are semelparous, i.e., they produce a single clutch of eggs in their lifetime. Field collections in both Asia and the United States are consistent in finding that the basic social structure is a male-female pair, together with a single cohort of offspring (Seelinger and Seelinger, 1983; Nalepa, 1984; Nalepa et al., 1997; Park et al., 2002).

Comparable to the flagellate *Lophomonas* noted above, the flagellates in *Cryptocercus* have a basic two-stage life cycle: the vegetative trophozoite, and the resistant cyst. Unlike *Lophomonas*, however, factors initiating encystment cycles of the flagellates in *Cryptocercus* are well characterized. Although it was recently noted that data on interactions between host physiology and gut microbiota are lacking (Macke et al., 2017), the work of L. R. Cleveland on the flagellates of *Cryptocercus* has been consistently overlooked; he clearly established the link between host hormones and the encystment cycles of the gut flagellates in *Cryptocercus* (reviewed by Nalepa, 2017). Rising titers of host ecdysone associated with juvenile hosts entering their molting cycle is the stimulus initiating encystment cycles in all taxa of cellulolytic hindgut flagellates examined. The timing of these physiological events in the host and symbionts is tightly synchronized, but the signal pathways activated and gene regulation and expression during the process are unexplored (see Jeelani et al., 2012; Gao et al., 2015). Although some of the resultant flagellate cysts are retained in the host hindgut and others are passed in the feces, there is no evidence that these cysts play a role in intergenerational transmission. The social structure of *Cryptocercus*, combined with the physiology of encystment of these particular protists mandates that flagellate transmission could occur only via proctodeal trophallaxis from parents (Nalepa, 1994). Adults pairs produce a single clutch of eggs in self-excavated galleries in a rotting log, then remain with that single cohort of offspring until parental death. Consequently, older nymphs are not present in galleries when adults reproduce, and cysts are never found in the feces of adults or intermolt juveniles (Cleveland et al.1934). Thus, coprophagy as a mechanism of intergenerational transmission is ruled out; adults do not molt and therefore do not excrete cysts, and older nymphs are absent from the social group. All protozoa in family members originate from the hindgut fluids of founding parents via parental care in the form of anal trophallaxis (**Figure 2**). After flagellates are established in the gut of neonates at the third instar (Nalepa, 1990), it is possible that cysts are exchanged among siblings via coprophagy (**Figure 2**). Nonetheless, all flagellates producing those cysts originated from parental hindgut fluids, because all juvenile siblings acquired them as hatchlings via parental trophallaxis.

## THE FLAW

Although the *Lophomonas* to *Cryptocercus* model may offer a plausible horizontal to vertical transition on the surface,

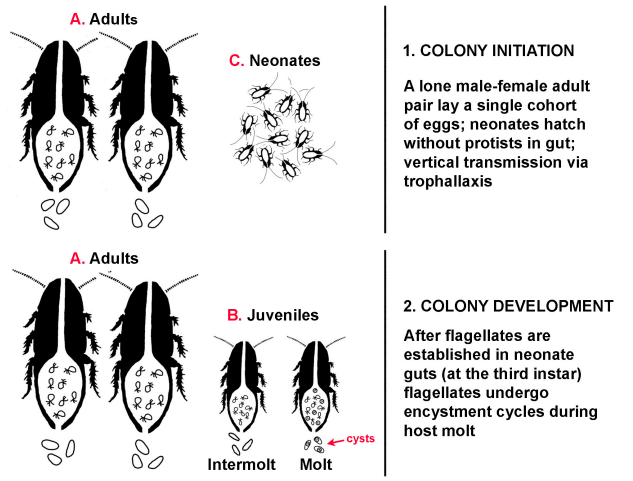


**FIGURE 2 |** Transmission dynamics during colony ontogeny in the subsocial wood feeding cockroach *Cryptocercus*. **(1)** Colony initiation. Adults **(A)** do not molt, trophozoites are present in the gut, no cysts are present in the feces. Neonates **(C)** hatch without protists in the gut. **(2)** Colony development. Trophozoites are present in the gut of adults **(A)** and juveniles **(B)**; cysts are present in the gut and feces of molting juveniles.

there are flaws in the argument. In *Cryptocercus*, there is tight integration of the encystment cycles of the flagellates with hormonal cycles in juvenile hosts, despite the cysts having little to do with intergenerational transmission. This suggests that the strong physiological connectivity between host development and the two-stage life cycle of the flagellates is an evolutionary contingency, a trait inherited from a gregarious ancestor (Nalepa et al., 2001). Such clear metabolic integration indicates that the host-flagellate relationship in the ancestor of *Cryptocercus* was unlikely to be commensal in the classical sense.

## Commensals or Parasites?

Although non-pathogenic protozoans are commonly referred to as commensals, this interpretation is problematic because they are physiologically dependent on the host to complete their life cycle and should be considered parasites (Bogitsh et al., 2018). Indeed, the very definition of a parasite indicates an obligate metabolic dependence on the host (Cheng, 1970; Goater et al., 2014); the host has maximum fitness without the parasite, but the parasite without a host has a fitness equal to zero (Combes, 2001). Even so, metabolic dependence on the host is a vague criterion for characterizing the symbiotic relationship of a protist living in the gut of a terrestrial insect, because wherever it may fall on the symbiotic spectrum, it depends on the host gut environment for the trophozoite stage of its life cycle. A flagellate always benefits from the gut environment; consequently, its symbiotic relationship may be better characterized in terms of fitness effects on the host (see Casadevall and Pirofski, 2015). It would be challenging if not impossible, however, to quantify adverse effects of the flagellates on their cockroach partners; in many parasites, negative effects on the fitness of the host are undetectable (Goater et al., 2014); the same can be said of cryptic benefits to the host. There may be a very narrow neutral zone where costs and benefits to the host are in balance and the relationship described as a true commensalism (**Figure 3**). A range of undetectable effects likely occur on a microscale and are mercurial depending on a number of subtle contextual factors, including those originating from the vast array of other microbial taxa in the gut, and host diet, digestive physiology, developmental stage, and immune responses. Parasitism, then, may be best described as the condition where host cost exceeds benefit, whether or not the cost is detectable. Similarly, mutualism may be thought to occur when the benefit to the host is greater than the cost of hosting the flagellates. Nonetheless, undetectable effects on the host are not amenable to measurement, thus making interactions that fall into the commensalism range (**Figure 3**) a vague theoretical construct (Zapalski, 2011).

The well-being of *Lophomonas* is known to be directly linked to the well-being of its host (Hoyte, 1961a); it can be eliminated from the gut by starving its cockroach partner, and benefits from a host diet of carbohydrates (Armer, 1944). Its nutritional needs are met by phagocytosing starch grains, fungi, spores, small flagellates and bacteria found in the surrounding fluid environment (Kudo, 1926b), but also may include metabolic products of the host. *Lophomonas* clearly benefits from the host gut environment, but detectable costs or benefits to its host await further study.

## Metabolic Connectivity

A commensal relationship is inconsistent with the physiological level of host–flagellate life cycle integration that must have existed in the gregarious predecessor of *Cryptocercus*, suggesting that the relationship was on the parasitic end of the symbiotic spectrum. The exquisite coordination of the complex encystment cycles of the flagellates with the molting cycle of their host (see Nalepa, 2017) suggests that parasitic flagellates in the *Cryptocercus*-termite clade were already present in a distant gregarious ancestor. A two-stage life cycle that is synchronized with host life history is a common feature among extant parasites (Møller et al., 1993; Chávez-Munguía et al., 2007), and steroid hormones in particular seem to stimulate the finely tuned developmental shift to the dormant transmission stage (Lawrence, 1986, 1991; Beckage, 1991). Encystment of *Opalina ranarum,* for example, is coordinated with the breeding season of its amphibian host via their gonadotropic and sex hormones (Bieniarz, 1950; El Mofty and Smyth, 1964). The orchestration of host and symbiont life cycles in the *Cryptocercus*-termite clade suggests that the flagellates were highly integrated with host physiology long before their host was dependent on the flagellates.

## Why Coordinate Developmental Cycles?

In the gregarious ancestor, parasitic flagellates evolved to respond to a shift in host hormonal titers in anticipation of an appropriate time to initiate physiological changes associated with dispersal as cysts outside the host body; encystment cycles are frequently associated with seasonality of the environment or other kinds of periodicity. Because parasite fitness is determined primarily by transmission success (Vickerman, 2000; Combes, 2001; Leung and Poulin, 2008), there had to be a selective

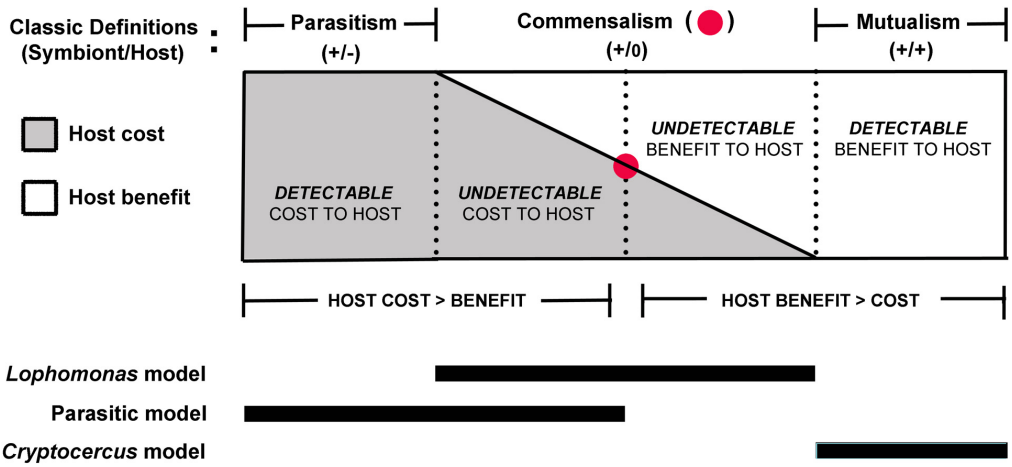


**FIGURE 3 |** Spectrum of symbiotic interactions in proposed models of the relationship of cockroaches to their gut flagellates. Parasitism and mutualism bookend an unprovable theoretical interval (commensalism) within the continuum of the symbiotic spectrum (see Zapalski, 2011); undetectable effects do not lend themselves to empirical proof. Because the protists always benefit from the relationship, the most relevant criteria for classification of the interaction are detectable damage or benefit in the host, described by the damage response framework (Casadevall and Pirofski, 2015). Regardless of the degree of detectable or undetectable negative impact on the host in the parasitic model, a change to vertical transmission would have strong potential to push the interaction to the mutualistic end of the spectrum.

advantage to coordinating encystment with the molting cycle of the host. That advantage lies in the life history and behavior of gregarious cockroaches. Aggregations are compatible with parasite transmission events because emitters (molting juveniles), vehicles (cyst-filled feces) and receivers (other potential hosts) co-exist in time and space (Møller et al., 1993; Baquero, 2017). Juvenile cockroaches are typically gregarious, coprophagous (Bell et al., 2007) and the stages that molt. Although the life cycle of the flagellates would reach a dead end in infected adults, from the parasite point of view any juvenile host would assure the production of cysts and therefore continuity of the lineage (**Figure 1**).

## Cost to Host

Gut flagellates must utilize host resources to meet their needs. In so doing alterations to the nutrient/energy budget of the infected host are expected. Parasites can cause anywhere from undetectable to drastic changes in host fitness, but harm can occur even with no measurable effect on the host. The cost may be slight, but it always exists (Combes, 2005; Poulin, 2007; Michalakis, 2009; Goater et al., 2014). To illustrate this point, Combes (2001) noted that an ant weighing 1 mg that flies on a Boeing 747 across the Atlantic costs the airline 10,000 molecules of fuel. The negative effect on host fitness is, however, more complex than the simple pilfering of host nutrients. An understanding of the mechanisms that link infection, host metabolism, immunity and life history are required to interpret the magnitude and direction of parasite-induced effects (Vickerman, 2000; Michalakis, 2009; Goater et al., 2014). Hosts may alter their physiology in a way that allows then to tolerate the presence of the parasite, and other microbes resident in the gut may play a role in shaping the way that the host body responds to infections. A variety of potentially virulent microbes are fairly harmless as long as they are part of a rich natural community of other symbionts in the same host (Poulin, 1995; Hudson et al., 2006). Avoidance of parasitism by the host is difficult because systems of recognizing parasites by hosts are limited (Perrot-Minnot and Cézilly, 2009), and in cockroaches, coprophagy has benefits that may outweigh any costs of parasitism. Cockroach feces offer fragmented, moistened and softened fare, and are enriched with lipids, carbohydrates, amino acids, unsaturated fatty acids, sterols, vitamins and cell debris originating from the excretor, its resident gut fauna, and the microbes that colonize the fecal pellet after its deposition in the external environment. The bacteria on feces 'predigest' recalcitrant foodstuffs, detoxify unpalatable chemicals, and are themselves utilized as food by the coprophage. Feces additionally serve as inoculum in the horizontal transmission of beneficial gut bacteria (reviewed by Nalepa et al., 2001; Bell et al., 2007). The presence of a mildly detrimental microbe may be 'tolerated' as a side effect of allowing beneficial species entry (Mushegian and Ebert, 2016). A parasitic flagellate of cockroaches that insinuates its cyst into this ingestible fecal bonanza has a high probability of finding a new host.

# TRANSITION FROM PARASITISM TO MUTUALISM

Symbioses are rarely static; they comprise a fluid spectrum of interactions from mutually beneficial to neutral to exploitative (Parmentier and Michel, 2013; Méthot and Alizon, 2014; Scharnagl, 2019). Although mutualistic relationships such as the one between *Cryptocercus* and its flagellates can arise in several ways from other interactions along the spectrum, many if not most cases of mutualism originate from an antagonistic,

parasitic relationship (Mayhew, 2006; Weiblen and Treiber, 2015; Scharnagl, 2019). Mutualism differs from parasitism in only one way: instead of one partner in the association exploiting the other without reciprocity, in mutualism each partner exploits the other (Combes, 2005). The most commonly cited catalyst driving the change from parasitism to mutualism is the switch from horizontal to vertical transmission. That shift in transmission mode can lead not only to a reduction in parasite virulence (Ewald, 1987; Yamamura, 1993; Read, 1994; Thompson, 1994; Poulin, 2007; Schmid-Hempel, 2017), but also to evolution of host-symbiont interdependence. Parasites become totally dependent on host reproduction for their own transmission, pushing the flagellates to evolve metabolically in a way more favorable to the host (Yamamura, 1993; Herre et al., 1999; Weiblen and Treiber, 2015). Novel interactions of vertically transmitted symbionts with their host can originate and progress rapidly under the pressure of adapting to the transformed relationship, adjusting them as a unit to each other and to their shared selective environment (McLaughlin and Cain, 1983; Thompson, 1994; Leung and Poulin, 2008; Gilbert et al., 2010; Gerardo and Hurst, 2017).

## Host Domination

Parasites are inextricably linked to the host that houses them, because the host is not only the resource base for a parasite, but also its habitat and vehicle (Combes, 2001). The establishment and maintenance of symbioses seem to be driven largely by the top–down influence of the host (Scharnagl, 2019). Although there are numerous examples of parasites driving changes in host behavior, these are predominantly in parasites with complex life cycles (i.e., those that require more than one host species to complete their life cycle) (Combes, 1991; Perrot-Minnot and Cézilly, 2009), not the basic two-stage life cycle of the flagellates in *Cryptocercus*. Selection may furthermore limit the evolution of host manipulation by any one microbial taxon in the gut microbiome, because of the large number of species and strains that compete with one another for space and resources (Johnson and Foster, 2018; Giudice, 2019).

## The Behavioral Change

The shift from horizontal to vertical transmission in the termite lineage lies squarely at the intersection between the evolution of host family life and the co-evolution of the host–symbiont relationship. The change from a gregarious social structure with coprophagy as a mechanism of microbial transfer, to family life with parents directly transferring gut microbiota to offspring in a liquid medium with fleeting, if any, exposure to oxygen is here proposed as the catalyst that drove the change from the parasitic to the mutualistic end of the spectrum in flagellates.

If the relationship between host and protists was originally parasitic then the direct transfer of the flagellates from parents to neonates via trophallaxis should be unexpected; hosts are typically under selection to avoid parasitism (Moore, 2013), particularly in the case of parents infecting vulnerable juveniles. However, if the parasites were of low virulence (discussed below) and if there were benefits to the direct transfer of hindgut fluids for reasons having little to do with the parasitic effect of the flagellates, then that host behavioral change is capable of initiating a cascade of evolutionary events that eventually results not only in host-flagellate mutualism but also termite eusociality (Nalepa, 2015). It is the presence of fitness benefits for either the host or the parasite that remains the crucial criterion determining whether a behavioral change is adaptive (Poulin, 1995).

## Host Fitness Benefits

A variety of fitness benefits accrue to a host that relies on trophallaxis rather than coprophagy to establish the gut microbiota in neonates. In addition to the above listed advantages of coprophagy, parental transfer of hindgut fluids would provide a more consistent transfer of the core prokaryotic assemblage associated with gregarious cockroach lineages (Schauer et al., 2014; Mikaelyan et al., 2016). Bacteria in cockroach guts display tremendous phylogenetic diversity, and include members of Bacteroidetes, Firmicutes, Fibrobacteres, and Proteobacteria (Dietrich et al., 2014; Mikaelyan et al., 2015b). The assemblage is dominated by obligate anaerobes, including those that break down structural polysaccharides of plant-based detritus (Bignell, 1977; Cruden and Markovetz, 1979, 1984, 1987), produce semiochemicals that influence host social behavior (Wada-Katsumata et al., 2015) and directly affect development by regulating gene expression in their host (Cruden and Markovetz, 1987; Jahnes et al., 2019). Another advantage of trophallaxis is that metabolic products of the parents and their resident microbial assemblage would not be compromised by exposure to the outside environment; these include hormones, enzymes, metabolites, and other chemicals that may serve as physiological or behavioral signals. Perhaps most importantly, vertical transmission of parasitic flagellate trophozoites would subject protists to the digestive activities of the cockroach at the receiving end of the interaction. While cysts of parasites associated with gregarious cockroaches evolved to withstand mastication, passage through the proventriculus (gizzard), and digestion by the host prior to reaching the hindgut, the large trophozoites are vulnerable and a potentially high quality, proteinaceous food source (Grassé and Noirot, 1945; Grassé, 1952; Machida et al., 2001; Nalepa et al., 2001; Brune and Ohkuma, 2011; Tokuda et al., 2014). This makes it impossible to separate symbiont transfer from nutrient transfer during gut fluid delivery (Nalepa, 2015) particularly when early juvenile stages are the recipients. The protozoan symbiosis is not established until the third instar in *Cryptocercus* (Nalepa, 1990); prior to that flagellate cytoplasm transferred from parents may help fuel their high nitrogen requirements for growth. Proctodeal trophallaxis also reinforces social structure because, unlike coprophagy, it requires physical contact and behavioral interaction (McMahan, 1969; Nalepa et al., 2001). Finally, the transfer of parental hindgut fluid frees young cockroaches from the necessity of seeking out fecal pellets, the distribution of which may be sporadic depending on the population dynamics and the size of the home aggregation.

## Trophallaxis as Parental Care

In insects, shifts in behavior play a central role in the regulation of microbial associations and are contingent on the evolutionary history of both the host and symbionts (Poulin, 1995; Ezenwa et al., 2012). Two aspects of cockroach evolutionary history are relevant here. First, because coprophagy was already established in the gregarious ancestor, the transition to proctodeal trophallaxis was seamless. It is not a huge leap from feeding on a fresh fecal pellet to feeding directly on hindgut fluids, making trophallaxis a simple shift in an existing behavior (Nalepa, 1994; Nalepa et al., 2001). Second, there are a number of independent origins of parental care in cockroaches where adults feed neonates on bodily secretions (Bell et al., 2007: Table 8.4). Given the recalcitrance of cellulose-based substrates, the onset of trophallaxis can be envisioned as the most efficient way to transfer nutritional resources to offspring (Nalepa, 1994). This suggestion is reinforced by the observation of adults of the wood-feeding cockroach *Salganea* feeding their young on oral fluids but without evidence of symbiont transfer (Shimada and Maekawa, 2011). Regardless of the selection pressure driving the evolution of trophallaxis-based parental care in the termite lineage, it had monumental consequences for the host–symbiont relationship and is a prime example of how host social organization can affect parasite transmission dynamics (Ezenwa et al., 2016).

# EVOLUTION OF THE MUTUALISM

## Ecological Basis

The sign and magnitude of a host–symbiont interaction is largely dependent on ecological context (Michalakis et al., 1992; Bronstein, 1994; Méthot and Alizon, 2014; Coyte et al., 2015; Bogitsh et al., 2018; Tipton et al., 2019): both that of the host within its habitat, and of the host as habitat for its microbiota (Bush et al., 2001; Goater et al., 2014). Trace fossils indicate that the early ancestors of cockroaches and termites fed on decaying plant matter, based on coprolite structure and distinctive pith borings in the stems of tree ferns (Labandeira and Phillips, 2002). Extant cockroach species have furthermore maintained their close association with rotting plant detritus in the natural environment, with plant structural polymers playing a significant role in their nutritional ecology (Roth and Willis, 1960; Nalepa and Bandi, 2000; Bell et al., 2007). Given that protists are major decomposers, contributing substantially to organic carbon mineralization and nutrient recycling (Wetzel, 2001; Corliss, 2002; Geisen et al., 2017), and that the liquid, anaerobic environment of the cockroach hindgut is not far removed from that of the anoxic sediments of water bodies, it seems likely that a shared capacity for utilizing cellulose-based material was the basis for the parasitic relationship in the gregarious ancestor. The flagellates would be preadapted for feeding and survival in the host digestive system, and the host gut would be a concentrated and continuous source of macerated plant detritus not only small enough for flagellates to phagocytose (Watanabe and Tokuda, 2010), but also accessible with few travel costs. The ingestion of particulate food via food vacuoles and its subsequent digestion by enzymes is thought to be a primitive feature of eukaryotic cells (Sleigh, 2000). Both the parasite and the gregarious host, then, likely possessed at least some capacity for cellulolytic digestion, either inherited from their respective ancestors or acquired via horizontal gene transfer from prokaryotes sometime during their evolutionary history (Lo et al., 2003b; Davison and Blaxter, 2005; Todaka et al., 2010; Sato et al., 2014). Bacteria are an important source of new genetic sequences for eukaryotes (Brugerolle and Müller, 2000; Sieber et al., 2017), and include those that confer the ability to degrade plant carbohydrates, to live anaerobically, and to adopt a parasitic life style. Each of these is documented in members of the Excavata: in *Giardia intestinalis, Trichomonas vaginalis*, *Leishmania* spp., and *Trypanosoma* spp. (Husnik and McCutcheon, 2018).

## Parasitism to Mutualism

The basis of the parasitic exploitation of the gregarious host by the flagellates is suggested to be the diversion of host-ingested detritus and associated prokaryotes for their own nourishment, while providing few metabolic products that increased fitness in the cockroach. If so, the parasites would be of relatively low virulence because hosts could compensate by changing their feeding behavior (Ponton et al., 2011; Goater et al., 2014). A modest increase in feeding rate by an ancestral gregarious cockroach may have been sufficient to provide adequate nourishment for both the host and its intestinal parasites.

If we accept this cellulolytic parasite scenario, then the mechanisms leading to mutualism were present before these single-celled organisms made the transition to that lifestyle. The flagellates were not only adapted to the gut environment but also dependent on the host, with their two-stage life cycle highly integrated into the physiological underpinnings of host development. It was a stable interaction, with the flagellate potentially obtaining more fitness benefits than the host. Cellulose digestion became a cooperative endeavor after the flagellates were taken into host custody via vertical transmission, pushing flagellates to the mutualism end of the symbiotic spectrum. Parasites are adept at changing metabolic pathways and evolve rapidly in response to new selective pressures (Poulin, 1998; Tachezy and Šmíd, 2008); they have a surprising stem cell network controlled by intrinsic and extrinsic mechanisms of cell conversion and differentiation (Niculescu, 2014). The importance of protists in biogeochemical nutrient cycling in the external environment (Geisen et al., 2017) furthermore suggests that they have a wealth of metabolic capacities that could be exploited by the host.

The currency exchanged (Wein et al., 2019) between the ancestral cockroach and its flagellates depends on knowing the physiological basis of the symbiotic partnership, a difficulty given the complex cocktail of metabolites involved and the derived nature of the microbiome in extant members of the lineage. Nonetheless, many animal groups produce cellulases on their own, but these are generally incomplete and must be supplemented by symbiotic microorganisms

(Lo et al., 2003b, 2011). Current evidence suggests that cellulose degradation in the lower termites is initiated by enzymes in the host salivary glands, then advanced in the hindgut by the flagellates; this dual system has been proposed as a model of efficient cellulose hydrolysis (Sugio et al., 2006; Ni and Tokuda, 2013). A reasonable assumption is that the flagellates shifted from exploiting cellulose in the host gut, to making its digestion a more efficient cooperative endeavor by integrating themselves into the cellulolytic metabolic pathways of the host. Vertical transmission (= proctodeal trophallaxis) was a first step leading to the extraordinarily complex division of labor in the termite-flagellate symbiosis, just as proctodeal trophallaxis (= vertical transmission) was the first step eventually leading to division of labor in the eusocial host (Nalepa, 2015).

## Downstream Effects

An expected consequence of vertical transmission is the initiation of a positive feedback loop between the newly minted host-flagellate digestive capabilities and host food choices. Increasing ability to metabolize lignocellulose would push the host to include more of it in its diet, feeding back on the metabolic contributions of the flagellate. A host diet high in structural polysaccharides and the development of the mutualistic partnership to fully digest it each establish the conditions for the development of the other. Eventually an ancestral cockroach that included rotted plant debris in its diet could make the transition to an exclusively wood diet, taking advantage of a relatively competition poor ecological niche and allowing the host–symbiont partnership to avail itself of the most abundant biomass on earth (Ni and Tokuda, 2013).

Another evolutionary consequence of the direct transfer of hindgut fluids was increased opportunity for protists to form symbiotic relationships with prokaryotes (Nalepa et al., 2001). Few symbiotic associations are recognized between bacteria and free-living obligate anaerobic flagellates (Fenchel and Finlay, 2010), but eukaryote-prokaryote relationships are both prevalent and increasingly well documented in the guts of *Cryptocercus*/lower termites. Their guts harbor a large pool of diverse bacteria associated with protists (Noda et al., 2009), and proctodeal feeding assures passage of established microbial consortia. The relationship between gut eubacteria and archaea with flagellates can be ecto- or endosymbiotic, and may be coevolved and stable or exhibit frequent host switches. Integration ranges from transient affairs to permanent, obligatory partnerships, each of which provides an opportunity for cross-feeding, for communication, and for genes to move to a new genome (Hongoh et al., 2005; Noda et al., 2007, 2018; Keeling and Palmer, 2008; Desai et al., 2010; Hongoh and Ohkuma, 2018).

Functionally, the cellulose-rich diet of the cockroach host ensures that the currency exchanged in these inter-microbe relationships would involve overcoming the deficits of food that has an excess of structural polysaccharides and a deficit of nitrogen. Cellulose digestion is advanced in the hindgut by the flagellates, but also by their associated bacteria (Tokuda et al., 2014; Yuki et al., 2015; Treitli et al., 2019), as well as by prokaryotes free-living in hindgut fluids (Ni and Tokuda, 2013). Gut bacteria acquire, conserve and recycle nitrogen, as well as serve as direct sources of protein (Thong-On et al., 2009; Desai and Brune, 2012; Tokuda et al., 2014; Tai et al., 2016). The duo of nitrogen-fixing bacterial endosymbionts and their cellulolytic protist host together enable the highly efficient growth of their host termite (Hongoh et al., 2008). Although the specifics of exchanged metabolites are yet largely unknown, recent studies are starting to reveal the astounding degree of complexity embodied in just one flagellate-bacteria relationship. Flagellates not known to engulf wood particles, like *Streblomastix strix* in the termite *Zootermopsis*, may have bacterial associates that fix nitrogen, provide amino acids and cofactors, and digest cellulose, some of which may fuel their flagellate host. The bacteria in turn lack at least one essential enzyme, which may be overcome by an exchange of intermediates with the flagellate. Other members of the gut flagellate community seem to play a role, and *Streblomastix* additionally internalizes and digests its epibiotic bacteria (Treitli et al., 2019). These insect-protist-bacterial relationships have been called triplex or tripartite (Brune and Stingl, 2005; Noda et al., 2007) but recent work indicates that additional terms may be needed. Utami et al. (2018) recently took the nested symbioses to a fourth level: *Treponema* spirochetes living on oxymonad protists in the gut of *Cryptocercus*/lower termites have ectosymbiotic bacteria of their own (see also Pramono et al., 2017).

## A Conundrum

If flagellates are so adept at switching metabolic pathways, it is a mystery as to why they retain their two-stage life cycle in *Cryptocercus*. The metabolic and genomic complexity of the encystment cycle suggests that it entails a substantial cost, and construction of a cyst wall requires considerable investment in protein and chitin (Eichinger, 2001). Cysts became largely defunct for intergenerational transmission at the onset of anal trophallaxis in the ancestral hosts, but the flagellates could not revert to a functional two-stage life cycle via horizontal transmission because of the concomitant change in host social structure (**Figure 2**). Nonetheless, vertical transmission of flagellates in *Cryptocercus* is unlikely to be as tightly coordinated as vertically transmitted, obligate intracellular symbionts (e.g., Sacchi et al., 2000; Arab et al., 2020). There may always be some noise in a transmission system that relies on host behavior, and vertical transmission in *Cryptocercus* should be considered high fidelity but not perfect (Tai et al., 2015). The life history of *Cryptocercus* does not rule out horizontal transmission but it is probably rare. Older, melanized juveniles may leave the natal log and shelter temporarily in abandoned or short, self-excavated galleries (Nalepa and Grayson, 2011). Because these subadults have a molt or two left before maturity, feces that contain cysts may be deposited outside their natal gallery. It is unknown how long these cysts are viable (but is amenable to testing).

It could be that a two-stage parasitic life-cycle is a difficult habit to break. The flagellates may be trapped in a maladaptive state because, like a variety of protozoan parasites (e.g., Clopton et al., 2016) cysts are an obligate stage in the life cycle. Ancient

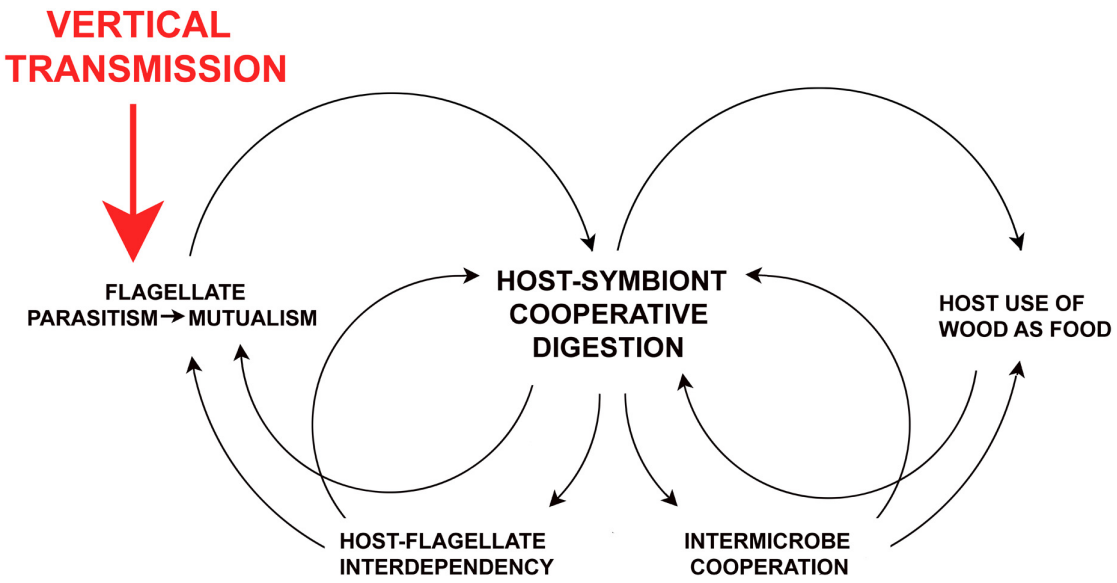


**FIGURE 4 |** Self-reinforcing dynamics of vertical transmission on host-gut symbiont relationships in the termite ancestors. Consequences of vertical transmission (proctodeal trophallaxis) on social evolution and life history of the host are addressed in Nalepa (2011, 2015, 2017).

events in phylogeny can commit a lineage forever, particularly if the protist is dependent on its host (Poulin, 2007). Although difficult to explain in evolutionary terms, there are other parasites with a dead-end in their life cycle (Vickerman, 2000; Mushegian and Ebert, 2016). In the human parasite *Entamoeba histolytica*, for example, those amoeba that end up in deep tissue sites are not further involved in the life cycle and reach a blind alley (Goater et al., 2014).

Because trophallaxis eases selection pressure to retain costly encystment cycles in the flagellates, a plausible explanation for their existence is that their deep integration with host physiology cannot be disengaged. This would accord with the idea of host domination in the evolution of the lineage, and with the 'flagellate as victim' hypothesis (Nalepa, 2017). Cysts in the feces of molting *Cryptocercus* nymphs, as well of vestiges of the encystment process in termites may be a legacy of their distant gregarious past. Response of protists to the molting hormones of the host nonetheless was strongly influential further along their shared evolutionary trajectory (Nalepa, 2017).

## THE POWER OF HOST BEHAVIORAL CHANGE

**Figure 4** illustrates some of the self-reinforcing co-evolutionary networks resulting from one host behavioral change: the shift from horizontal to vertical transmission. The principle outcomes are the transition from parasitism to mutualism in the flagellates, host-flagellate interdependence, and the origination of their cooperative partnership in processing lignocellulose. The latter feeds back on host food choices, and continuous residency of the flagellates in host gut fluids allowed for increased multilayered prokaryotic-eukaryotic integration and collaboration. Such feedback processes cement mutualisms and can accelerate during co-evolution (Biedermann and Rohlfs, 2017).

It should be noted that the host behavioral change from coprophagy to trophallaxis also had potent evolutionary consequences for host social structure, as it was the genesis of obligate subsociality and trophallactic exchanges in the lineage. Hosts became dependent on flagellates, neonates became dependent on parents, and eventually, termite colony members became dependent on each other (Nalepa, 2015, 2017). Vertical transmission additionally instigated or facilitated division of labor on three known levels: between hosts and their gut microbiota, among the diverse array of microbes that settled into the host gut, and eventually, among the members of the host social group. Arguably, then, it was the single key event in the genesis of termites from cockroaches, reinforcing the idea of behavioral change as a potent influence on the evolutionary trajectory of host-symbiont lineages.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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# Defensive Symbioses in Social Insects Can Inform Human Health and Agriculture

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Social animals are among the most successful organisms on the planet and derive many benefits from living in groups, including facilitating the evolution of agriculture. However, living in groups increases the risk of disease transmission in social animals themselves and the cultivated crops upon which they obligately depend. Social insects offer an interesting model to compare to human societies, in terms of how insects manage disease within their societies and with their agricultural symbionts. As living in large groups can help the spread of beneficial microbes as well as pathogens, we examine the role of defensive microbial symbionts in protecting the host from pathogens. We further explore how beneficial microbes may influence other pathogen defenses including behavioral and immune responses, and how we can use insect systems as models to inform on issues relating to human health and agriculture.

**Keywords: defensive symbiosis, social insects and humans, gut microbiome, colonization resistance, model systems, social immunity, insect agriculture**

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## INTRODUCTION

Some of the most successful species on the planet in terms of number of species generated over time, ability to inhabit diverse ecosystems, and maintenance of high population densities are social animals (Wilson, 1987). Social lifestyles, however, come at the cost of increased exposure to pathogens. Both modeling and experimental results indicate that population size and density correlate with pathogen prevalence and diversity (Anderson and May, 1979, 1982; Altizer et al., 2003; Schmid-Hempel, 2017). The 10-fold expansion of the human population in the last 200 years with similar population density increases has caused concerns around the risk of spreading infectious diseases (Cohen, 2003). Social insects have faced the same challenges successfully, maintaining high population densities over millions of years and are simple models to gain a better understanding of how to mitigate pathogen burden and spread (**Figure 1**).

While social living may enhance pathogen spread, social living also enables the spread of beneficial microbes (Biedermann and Rohlfs, 2017). For instance, after termites molt, they must replace their gut symbionts from other nest mates through trophallaxis and coprophagy. This "social gut" is suggested to contribute to nestmate recognition as well as development, nutrition, and defense (Breznak and Brune, 1994; Matsuura, 2001; Nakashima et al., 2002; Adams and Boopathy, 2005). Many microbes benefit the host by providing protection against predators, parasites, pathogens, or environmental stresses, also known as defensive symbiosis (White and Torres, 2009). In a mutualistic relationship, the host provides shelter and/or nutrients in exchange for defense. Understanding interactions between hosts, pathogens, and beneficial microbes can inform on the potential use of beneficial symbionts in systematically targeting certain pathogens.

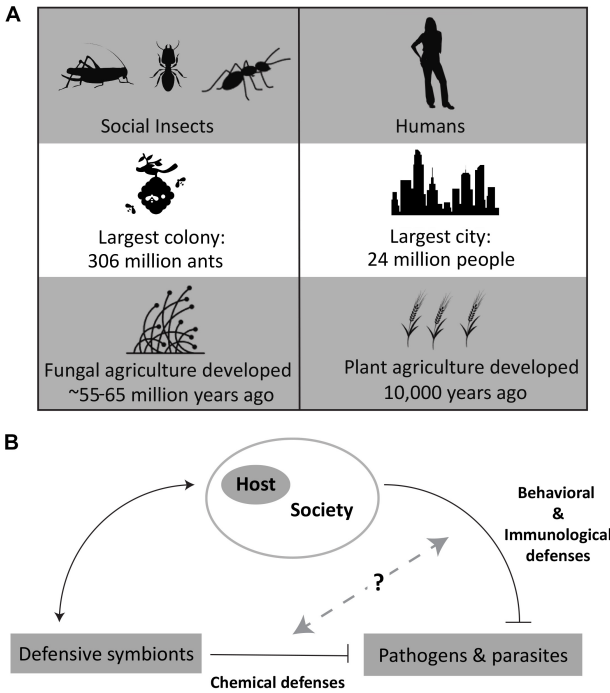


**FIGURE 1 | (A)** Comparisons of human and insect societies, based on social grouping sizes (Burchill and Moreau, 2016; Sawe, 2018) and history with agriculture (Pringle, 1998; Schultz and Brady, 2008). **(B)** Overview of the relationship of defensive symbionts with host and pathogens. Specific image credit from the Noun Project (https://thenounproject.com/): Woman by Lluisa Iborra, Locust by OCHA Visual, Termite by Heberti Almeida, Ant by Jacob Eckert, City by sumhi_icon, Beehive by Juraj Sedlák, Barley by Nathan Stang, and Fungi by CombineDesign. All images used and modified under the Creative Commons License, Attribution 3.0.

In interactions between social animals, their microbial defensive symbionts and pathogens, many different selective pressures may be operating simultaneously. Pathogen pressures can impact host and symbiont (King and Bonsall, 2017; Engl et al., 2018). Beneficial symbionts may influence social behavior to facilitate their horizontal transmission, but core microbiota may be influenced by diet or other factors (Sherwin et al., 2019). The evolutionary and ecological dynamics of microbial symbiont relationships with social animals are not well understood. To deconvolute these interactions, social insects are interesting models to compare social and solitary relatives (e.g., bees, discussed below) or comparing changes in microbiota of species that alternate between gregarious and solitary lifestages may also be useful (Lavy et al., 2018).

In this review, we discuss the role of microbial defensive symbionts in pathogen mitigation within social communities and their associated agricultural systems. We also consider how defensive symbiosis intersects with immunological and behavioral defenses. We compare examples from insects with defensive symbionts in humans and highlight how insect models can advance understanding the social impacts of defensive symbionts.

## INSECT DEFENSES AGAINST PATHOGENS

While defensive symbionts can benefit both social and solitary animals, social living may better enable sharing defensive symbionts than solitary lifestyles. For example, eusocial bees (e.g., *Apis mellifera* and *Bombus* spp.), have a consistent core microbiota that defends against the trypanosome gut parasite *Crithidia bombi*, whereas solitary bees do not have a consistent core community (Koch and Schmid-Hempel, 2011). Several core microbiome members, including *Gilliamella apicola* and *Lactobacillus* spp., correlate with decreased susceptibility to *C. bombi* (Cariveau et al., 2014; Mockler et al., 2018; Näpflin and Schmid-Hempel, 2018). Additionally, experiments disrupting the core bee microbiota support the hypothesis that the gut microbiota plays a role in protecting against opportunistic pathogens (Raymann et al., 2017) and another common parasite, *Lotmaria passim* (Schwarz et al., 2016). Biofilm formation by the core strains is the suggested protective mechanism against this pathogen, as indicated by fluorescent *in situ* hybridization (FISH) imaging (Martinson et al., 2012) and the enrichment of secretion systems and surface proteins in bee gut metagenomes (Engel et al., 2012). As biofilm formation and colonization resistance are broad defensive mechanisms, it is unclear whether solitary bees have microbes with similar functionality. Likewise, social bee gut microbes may confer other functions affecting fitness.

Social animals need to not only protect themselves from disease, but also their shared food sources. Three lineages of eusocial or subsocial insects demonstrate agricultural behavior: ants (Myrmicinae: Attini), termites (Macrotermitinae), and ambrosia beetles (Xyleborinae and others). All of these insects live in gregarious communities supporting the hypothesis that sociality allowed for evolution of insect agriculture (Mueller et al., 2005). Fungus farming termites cultivate basidiomycete fungi, *Termitomyces* spp. as a food source that are either vertically or horizontally acquired depending on termite species (Johnson and Hagen, 1981; Korb and Aanen, 2003). Some termites (*Macrotermes natalensis*) harbor *Bacillus* sp. that produce bacillaene which has antifungal activity and helps protect the fungal cultivar (Um et al., 2013). Xyleborine ambrosia beetles cultivate an assemblage of fungi, rather than a single fungal cultivar, which comprises mycelial fungi, yeasts, and bacteria (Norris, 1965; Hulcr and Stelinski, 2017). A cycloheximide-producing *Streptomyces* phylotype has been isolated from two species of ambrosia beetles as a possible defensive symbiont (Grubbs et al., 2019).

In the fungus-growing ants, microbial associations range from mutualistic to parasitic and are well-described. The ants grow a fungal cultivar as their primary food source in a monoculture, which makes it highly susceptible to the specialized fungal pathogen *Escovopsis* (Ascomycete; Hypocreales). To protect their food source, the ants evolved several defense mechanisms, including a mutualism with *Pseudonocardia* spp. (Currie et al., 1999b, 2003). *Pseudonocardia* produces antimicrobial molecules that are active against *Escovopsis* (Currie et al., 1999b, 2003; Poulsen et al., 2010). Growing *Pseudonocardia*

and *Escovopsis* together reveals patterns of inhibition and resistance between the two organisms suggesting population and interaction dynamics at fine phylogenetic scales (Poulsen et al., 2010; Cafaro et al., 2011). Several of the antibiotics produced by *Pseudonocardia* have been characterized (Oh et al., 2009; Carr et al., 2012; Van Arnam et al., 2016) although the full diversity of antibiotics used is unknown.

## INTERACTIONS OF DEFENSIVE SYMBIONTS WITH HOST DEFENSES IN INSECTS

Other methods of pathogen resistance, such as behavior and immunity, aid in disease resistance and can be influenced by microbes (Nyholm and Graf, 2012; Lizé et al., 2014; Flórez et al., 2015). Host and symbionts may adapt to each other in different ways: symbionts may avoid triggering immune function (Trappeniers et al., 2019); hosts may diversify immune pathways (Maire et al., 2019) or hosts may potentially reduce immune function (International Aphid Genomics Consortium, 2010; Douglas et al., 2011). Further examples of innate immunity in social insects can be found in the following review (Otani et al., 2016).

Social insects can coordinate defensive behaviors, some of which may be triggered or helped by beneficial microbes. Many of the defensive behaviors in social insects are aimed at maintaining sanitation of the nest as well as the individuals within the nest. This phenomenon of collective actions to mitigate pathogen spread/exposure is known as social immunity, which is defined as the control or elimination of potential pathogens by cooperation of individuals through behavioral, physiological, and/or organizational means (Cremer et al., 2007; Meunier, 2015). For example, subsocial aphid *Nipponaphis monzeni* soldiers respond to attacks on their colonies by swarming and exploding their abdomens. Their abdomens are swollen with hemocytes and tyrosine that seal and protect the colony. The endosymbiotic bacterium, *Buchnera*, regulated by aphid host genes, helps overproduce tyrosine (Kutsukake et al., 2019). This example highlights the complex interplay occurring between host, beneficial symbionts, immune system, and social structure of an organism. Other examples of social immunity include grooming, removing waste material and weeding nests and fungal gardens. Further experimentation using antibiotics or probiotics could explore the manner in which microbes may influence behavior and fitness (Alberoni et al., 2018).

Defensive behaviors can also be facilitated by the microbial production of chemical signals or chemical defenses. Social insects participate in extensive grooming behaviors categorized as autogrooming (i.e., self-grooming) and allogrooming (i.e., grooming among nestmates), which serve not only to remove foreign substances from the body surface, but can also provide lasting antimicrobial defenses (Zhukovskaya et al., 2013). In terms of using microbes for production of chemical defenses, many examples in the above defensive symbioses fit this description (e.g., antimicrobial phenols from locust symbionts, antibiotics from fungus-farming ant symbionts). Microbes are also capable of producing chemical signals, such as the intestinal microbes of subterranean termites (*Reticulitermes speratus*), which allow recognition of nestmates from non-nestmate intruders (Matsuura, 2001). The diversity of interactions between defensive microbes and host behavior remains an open area of exploration.

## HUMAN DEFENSES AGAINST PATHOGENS

As in insects, the microbiota provides defense against various pathogens in humans, but is more complex than insect microbiomes. While different sites, such as the vagina and nasal cavity can support symbionts with abilities to produce defensive compounds (Donia et al., 2014; Zipperer et al., 2016), most of the potential defensive microbes described reside in the gut. Unlike many insect gut microbiotas, the human gut microbiota may contain hundreds of species (Qin et al., 2010). Adding further complication, whereas in bees and other hosts a core community is evident, a consistent core community has not been identified in humans, although a core functionality appears more conserved than particular strains (Turnbaugh and Gordon, 2009; Human Microbiome Project Consortium, 2012). Although humans lack an equivalent solitary lifestyle to insects, evidence suggests that humans in close social relationships may share a variety of bacteria with one another and have greater richness and diversity than humans living alone (Dill-McFarland et al., 2019).

Many different mechanisms for microbial defense exist and understanding the microbiota's functions may lead to improved therapies. For example, fecal microbiota transplants for treating *Clostridium difficile* infections that are non-responsive to antibiotics have cure rates of 90% (Bakken et al., 2011; Youngster et al., 2016). Several mechanisms have been suggested including that the microbiota outcompete the pathogen for nutrients, microbially produced antibiotics target *C. difficile*, microbially produced secondary bile acids inhibit *C. difficile*, and microbial interactions with the immune system help repair the gut barrier (Khoruts and Sadowsky, 2016). Human gut microbes have also been linked to defense against *Vibrio cholerae*, where correlations have been found between microbiota taxa present in the gut and resistance to cholera (Hsiao et al., 2014; Midani et al., 2018). Likewise, human microbiota strains compete with *Salmonella* for nutrients and produce metabolites that potentially inhibit *Salmonella* (Antunes et al., 2014; Bratburd et al., 2018; Zhang et al., 2018). Although many interactions and correlations have been suggested between defensive symbiotic bacteria and pathogens in humans, the challenge remains to explore these symbionts on a society-wide scale to understand the benefits not only to individuals but to public health.

Although humans do not have ancient history (on an evolutionary time scale) with agriculture, many crops used by humans associate with defensive microbes against certain pathogens. One example of an agricultural defensive symbiont is *Pseudomonas fluorescens*, a bacterium that produces the antibiotic 2,4-diacetylphloroglucinol, which can inhibit the

causative agent of take-all disease in wheat (Keel et al., 1992). This bacterium can be found naturally in soils and is a prominent example of suppressive soils, where soil harbors a community or certain strains that inhibit plant pathogens, analogous to the idea of colonization resistance in animals. Beneficial microbes may provide an environmentally sustainable alternative to chemical control of pathogens and vectors, but will require maintaining beneficial microbes in agricultural settings and consideration of microbial interactions in plant breeding beyond the host's pathogen resistance (see the following review for more detail (Syed Ab Rahman et al., 2018).

## INTERACTIONS OF DEFENSIVE SYMBIONTS WITH HOST DEFENSES IN HUMANS

The role of the immune system and behaviors is increasingly recognized as not only defending against harmful microbes, but also fostering the establishment and maintenance of bacterial symbionts. We direct the reader to other reviews for further exploration of the numerous interactions between the microbiota and the immune system (Belkaid and Harrison, 2017) and behavior (Vuong et al., 2017; Johnson and Foster, 2018).

Humans have been practicing their own social immunity with hygienic behaviors throughout history. This includes early ritualistic behaviors, quarantine and sanitation, and after the rise of the germ-theory of disease, water treatment, vaccinations, and vector control (Institute of Medicine (US) Committee for the Study of the Future of Public Health, 1988; Curtis, 2007). While humans have taken advantage of antimicrobial compounds from a variety of sources for hundreds of years (Aminov, 2010; Harrison et al., 2015), large scale antibiotic discovery, often microbially derived, took off in the 1900's and enabled treating a wide variety of pathogens in people as well as in agriculture (Aminov, 2010). Unfortunately, broad-spectrum antibiotics can have lasting impacts on the microbiota affecting the many interactions discussed above (Jernberg et al., 2007). While efforts to eliminate pathogens have substantial impacts, most notably with vaccines eliminating smallpox and reducing other disease to 99% fewer cases (Orenstein and Ahmed, 2017), practices for sharing beneficial microbes could also be valuable for medicine and agriculture. These practices may include fecal microbiota transplants, probiotic and prebiotic supplementation (George Kerry et al., 2018; Sonnenburg and Sonnenburg, 2019), creating built environments that favor beneficial microbes (Kembel et al., 2012); however, besides perhaps fecal microbiota transplants for treating *C. difficile*, these practices currently lack substantial evidence of efficacy.

## WHAT CAN WE LEARN FROM INSECTS?

Insects are useful models to address societal-wide impacts of defensive symbionts (**Table 1**). Given the vast complexity in the human gut, insects can be a simple model to dissect various mechanisms of microbial defenses since insects tend to have simplified microbiomes relative to humans. Comparisons between social and solitary insects (whether in different life stages as described above with locusts, or among related social and solitary members as described with bees) can shed light on what roles, if any, defensive symbionts have played in the evolution of sociality. Insect colonies are well-defined social units for replication, tend to have limited within colony genetic variation, and can be reared in controlled conditions. The insects themselves often have relatively fast life cycles, which is useful for examining fitness and intergeneration effects defensive microbes may have. Social insects also engage in behaviors of interest, like farming. In the most direct sense, natural products from insect symbioses may be useful as leads for new antibiotics themselves (Stow and Beattie, 2008; Ramadhar et al., 2014; Chevrette et al., 2019) and insects have inherent practical value as many species are important pollinators or pests; however, we also want to highlight using insect models to explore the societal impact of gaining or losing beneficial symbionts. We detailed many benefits of insect models above, but these models come with drawbacks. The simplicities of social insect models limit conclusions relevant for humans to basic ecological dynamics. Insect models lack many features that mediate host-microbe interactions in humans, including an adaptive immune system or complex nervous systems. While much microbiome research has focused on the impact to the individual host, social insects can be used to address basic ecological and evolutionary dynamics including (i) how resilient societies transmit beneficial microbes to other individuals; and (ii) the larger impact of beneficial microbes at the population level.

Social insect models can address how social animals maximize beneficial microbe transmission while minimizing pathogen spread. Disrupting transmission of beneficial microbes can render hosts more susceptible to disease (Bohnhoff et al., 1954; Currie et al., 1999a; Raymann et al., 2017). In some

**TABLE 1 |** Comparison of social insect and human models for defensive symbiosis.

| Advantages of insect models | Human alternatives |
|---|---|
| Control of variables (diet, environment, etc.) | Diets and environment generally not experimentally manipulated; metadata may be limited or subject to self-reporting inaccuracies |
| Defined units of replication for social group (e.g., one colony) | Units could be family, geographical region, etc. |
| Relatively simple microbiomes | Complex gut microbiomes, other sites varying complexity |
| Shorter life cycles | Long life cycles |
| Genetic variation within a colony lower than from a general population | Variable genetic variation |
| Lifestyle variation exists, including solitary, social, and eusocial members | Different types of social groupings, but all social |

human societies, transmission and maintenance of microbes has changed dramatically with the introduction of antibiotics, hygiene practices, and diet changes (Bokulich et al., 2016; Vangay et al., 2018). Disruptions in microbiota transmission are hypothesized to have health impacts, including obesity (Principi and Esposito, 2016). In both social insects and humans we have limited understanding of how beneficial microbes are effectively transmitted. In the leaf-cutter ant system, we know that the defensive symbiont *Pseudonocardia* is generally vertically transmitted, acquired during a narrow time window (Marsh et al., 2014) and may use certain host structures (Li et al., 2018), but we do not know what limits bacterial acquisition to certain strains and microbial adaptations to the host. Analogously in humans, we know microbial acquisition begins at birth but the roles and extent of vertically versus horizontally acquired microbes is still debated (Ferretti et al., 2018; Korpela and de Vos, 2018; Moeller et al., 2018; Brito et al., 2019). One drawback of insect models is that specific mechanisms enabling transmission and colonization of beneficial microbes likely differ considerably between insects and humans (e.g., coprophagy is normal behavior for all termite colony members, while fecal microbiota transplant in humans is a medical procedure for the sick). Similarly, humans may travel further and interact with other communities introducing complicated interactions that may not be captured with insect models. However, the defined social structures of eusocial insects may be useful for understanding and manipulating microbial transmission later in life. Reproductive queens have limited contact with other adult workers, for instance, and understanding when and how they share microbes with other castes could illuminate the social elements of microbial transmission (Otani et al., 2019). Microbiomes of distinct nest structures provide an interesting comparison to the idea of built environments (Sharma and Gilbert, 2018).

Additionally, social insect models may address how environmental perturbations such as diet or temperature change the overall community response to pathogens and illuminate fitness effects in different contexts. For example, different substrates used in leafcutter ant fungal gardens impacts overall colony survivorship (Khadempour et al., 2016). While some leafcutter ants associate with defensive symbionts as described above, others rely on their own chemical defenses (Fernández-Marín et al., 2009). The leafcutting ant model could be used to explore how resilient different defensive strategies (chemical or biological control) are to perturbations such as the availability of different substrates. Fisher et al. (2019) predict how other social insect characteristics (including degree of specialization and nest architecture) may enhance susceptibility or resilience to various climate perturbations. The relative simplicity of insect models could help test and reveal basic principles to understand how microbial defenses change in different contexts.

## CONCLUSION

How societies effectively address risk of pathogen exposure is of increasing concern, especially as the human population size and density rises. Social insects provide a window to explore disease management on a society-wide scale. Increasingly, defensive symbionts are recognized for their valuable role in mitigating pathogens, in insects as well as in humans. Social insects can act as useful models to address the role of defensive symbionts in societies and their interactions with physiological, chemical, and behavioral defenses. Examples from insects provide insight for microbiome-based therapies and agricultural products, as well as help address basic questions on how beneficial microbes are transmitted, maintained, and perturbed in social animals.

## AUTHOR CONTRIBUTIONS

JB, HH, and RA wrote the manuscript. All authors contributed to the manuscript revision and approved the submitted version.

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# The Internal, External and Extended Microbiomes of Hominins

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The social structure of primates has recently been shown to influence the composition of their microbiomes. What is less clear is how primate microbiomes might in turn influence their social behavior, either in general or with particular reference to hominins. Here we use a comparative approach to understand how microbiomes of hominins have, or might have, changed since the last common ancestor (LCA) of chimpanzees and humans, roughly six million years ago. We focus on microbiomes associated with social evolution, namely those hosted or influenced by stomachs, intestines, armpits, and food fermentation. In doing so, we highlight the potential influence of microbiomes in hominin evolution while also offering a series of hypotheses and questions with regard to evolution of human stomach acidity, the factors structuring gut microbiomes, the functional consequences of changes in armpit ecology, and whether *Homo erectus* was engaged in fermentation. We conclude by briefly considering the possibility that hominin social behavior was influenced by prosocial microbes whose fitness was favored by social interactions among individual hominins.

**Keywords: fermentation, primates, prosocial microbes, feces, food, armpits, alcohol**

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## INTRODUCTION

As part of an article collection on the drivers of sociality we were asked to consider the influence of hominin microbiomes on the evolution of hominin social behavior. As a starting point, we consider how large-scale physical, social, and behavioral changes that occurred during human evolution have (or might have) affected our interactions with microbes. We focus especially on the last six million years or so, starting from when we last shared a common ancestor with chimpanzees (*Pan troglodytes*) and bonobos (*Pan paniscus*), our last common ancestor (LCA), and before the industrial revolution (at which point many changes in human lifestyle appear to have begun to precipitate rapid changes in microbiomes). We use the word "hominins" to include all of the species after the split from the LCA, fossil species more closely related to human ancestors than chimpanzees or bonobos, and our own species, *Homo sapiens*. We use the word "hominids" to describe the broader lineage that includes the common ancestor of all great apes along with hominins.

Reconstructing the microbiomes of ancient hominins will ultimately rely on two main sources of data: (i) ancient microbial DNA from humans and non-human primates (Compton et al., 2013; Weyrich et al., 2017), and (ii) comparisons of modern genes, phenotypes and microbiota among humans, great apes, and other non-human primates, mammals and birds. Here we leverage the

second of these sources to explore the complex interplay between human societies and behavior, microbiomes, and evolution. We consider four features of hominin bodies and lifestyles that have changed in the time since that LCA in ways that might both influence the microbiome and influence the effects of the microbiome on human social behavior. We begin with the stomach.

## THE STOMACH

The stomach plays two key roles in mammals. One of those roles is in the degradation of protein (and, in some cases, chitin). This role has received disproportionate research attention (and is the focus, for example, in medical texts). The other role is as a kind of ecological filter, allowing some species into the intestines but not others. Like a bouncer at the door to the intestines' microbial party, the stomach (acting as a filter) can be more or less restrictive. When very acidic, the stomach prevents most microbes ingested in food (apart from the most acid-tolerant) from arriving intact in the intestines. When more neutral, it allows most microbes through to the intestines alive. In primates that exclusively ingest fruits and leaves, the cost of allowing food borne microbes into the intestines is modest. Indeed, such microbes, in as much as they have already begun to degrade the food on which they are found, may be especially likely to aid in the breakdown of that food. This is particularly true in foregut fermenters (in which the fermentation chamber is the first chamber of the gut and serves as a gastrointestinal analog of the brewer's tank; **Figure 1**). However, in omnivorous primates that include raw meat in their diets, the potential to ingest food borne pathogens is relatively great and hence the stomach might be expected to be more acidic (Ragir et al., 2000). In general, across mammals and birds, these patterns bear out. The more carnivorous an animal it is, the more likely its stomach is to be more acidic. But as can be seen in **Figure 1**, humans appear to be an outlier even within this schema. Humans have stomachs with a mean pH of 1.5 (Beasley et al., 2015). The extremely acidic stomachs of humans are unlike those of any other primate so far sampled, and find their closest analogs not in other primates but, instead, in the stomachs of vultures (which are similarly acidic) and potentially hyenas. No studies have documented the pH of hyena stomachs, but digestive anecdotes from hyena researchers (Christine Drea, pers. comm.) suggests hyenas have stomachs that are similar in acidity to vultures and humans. Vultures and hyenas have a good reason to have very acidic stomachs. They employ their stomachs as defenses against the bountiful food borne pathogens they ingest daily. Amazingly, however, while some vulture species, such as the white-backed vulture (*Gyps africanus*, pH of 1.2) have stomachs that are more acidic than those of humans, others actually have stomachs that are less acidic than humans. In the primate story, the stomachs of humans are unusual.

The acidity of the human stomach poses two mysteries. The first relates to the timing of the origin of the acidity. The stomachs of chimpanzees and bonobos have been poorly studied, which is remarkable given the long history of the use of chimpanzees as laboratory animals. It is possible that the stomachs of chimpanzees and bonobos are like those of humans, very acidic (which might suggest that such acidity evolved in one of our common ancestors). That the stomachs of chimpanzees are (or can be) at least somewhat acidic is suggested by the observation that the bacteria species *Helicobacter pylori* more readily establishes in the stomachs of chimpanzees in the laboratory after they have been given antacids (Hazell et al., 1992), as is also the case in humans. If the stomachs of chimpanzees are as acidic as those of humans, one needs to explain why our common ancestors evolved acidic stomachs prior to six million years ago. On the other hand, the stomachs of chimpanzees might also be closer to neutral in pH, as is the case for other fruit eating primates. The truth is we don't know enough to distinguish these possibilities yet. Two anecdotes, however, are intriguing. The first is the study of the stomach pH of a single captive chimpanzee. That chimpanzee is reported to have had a stomach pH that was approximately neutral (Brodie and Marshall, 1963). Several strong caveats exist, of course. The captive animals in the study were fed a processed primate mash, supplemented with vitamins, and lived in a captive environment completely dissimilar to the wild. Yet, despite these caveats the observation is of interest. The second anecdote relates to reports of dissections of recently dead, captive chimpanzees that note "yeast overgrowth," in their stomachs (Migaki et al., 1982). Very few yeast species are able to grow in hyper-acidic environments. As a result, the observation is reconcilable with the idea that the stomachs of chimpanzees are not as acidic as those of humans.

We hypothesize that the stomachs of chimpanzees are likely somewhat acidic, but less so than those of humans. We also propose that the extreme acidity of human stomachs evolved after our split with the LCA with chimpanzees. If this is the case, it raises the question of what factors favored such acidity. One possible explanation is scavenging prey items abandoned by carnivores and/or the consumption of prey items too big to eat all at once. Chimpanzees in all habitats where they are found in the wild eat meat (Moore et al., 2017), as do bonobos (Wakefield et al., 2019), leading many to think that the LCA did as well. Sometimes the meat chimpanzees consumed Is scavenged (Nakamura et al., 2019) but relatively rarely (compared to other foods in their diet). More often the meat is eaten fresh from kills, though chimpanzees exhibit great variability between communities in success, technique, and seasonality of hunting behavior (Moore et al., 2017; **Figure 2**). Given that several chimpanzee communities target mammalian prey, and may do so using tools (Pruetz and Bertolani, 2007; Nakamura and Itoh, 2008), it is likely that species of *Australopithecus*, Homo *habilis* or Homo *erectus* also targeted and consumed meat, but also that how much meat they consumed, how fresh the meat was and how much was excess varied. While there is broad consensus among paleoanthropologists and evolutionary anthropologists that meat-eating played a role in the evolution of *Homo,* the relative importance of hunted and scavenged meat is contested. At least some of the meat that early hominins were eating was carrion (Pante et al., 2018). Some bones, for example, from the time during which *H. erectus* was extant, show evidence both of cut marks by stone tools and, in a layer beneath the cuts

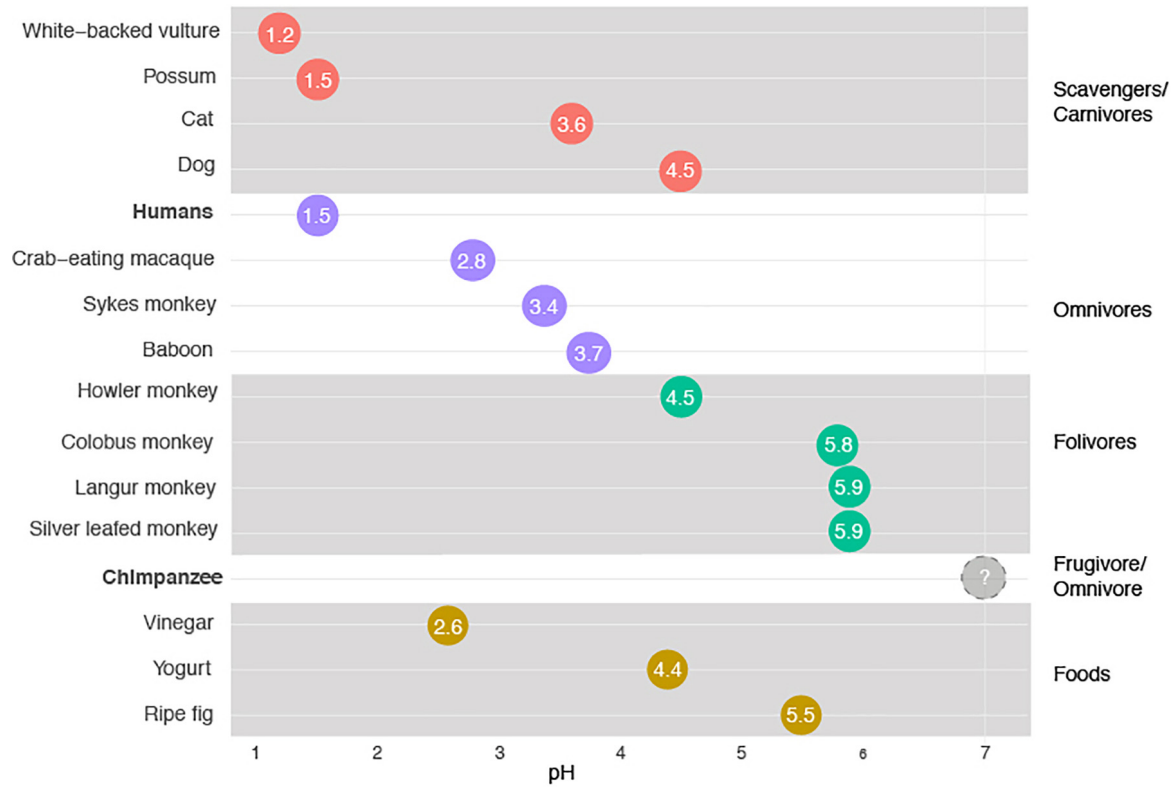


**FIGURE 1 |** Stomach pH as a function of animal species. A pH of 7 is neutral. Data for chimpanzees come from a single laboratory study of a single individual chimpanzee. A sampling of acidic foods eaten by modern humans is included for reference.

from those tools, tooth marks from hyenas (Blumenschine, 1995). The obvious inference is that such bones were scavenged by our ancestors after being killed by another mammal (maybe hyena, maybe something else). Any hominins that scavenged for prey before the advent of fire may have avoided food borne pathogens if their stomachs were acidic. As a result, it is possible that the acidity of the hominin stomach may have played a role in human foraging behavior and diet. That said, we note that the question of how much hominins scavenged, and how central it was to social evolution, is the subject of intense debate (Dominguez-Rodrigo and Pickering, 2017). An alternate (but not mutually exclusive) hypothesis is that acidic stomachs became advantageous once our ancestors began to hunt large prey. This might be expected if the meat from such a prey items was often more than could be eaten in a sitting such that meat was eaten later (after it had begun to rot) even though it had not been scavenged.

## THE INTESTINES

At some point in the last six million years, in addition to the potential changes in stomach acidity, the guts of our ancestors changed in other ways. The large intestine became shorter relative to the small intestine, while total intestine length also declined relative to body size. That this shift and shortening happened is suggested based on comparisons between the guts of chimpanzees, bonobos, and humans as well as the relatively smaller rib cage (and hence space available for the intestines) in the genus *Homo* compared to earlier hominin species (Aiello and Wheeler, 1995). However, it is worth noting that even within humans that the length of the large intestine varies even among individuals with similar genetic backgrounds. In one study of one hundred individuals, the shortest small intestine observed in any individual was half the length of the longest small intestine. Similarly, the ratio of small intestine to large intestine varied from 2.6 to 4.5. Given that gut morphology differs within populations of modern humans, it is possible (indeed likely) that variation among modern human populations is even greater (Underhill, 1955). To date no studies have considered such variation. The mean ratio of the small to large intestine length for chimpanzees is 1.0 (such that the chimpanzee large intestine is equal in length, on average) to the small intestine (Chivers and Hladik, 1984). But undoubtedly this value varies among chimpanzees as well, such that it is not inconceivable that some human populations and some chimpanzee populations actually have far more similar gut morphologies than tends to be assumed.

The shortening in the relative size of the human large intestine, whatever its consistency and magnitude, raises two questions: why the shortening occurred and what its consequences might have been for digestive physiology and the gut microbiome. In general there seems to be an emerging consensus that the use of tools, especially stick and stone kitchen tools of various sorts,

to obtain and process foods made our ancestors less reliant on the fermentation that occurs in the large intestine. Cooking is likely one of the tools that our ancestors had at their disposal. Recent work has shown that cooking plant food reshapes the gut microbial environment (Carmody et al., 2019), suggesting that the use of fire, despite mixed evidence for its impact on starch digestibility (Schnorr et al., 2016), may have made nutrients in some types of food more available and also eased the chewing necessary to break down food (Wrangham, 2009). Fire may have also made it possible to smoke hives and therefore easier to harvest large quantities of honey with its easy to digest calories (which do not necessarily require gut microbes; Marlowe et al., 2014). In addition, fishing techniques and tools might have made fish and shellfish protein available which, even raw, is very easy to digest. Pounding tools, such as those employed by chimpanzees, would have made roots and tubers also easier to digest (Crittenden, 2016). Similar tools are used by many small-scale societies around the world, including contemporary subsistence foragers (Benito-Calvo et al., 2018) as well as by chimpanzees (and hence likely our LCA; **Figure 2**). All of this is to say that as our ancestors invented more kitchen implements they would have been able to pre-digest and pre-process some of their foods, allowing them to rely less on microbes in their guts to break down recalcitrant components of their diets, such as cellulose. They could get by with smaller guts and invest their bodily energy elsewhere, for example in big brains (an idea called the expensive tissue hypothesis; Aiello and Wheeler, 1995).

The shorter average large intestine length of species of *Homo* compared to those of their ancestors would have had at least two potential consequences for microbiomes. The shorter larger intestine would have sustained a smaller biomass of microbes relative to their body mass (simply because of the reduction in volume). In addition, the retention time of foods in the gut may have been reduced (Ragir et al., 2000). Some features of microbiomes, however, seem likely to have been similar between hominins and our LCA with chimpanzees despite changes in gross intestinal morphology. For example, the taxonomic classes of bacteria found in the guts of both chimpanzees and humans (from urban and rural settings) tend to overlap. What is more, the same families and genera of bacteria tend to occur in similar proportions (Moeller et al., 2012). This overlap is hypothesized to pre-date the human-chimpanzee split (and hence to be characteristic of our LCA). Furthermore, humans in small-scale, non-industrialized populations host a handful of microbial taxa that appear to be genetically equivalent to those in great apes at the level of operational taxanomic units (OTUs) or strains (Amato et al., 2019b). The same humans also share a range of bacterial metabolic pathways with other extant apes, including those involved in vitamin and amino acid synthesis. These results suggest that despite the reduction in length of the human intestines, enough physiological similarities remain between humans and apes such that the composition and function of their microbiomes is similar.

Nevertheless, despite these similarities, it is important to point out that the gut microbiomes of modern humans diverge in important ways from those of extant apes. These differences do not, however, appear to relate to gross morphological features of the gut but instead to diet. The gut microbiomes of humans, while similar to those of modern chimpanzees, appear to be even more similar to those of cercopithecine monkeys, such as baboons (genus *Papio*; Amato et al., 2019b; **Figure 3**). Differences in gut microbiome composition are greater between humans and apes (PERMANOVA $F_{1,55} = 14.4$, $r^2 = 0.21$, $p < 0.01$) than between humans and cercopithecines (PERMANOVA $F_{1,57} = 10.0$, $r^2 = 0.15$, $p < 0.01$). Differences in gut microbiome functional potential are similar between humans and apes (PERMANOVA $F_{1,35} = 5.4$, $r^2 = 0.16$, $p < 0.01$) and humans and cercopithecines (PERMANOVA $F_{1,35} = 7.4$, $r^2 = 0.18$, $p < 0.01$). While humans are genetically far more similar to chimpanzees than to baboons, baboons are more similar in diet (and habitat use) to ancestral *Homo* species than are chimpanzees. Baboons eat diets that are highly omnivorous and relatively high in starch content. Since the gut microbiome plays an important role in processing host dietary compounds, particularly resistant carbohydrates (and in some cases, specifically fibrous plant foods, see Schnorr et al., 2014) it is likely that the same microbial lineages and metabolic pathways nutritionally benefited both our hominin ancestors and extant cercopithecines. Given that the human shift toward habitats and diets like those of modern baboons are often linked to tool use, cooking, and ultimately, reductions in human intestinal length, it seems reasonable to suggest that this suite of changes altered the human gut microbiome. The result appears to be a "characteristic" human microbiome composed of both "ape" and "cercopithecine" traits.

Beyond these "characteristic" human microbiome traits, however, substantial variation exists in the composition and function of the human microbiome. Whether or not this variation is associated with variation in gut length and more generally morphology is unknown, but at broad scales it correlates strongly with geography and lifestyle (Yatsunenko et al., 2012; Obregon-Tito et al., 2015; Gomez et al., 2019). This pattern suggests that the human gut microbiome has the potential to play a role in local adaptation. If local populations of *H. erectus* tended (as with modern baboons or chimpanzees) to be more likely to share microbes with each other than with geographically isolated populations, they might also be more likely to share microbes able to digest or detoxify the foods they were eating

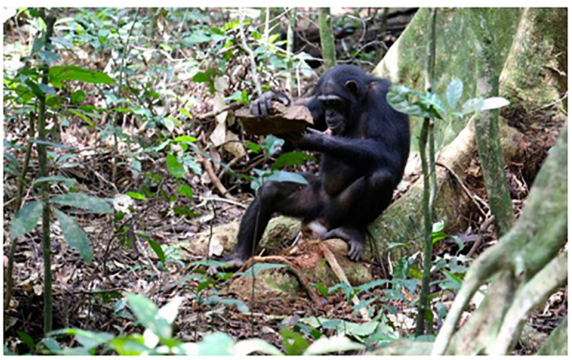

**FIGURE 2 |** Chimpanzee pounding a nut with a stone hammer. Photo by Liran Samuni as part of the Tai Chimpanzee Project.

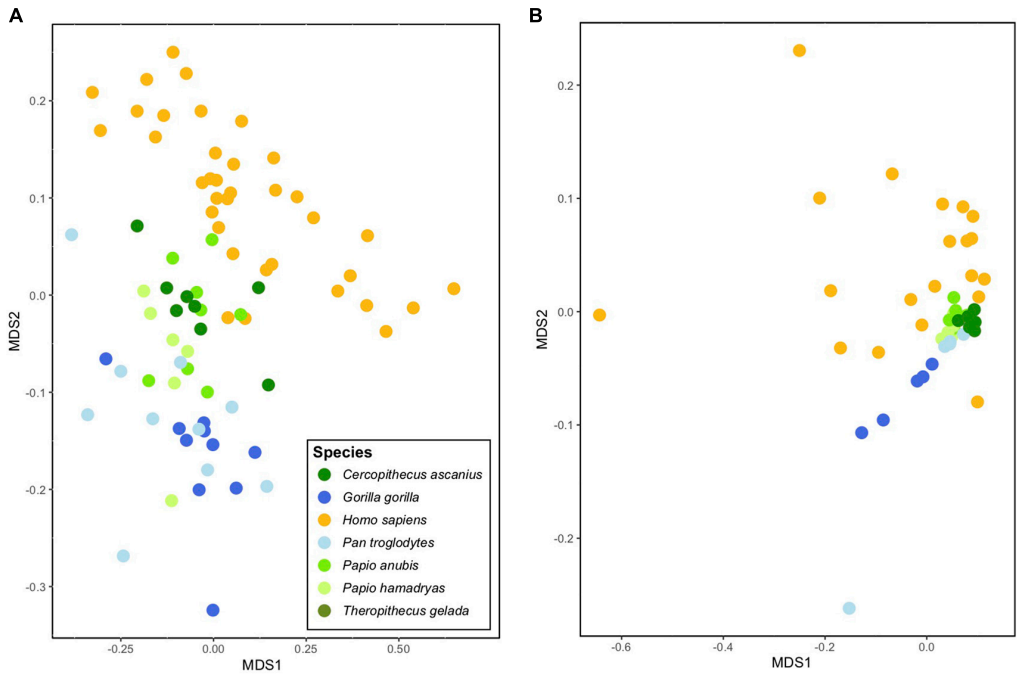


**FIGURE 3 |** Ordination plot indicating similarities in the gut microbiomes of select primate species based on **(A)** taxonomic composition and **(B)** functional potential (data from Amato et al., 2019b). Each point represents the gut microbiome of a single individual, and clustering of points indicates similarity. Note that redtail monkeys (*Cercopithecus ascanius*) and baboons (*Papio anubis* and *P. hamadryas*) generally cluster closer to humans than do chimpanzees (*Pan troglodytes*) and gorillas (*Gorilla gorilla)* in both plots. Note also the relatively higher variation among individual humans (even with the small sample size included here) in functional bacterial genes (scatter of yellow points at right).

in a local region. Or perhaps they shared microbial taxa that increased resistance to endemic infectious diseases. Either way social life (and sharing microbes within social groups) might facilitate digestive plasticity in response to new conditions. More to the point, the social sharing of microbes, might have led to local microbial adaptations to environments, even without changes to host genomes. It has been recently shown is that in some modern human populations, but also other mammals, microbes can contribute to ecological niche differentiation and expansion. For instance, gut microbes enable woodrats to consume a diet high in tannins, allowing them to gain food resources inaccessible to mammals that do not have this gut microbial adaptation (Kohl et al., 2016). In modern humans, such microbial local adaptation appears to allow, for example, populations that consume a diet rich in seaweed to extract normally inaccessible complex carbohydrates (Hehemann et al., 2010). Fewer studies have examined microbiome adaptations to local infectious disease profiles, but one can imagine similar dynamics. And whatever these effects they will ultimately be (and have been) strongly influenced by the social behavior of hominins.

'We hypothesize that microbially facilitated local adaptations were critical to the human evolutionary trajectory. A defining feature of the first humans (be they *H. habilis* or *H. erectus*) was the extent to which they moved, which happened in two ways. First, early members of the genus *Homo* roamed the African landscape bipedally. In doing so, they confronted more food choices than had their ancestors. An abundance of carbon isotope data show a variability in diet including plants such as grasses and sedges, as well as the animals who consume these plants (Sponheimer and Dufour, 2009). Further, the manufacture of stone tools and their uses for targeting both plant foods as well as terrestrial and aquatic animals is well documented (Braun et al., 2010; Lemorini et al., 2014), suggesting that our early ancestors enjoyed a diet far more diverse than our great ape counterparts. Second, *H. erectus*, a very successful hominin by all accounts, moved into new geographic areas. *H. erectus* would eventually arrive as far north as Spain and as far east as China. In doing so, *H. erectus* used a diversity of approaches to eat: in different places, different foods, and utilizing a variety of different tools, both as reflection of what was available in those environments but, by analogy to modern chimpanzees (**Figure 4**), probably also due to differences in culinary culture. It was also likely exposed to novel disease landscapes. A plastic microbiome that could shift rapidly both within and across individuals and populations could have facilitated dietary diversity by contributing key metabolic pathways to maximize nutritional output from a range of foods and may have also increased the ability of *H. erectus* to endure new diseases (Amato et al., 2019a). While we cannot assess *H. erectus* microbiomes directly, modern human microbiomes exhibit more inter-individual variation compared to closely related non-human primates (Schnorr et al., 2016; Amato et al., 2019b).

It is reasonable to imagine that this microbiome diversity is tied directly to the vast dietary and pathogen exposure diversity

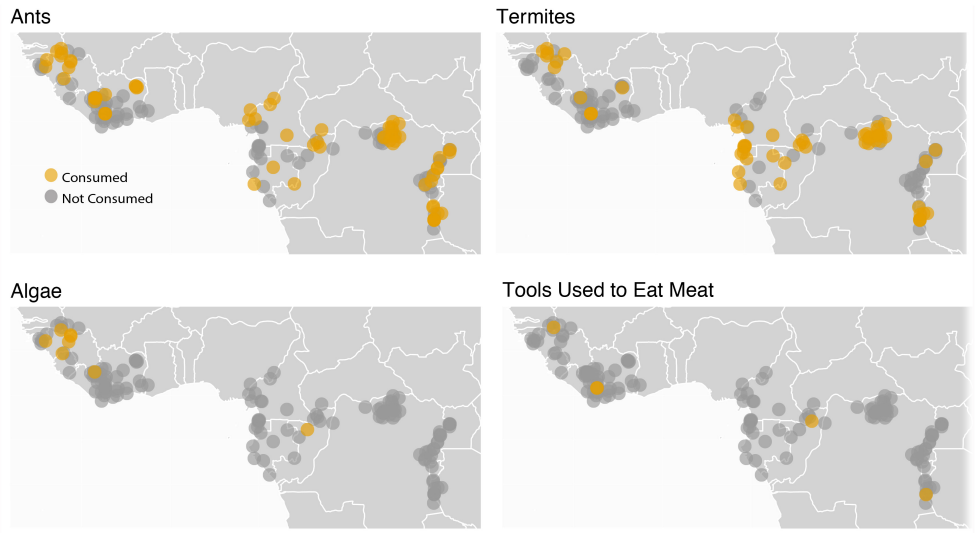


**FIGURE 4 |** The variation in diets among *H. erectus* populations is likely to have exceeded that of any hominin that lived previously, both because of the diversity of tools used by *H. erectus* and because of its large geographic range. However, this is not to say other primates do not also vary in their diets geographically. The map above shows sites at which chimpanzees have and have not been observed feeding on ants, termites, algae, and meat using tools (data from Kühl et al., 2019), one measure of chimpanzee dietary diversity. It is of note that the animal species chimpanzees use tools to eat differ among communities even in cases in which the environment does not differ, due to chimpanzee culinary cultures. For example, the chimpanzees at Gombe in Tanzania use tools to eat driver ants (*Dorylus* spp.) and acrobat ants (*Crematogaster* spp.), but the chimpanzees at nearby Mahale in Tanzania (Nishida and Hiraiwa, 1982), where both driver ants and acrobat ants are present, use tools to feed on carpenter ants (*Camponotus* spp.).

represented by humans globally, which began to emerge with *H. erectus*. Additional research is necessary to explore this idea further. Ancient DNA studies may allow some insights. Some insight might also be garnered from the study of modern chimpanzee populations, especially given that the tool use and culinary cultures of chimpanzees differ greatly among communities (**Figure 4**). We can predict that if microbiome composition was associated with dietary differences among *H. erectus* populations that the same should also be true among extant chimpanzee populations. This has yet to be tested, but is testable. Assuming local microbial adaptations facilitated human dietary niche expansion and subsequent human success in a range of environments around the world, human social structures likely played an important role in establishing and maintaining geographically specialized microbiomes.

## SKIN

Human bodies have several kinds of "sweat" glands. One kind, eccrine sweat glands, is associated with evaporative cooling. However, humans also have a second important kind of "sweat" glands, apocrine glands. In some non-humans, such as camels (genus *Camelus*), the primary function of apocrine glands is to produce sweat and to function in the way that eccrine glands function in humans (Folk and Semken, 1991). But in humans and apes apocrine glands appear to play other roles. They are located primarily in armpits (and to a much lesser extent around the genitals and anus), where they collectively form what have been termed axillary organs (Ellis and Montagna, 1962). In extant hominids (humans and living apes) the apocrine glands produce a white, milky substance that feeds slow-growing bacteria species living in the glands themselves and on the surface of the skin. It is these bacteria that are responsible for the main body odors associated both with the armpits and the genitals (Shelley et al., 1953).

It is thought that the primary function of apocrine glands in primates is to help convey chemical signals among individuals within a species. Aroma wicks up the hair associated with the apocrine glands (which in gorillas, chimpanzees and humans has a different morphology than does ordinary body hair; Weiss, 2009) and travels to the noses of conspecifics, much as occurs (whether one likes it or not) between one human and another in, say, a crowded elevator. The key question is just what the aromas produced by apocrine glands convey. Of course, they might convey different types of information depending on species and context. In lemurs, aromas from apocrine glands can signal individual identity ("It is me!"; Scordato et al., 2007), as well as relatedness ("I am not your brother."; Charpentier et al., 2008). In Western lowland gorillas (*Gorilla gorilla gorilla*), the same seems possible, in as much as humans are able to distinguish the aroma of individual gorillas (Hepper and Wells, 2010), and one imagines that gorillas are better at distinguishing among gorillas than are humans. Because apocrine glands produce their secretion in response to stressful situations and arousal, the aromas produced by the bacteria in these glands might also signal fear, arousal or stress. Finally, the products of the glands include both proteins and fats and where nutrients are scarce must be relatively expensive to the host (Zeng et al., 1992). Some aromas might thus be reliable signals that an individual is

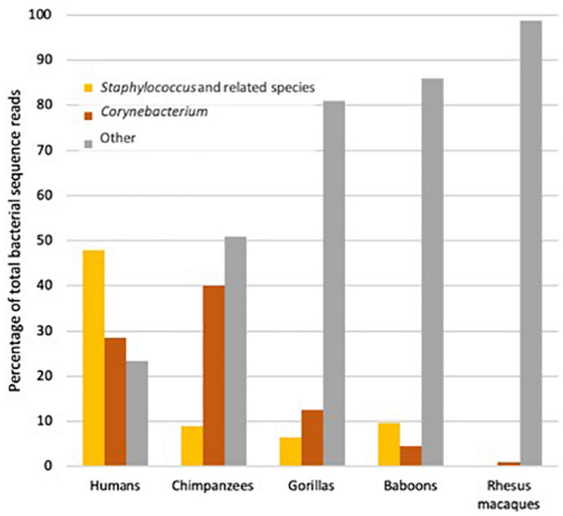


**FIGURE 5 |** The proportion of bacterial reads in samples of armpits of humans (*Homo sapiens*), chimpanzees (*Pan troglodytes*), gorillas (*Gorilla gorilla*) and two monkey species, Hamadryas baboons (*Papio hamadryas*), and Rhesus macaques (*Macaca mulatta*) that were from species of slow-growing, odor-producing *Corynebacterium*, weedy fast-growing species of *Staphylococcus* and relatives or other taxa (Data from Council et al., 2016).

sufficiently well-fed to produce apocrine substances, and hence also healthy. Conversely, unusual apocrine aromas might reliably signal infection or poor nutrition. In this framing, armpit aromas might be one hominid equivalent of a peacock tail (as has been suggested to be the case for similar glands in some lemur species; Walker-Bolton and Parga, 2017). Realistically, however, the social role of these organs, while likely to have been important in our ancestors, has been poorly considered. We know both that human armpit odors and microbes tend to be different than those of chimpanzees and gorillas, but also very variable among humans (Council et al., 2016; **Figure 5**). This variation is intriguing in light of the discovery of the influence of the ABCC11 gene on the apocrine glands.

One variant of the ABCC11 gene, with a single nucleotide substitution, is much more common in some human populations than in others, particularly east Asian populations but also in some populations from cold habitats (Ohashi et al., 2010). This version of the gene is noticeable for two reasons. In the homozygous form it produces dry earwax (ear wax is also produced by an apocrine gland, albeit a highly modified one). In addition, that same variant leads to apocrine glands in the armpit that produce very little secretion. As a result, individuals with this variant of the ABCC11 gene have microbes that are very different from those with the ancestral variant. This single nucleotide substitution explains much of the variation in skin microbe compositions among individual humans (with additional variation explained by whether or not individuals use antiperspirant; Urban et al., 2016). The effect of ABCC11 is seen not just in the armpit but also more generally (Coyle, 2018). What is most remarkable is that this single nucleotide substitution, which arose roughly forty thousand years ago appears to have been under extraordinarily strong selection in temperate Asia, for reasons that remain enigmatic but could relate to the ways in which changes in human social systems impacted the value of the odors being produced by armpit microbes to human survival (Ohashi et al., 2010).

If we are to target for study bacterial taxa on the skin that might have been influenced by or influence hominin social behavior for further study, bacteria of the genus *Corynebacterium* are of interest, but so too are those of the genus *Staphylococcus*. Species of *Staphyloccoccus* have long been thought to be the medically normal beneficial skin bacteria of humans, bacteria able to help defend the skin against pathogens and perform other functions. Attempts were even (successfully) made to innoculate the skin of newborn babies with particular strains of *Staphylococcus* bacteria so as to ward off pathogens (see history reviewed in Dunn, 2018). However, the study of the skin bacteria of other primates suggests that the dominance of *Staphylococcus* bacteria on human skin is unusual (**Figure 3**). This raises the question of when *Staphylococcus* bacteria began to dominate human skin microbiomes. One intriguing observation was made in a recent study by Ashley Ross and colleagues of skin bacteria across mammals. The study found that *Staphylococcus* was a minor player in the skin microbiota of most mammals. However, there were exceptions. *Staphylococcus* was the most common genus of bacteria on the skin of some wild sheep (*Ammotragus lervia*), goats, cows (all of which are domesticated animals or relatives of domesticated animals) and humans. This observation raises the possibility that skin *Staphylococcus* spread among humans and domesticated animals during cohabitation and, in doing so, changed the dominant skin microbial taxa. One can imagine a scenario in which the dominance of this bacteria across both humans and domesticates then also facilitated domestication in as much as it would increase the similarity of the aroma of domestic animals to humans and vice versa. As cohabitation with domesticated animals became more common, and human communities benefited from these associations, this may have led to the further spread of *Staphylococcus* dominated skin communities. On the other hand, it may be revealed that the skin microbes of zoo animals are unusual and record recent sharings of taxa and, in doing so, obscure ancient ones.

## THE EXTENDED MICROBIOME

Recent research has highlighted the role of food processing in human evolution and the evolution of human sociality (Henrich, 2017). Food processing has a potentially large impact in as much as it reduces the calories needed for digestion, and the amount of chewing necessary for a given food item (Zink and Lieberman, 2016). In addition, food processing is thought to lead to an increased probability of the use of key sites on the landscape as home bases. The types of food processing that have received the most attention to date have been those that chimpanzees undertake, such as the pounding of nuts and seeds

(Boesch and Boesch, 1983), the use of sticks to access tubers, insects, and even animal prey, and roots (Bogart and Pruetz, 2008). Additional types of processing that our ancestors may have engaged in included the use of fire to cook food, the use of fire and smoke to calm bees (Crittenden, 2011), and in doing so allow the extraction of large quantities of honey and the use of new kinds of tools to cut into, dismember and divide meat, whether from animals that have been killed or those that have been scavenged. Some of these forms of food processing involve microbes to a degree, but typically as supporting characters. For example, one of the advantages of cooking is that it kills off potential pathogens in meat. Another is that it makes nutrients in tubers and roots more available to microbes in the large intestine (and easier for them to metabolize). But at some point microbes began to play a more central role, once humans began to actively control fermentation.

The use of technologies to control rot allowed humans to begin to favor microbes with traits that were desirable. Those traits might include aromas, flavors, acids or alcohol (as well as nutritional properties that these attributes might portend). Simultaneously, fermentation allowed other microbes to be disfavored, thanks to the allelopathic effects of alcohol, acids and other products of fermentation. Finally, fermentation could enrich certain vitamins in foods and begin the process of processing (ultimately, digesting) food such that more nutrients would be available (Speth, 2017).

The timing of the first controlled fermentation by humans is unknown. It is possible that *H. erectus* fermented foods. Some fermentations require vessels, but not all. Food can be fermented in animal stomachs (Frink and Giordano, 2015). In addition, food can be fermented by submerging it in slow-moving streams or by burying it underground. Many carnivore species ferment food. In hot regions, Hyenas appear to store (and to some extent ferment) food items by putting them in water (Selvaggio, 1998). In cold regions, foxes and other carnivores store and ferment foods by burying them (Vander Wall and Smith, 1987). All of this is to say that neither technical nor intellectual barriers would have prevented *H. erectus* or their relatives from fermenting at least some kinds of foods. Recently Speth has suggested that Neanderthals may have fermented meat and nothing in his argument precludes far earlier uses of fermentation (Speth, 2017). In as much as fermentation requires very little in terms of persistent tools, it is difficult to know what archeological evidence would support (or refute) the idea that *H. erectus* or later hominins fermented foods, or to estimate the timing of the first fermented foods. The evidence that does exist relates to two genetically encoded human traits, those associated with sour taste receptors and those associated with the enzyme alcohol dehydrogenase and its function.

In nearly all primates that have been studied to date, even slightly acidic foods are perceived as sour and aversive. In general, it is thought that sour taste receptors evolved in mammals so as to lead them away from foods (be they fruit or meat) that had begun to rot due to the presence of lactic acid bacteria or acetic acid bacteria or fruits that were unripe and hence might contain plant defensive compounds. In studies to date, there appear to be only two or three primate species that respond differently to sour foods. Night monkeys (genus *Aotus*), which forage in the dark and so must smell foods as much as they see them, are able to detect acidic substances and perceive them as sour. Additionally, unlike most other non-human primates, unless these foods are highly acidic, night monkeys perceive them as pleasant (Glaser and Hobi, 1985). The other clear exception is humans. Adult humans, like night monkeys, perceive slightly acidic foods as pleasant and can learn to enjoy even very acidic foods; Liem and De Graaf, 2004; Breslin, 2013. Therefore, at the moment, our picture of sour taste preference is one in which two lineages, that of night monkeys and that of our own species, evolved a preference for sour foods. This portrait of the past is obviously heavily contingent on how poorly studied sour taste has been in primate species. It is possible the preference for sour tastes is more common than is so far appreciated. For example, Toshisada Nishida found that a relatively high proportion of the fruits ingested by the Mahale chimpanzees tasted sour to him (Nishida et al., 2000), such that it seems plausible that the Mahale chimpanzees enjoy such fruits (whether they be sour due to unripeness or rot). While it is possible that the chimpanzees have learned to enjoy sour fruits rather than innately enjoy them, it seems less likely. Assuming that both chimpanzees and humans innately prefer sour foods, it is reasonable to hypothesize that the preference occurred in our common ancestor. Unfortunately, the workings of and genes associated with sour taste receptors have not yet been well characterized (although see Montell, 2018). Regardless, once our ancestors evolved a preference for sour foods it would have been much easier to learn to control fermentation in as much as one of the key products of fermentations (acids) tasted pleasant.

A second evolutionary change that certainly influenced the ways in which our ancestors fermented foods is the evolution of alcohol dehydrogenase. Alcohol dehydrogenase facilitates the first key step in the breakdown of alcohol, yielding toxic acetaldehyde that must be further degraded. While the other genes in this pathway have yet to be explored, humans and apes possess a variant of the alcohol dehydrogenase gene that is forty times more efficient than that of almost all other primates (Carrigan et al., 2015). Given that ethanol is a necessary byproduct of the fermentation process, changes in human alcohol dehydrogenase are often linked to human consumption of fermented foods, particularly fermented beverages. Recent reconstructions, however, suggest that this gene evolved roughly ten million years ago, in line with what might be expected if it evolved when early apes began to spend more time on the ground and began to encounter and consume fermented fallen fruit that was more ethanol-rich than ripe fruit picked directly from trees (Carrigan et al., 2015). Some modern chimpanzee communities (like modern humans) enjoy a tipple, and even make tools with which to access alcohol (Hockings et al., 2015). The same may well have also been true of our common ancestors.

Regardless of when fermented food use first emerged in the human lineage though, the fermentation process ultimately

allowed our ancestors to begin to store food (and also to stay in one place for more time). It would have facilitated the persistence of larger groups of individuals living together. In addition, it set up a potential feedback. Individuals who fermented foods very often relied upon bodily microbes to do so. Sometimes they were microbes found on the bodies of insects, as is the case with brewer's yeast (Madden et al., 2018). In other cases, they were microbes associated with human or other mammal bodies. Modern examples of the latter include the use of salivary microbial communities to initiate the fermenting process in chicha production in Peru, and similar fermented beverages around the world (Freire et al., 2016), the use of skin microbes to produce bodily aromas in some semi-soft cheeses (Pham et al., 2017), the use of body associated *Lactobacillus* species in sourdough breads (Gänzle and Ripari, 2016) or the use of the ancestrally mouth-associated bacteria species, *Streptococcus thermophilus*, in the production of yogurt (Goh et al., 2011). Once they began using body and other microbes to ferment foods, our ancestors extended their genomes and ultimately their phenotypes in much the way that beavers do in building a dam that yields a pond (Carthey et al., 2018). More specifically, by co-opting body microbes, they extended their guts, allowing digestion to begin to happen where food was fermented. Furthermore, when those food items were and are consumed, they can re-inoculate consumers, becoming even more common within the communities of individuals that rely upon them. For individuals who ate together, this would have been a mechanism through which microbes and microbial genes within groups became more similar than between groups. As a result, the complex dynamics of social networks interacting with microbes would intensify in these contexts.

## PROSOCIAL MICROBES

Ultimately, what we are left with in regard to the potential influence of microbes on the evolution of hominins is a sketch. It is a rough sketch, subject to revision. And it is a sketch based on what we can observe today. We conclude by considering, even more speculatively, what might be. More specifically, we consider the possibility that some of the microbes associated with hominin microbiomes (be they those of stomachs, intestines, skin, the extended microbiome, or other microbiomes such as the breast milk microbiome or the vaginal microbiome, which differs greatly among primate species; Miller et al., 2016) might have directly favored particular kinds of social behavior and, in as much, account for two of the major social transitions in hominins: the transition to larger more sedentary populations and the transition to urban living.

As we have already noted, human control over microbial populations might have facilitated such transitions (e.g., by allowing food storage and turning pathogenic water into non-pathogenic booze). In turn, could microbes have controlled human behavior? Recently, a number of microbes have been shown to control the social behavior of their hosts in ways that increase microbial fitness. The eukaryote, *Salpingoeca rosetta*, for instance, can lead a solitary or multicellular lifestyle. The transition between these two lifestyles is mediated by lipids produced by a *Algoriphagus machipongonensis*, its bacterial commensal. In other words, the products of the microbiome of this eukaryote determine whether or not it is social (Woznica et al., 2016). Or consider leaf cutter ants such as the species *Acromyrmex echinatior*. Leaf cutter ants, like most social insects, recognize each other on the basis of their cuticular hydrocarbons. Those cuticular hydrocarbons are produced, in part, by bacteria on the exoskeletons of the ants (perhaps a phenomenon not so very different than apes recognizing each other on the basis of their armpit odors). If the ants are treated with antibiotics, their nestmates attack them (Teseo et al., 2019). In considering the evolution of hominins, these examples raise the question: is it possible that at critical junctures in hominin social evolution that some microbes were favored by social interactions and evolved in association with hominin populations? If such microbes lived persistently on or with their host (and so were disadvantaged by the death of the host) and spread human to human through social interactions, they might increase their fitness if they caused their hosts to behave more socially, live in larger groups and interact more frequently. It is now well-documented that the malaria parasite can influence its hosts so as to make transmission more likely (by making hosts more attractive to vector mosquitoes; De Moraes et al., 2014). It doesn't seem much more outlandish to imagine a microbe (be it a species of bacteria, fungus, protist or even virus) that would make its own spread more likely by making humans more social. In concluding with this example, we pose the question to the field of how we might even look for such a microbe. One might argue that the spread of yeast strains that produce more alcohol represents a relatively recent example of such a scenario (in which alcohol producing yeasts lead us to addiction, drunken social interactions and the desire for more products of such alcohol producing yeasts). But we can't yet preclude far more ancient influences of microbes on the ways in which we interact.

## DATA AVAILABILITY STATEMENT

All data are from previously published papers (Yatsunenko et al., 2012; Beasley et al., 2015; Clemente et al., 2015; Obregon-Tito et al., 2015; Smits et al., 2017; McDonald et al., 2018; Amato et al., 2019c; Kühl et al., 2019) and are available through NCBI as Bioproject PRJNA281417, https://science.sciencemag.org/content/suppl/2019/03/06/science.aau4532.DC1 and from the EBI with the following Project IDs: ERP104379 (Amato et al., 2018), ERP008799 (Clemente et al., 2016), ERP008694 (Clemente et al., 2016), ERP014589 (Obregon-Tito et al., 2016), PRJEB3079 (Yatsunenko et al., 2016), ERP109605 (Smits et al., 2018), and ERP012803 (McDonald et al., 2016). They are also publicly available on Qiita (Gonzalez et al., 2018) with the following Qiita IDs: 11212, 10052, 1448, 850, 11358, and 10317.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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CORRECTION
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# Corrigendum: The Internal, External and Extended Microbiomes of Hominins

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**Keywords: fermentation, primates, prosocial microbes, feces, food, armpits, alcohol**

## A Corrigendum on

**The Internal, External and Extended Microbiomes of Hominins**
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In the original article, there was a mistake in **Figure 1**. Colobus monkeys and Langur monkeys were misclassified as omnivores rather than as folivores; and "Sykes monkeys" was misspelled and misclassified as an herbivore rather than as an omnivore. The corrected **Figure 1** appears below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

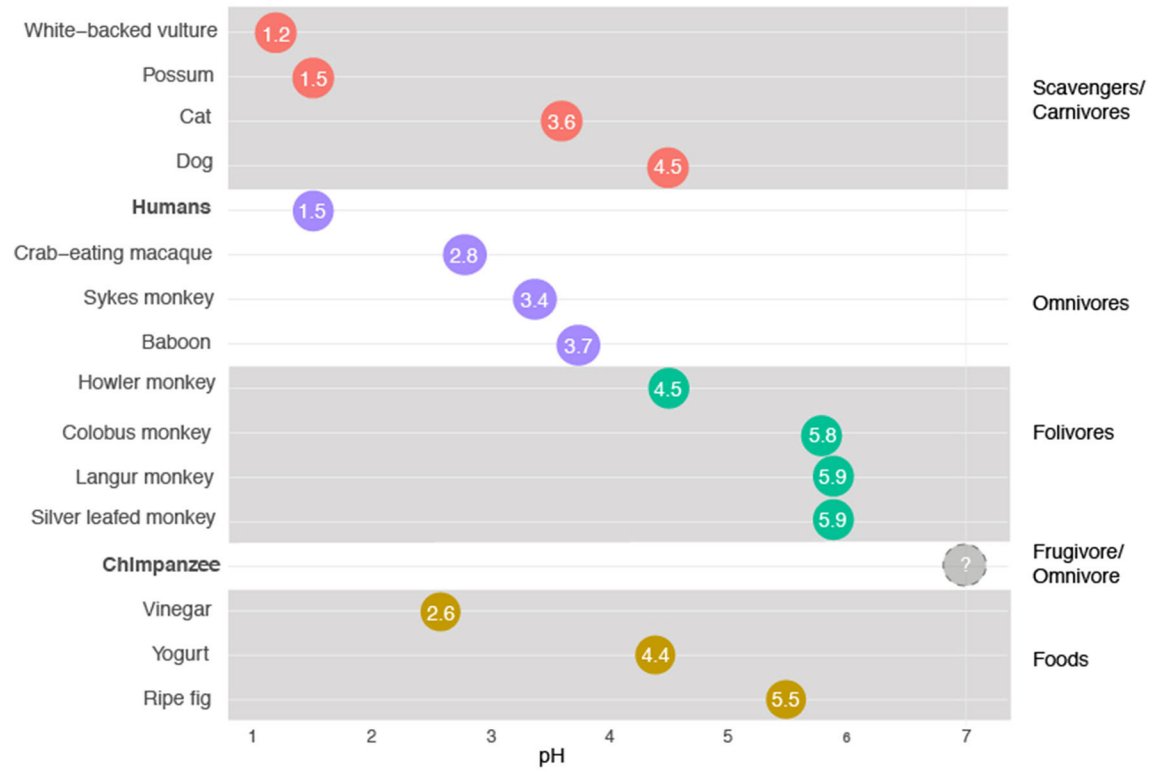


**FIGURE 1 |** Stomach pH as a function of animal species. A pH of 7 is neutral. Data for chimpanzees come from a single laboratory study of a single individual chimpanzee. A sampling of acidic foods eaten by modern humans is included for reference.

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ORIGINAL RESEARCH
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# No Evidence for Single-Copy Immune-Gene Specific Signals of Selection in Termites

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Selection pressures from pathogens appear to play an important role in shaping social evolution. Social behavior, in particular brood care, is associated with pathogen pressure in wood-dwelling "lower" termites. Yet, generally pathogen pressure is predicted to be low in wood-dwelling termite species that never leave the nest except for the mating flight. In comparison, pathogen pressure is predicted to be higher in species that leave the nest to forage, and thus constantly encounter a diversity of microbes from their environment. We hypothesized that such differences in predicted pathogen pressure are also reflected by differences in the intensity of natural selection on immune genes. We tested this hypothesis in a phylogenetic framework, analyzing rates of non-synonymous and synonymous substitutions on single-copy immune genes. Therefore, we leveraged recent genomic and transcriptomic data from eight termite species, representing wood-dwelling and foraging species as well as 14 additional species spanning the winged insects (Pterygota). Our results provide no evidence for a role of pathogen pressure in selection intensity on single-copy immune genes. Instead, we found evidence for a genome-wide pattern of relaxed selection in termites.

**Keywords: immunity, social insects, termites, selection, comparative genomics**

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## INTRODUCTION

Like in other organisms, pathogens seem to be important drivers of evolution in social insects. On the one hand, social insects are a "desirable" target for pathogens as an insect colony represents a large source of many potential hosts, all with a similar genetic background. Thus, if pathogens manage to enter a colony, they can exploit many individuals. However, social insects are also well protected as they evolved "social immunity" (Traniello et al., 2002; Cremer et al., 2007), a repertoire of defensive mechanisms that work at the colony level. For instance, behavioral task division limits contact to potentially infected individuals and can lead to their eviction. Also molecular mechanisms of social immunity exist, for example indirect immunization of colony members (e.g., Traniello et al., 2002; Cremer et al., 2007; Masri and Cremer, 2014) or impregnation of the nest walls with fungicidal compounds (Bulmer et al., 2009; Rosengaus et al., 2011). Thus, social immunity can be considered a selected emergent property of insect colonies where the whole is more than the sum of the individual parts (Rosengaus, personal communication). The evolution of social immunity aligns with the complexity of social organization, suggesting that selection pressure by pathogens could be a driver of complex social organization.

In termites, there is evidence for an association between selection pressure from pathogens, ecology, and social complexity. Although all termites are eusocial, the degree of worker altruism differs between termite lineages and aligns with ecology (Korb et al., 2012). Species of most early branching lineages of the termite phylogeny share a similar ecology called "one piece nesting" or "wood-dwelling" life type (Abe, 1987; Korb, 2007). Wood-dwelling termites nest in a single piece of wood that serves as food and shelter. "Workers" of wood-dwelling termites are developmentally totipotent immatures that become reproductives, and thus are sometimes considered "false" workers. In particular, species of the family Kalotermitidae display little brood care by false workers and form less complex societies, where the interactions are generally not altruistic but cooperative (Korb, 2007). Yet, there are differences in the degree of brood care between wood-dwelling species. Brood care appears to be malleable depending on nesting ecology (Korb et al., 2012). Brood care is negligible or absent in several dry wood termites such as *Cryptotermes secundus* (Kalotermitidae) that nest in sound dry wood. More intensive brood care (especially allogrooming) occurs in the dampwood termite (Archotermopsidae), *Zootermopsis nevadensis*, which nests in rotten, decaying wood (Korb et al., 2012). Brood care is also present in *Zootermopsis angusticollis* (Rosengaus and Traniello, 1993). Looking beyond termites having a wood-dwelling life type, brood care is more intensive in termite lineages that have altruistic workers with reduced developmental options (true workers) and complex social organization (Roisin and Korb, 2011) like *Macrotermes*. These species are central-place foragers (multiple-pieces nesting, see Abe, 1987; Korb, 2007) with nests separated from the foraging ground.

The increase in social complexity and changes in ecology seem to align with the degree of immune challenge in two ways. First, wood-dwelling termites are confined within their nests, and hence it seems reasonable to assume that they are not exposed to microbial challenges as frequently as foraging termites. Second, within the group of wood-dwelling species, the nests of termites that live in sound drywood can be assumed to be microbe- and pathogen-poor when compared to nests of species that live in decaying dampwood. Rosengaus et al. (2003) provide evidence for the latter if we assume that pathogen pressure can be extrapolated from the cultivable fungal and bacterial loads that these authors measured. In fact, the dampwood termite, *Z. angusticollis* that was investigated in Rosengaus et al. (2003) seems to have huge constitutive investments in immune defense at different levels. This includes the individual as well as the colony level, potentially with socially acquired immunity (Rosengaus et al., 1999, 2011; Traniello et al., 2002; Cole and Rosengaus, 2019). The investment in immunity in dampwood termites of the genus *Zootermopsis* is also visible at the genome level. In the genome of *Z. nevadensis*, six copies of Gram-negative binding proteins (GNBPs) were found, more than in many other insect species (Terrapon et al., 2014). GNBPs can serve as microbial detectors and effectors alike. Four of these presumably termite-specific genes were also found in the genome of the fungus-growing termite *Macrotermes natalensis* (Termitidae, see Poulsen et al., 2014; Korb et al., 2015). *M. natalensis* is a foraging species with intensive brood care and a complex social organization. For other central-place foraging termites, such as several Australian *Nasutitermes* species (Termitidae), which also have a complex social organization, as well as for *Reticulitermes*, GNBPs had previously been shown to be under positive selection (Bulmer and Crozier, 2006). Additionally, selection on three immune genes (GNBP1, GNBP2, and Relish) differed between Australian Nasutitermitinae. The rate of adaptive evolution of GNBP2 and Relish were increased during the transition from feeding on dry grass stored in epigeal nests to feeding on decaying wood (Bulmer and Crozier, 2006; Rosengaus et al., 2011), providing additional evidence for immune challenges that are specific to species that live in decaying wood.

Based on these results, we hypothesized that selective pressure on immune defense genes (IGs) differs across termites depending on their life type (Korb et al., 2015). We tested the hypothesis that wood-dwelling termites, which do not leave the nest to forage outside show relaxed selection on IGs and fewer signs of positive selection compared to soil-foraging species. Additionally, we tested whether within the wood-dwelling life type, the dampwood termite *Z. nevadensis* had stronger signs of selection than other wood-dwellers that nest in sound wood.

In order to test for differences in selective forces acting on IGs between wood-dwelling and foraging species, we analyzed a set of 81 previously identified single-copy IGs (see section "Materials and Methods") in eight termite species (published data see Misof et al., 2014; Poulsen et al., 2014; Terrapon et al., 2014; Harrison et al., 2018; Evangelista et al., 2019; data sources are provided in **Supplementary Table S1**). Four of the species are wood-dwelling species: *C. secundus, Incisitermes marginipennis, Prorhinotermes simplex*, and *Z. nevadensis*. The remaining four species are foraging species: *Mastotermes darwiniensis*, *Reticulitermes santonensis* (i.e., *Reticulitermes flavipes*), *Coptotermes* sp. and

*M. natalensis*. These were analyzed in a phylogenetic framework of 22 species (one mayfly, 12 polyneopteran insects including above listed termites and their closest relatives Cryptocercidae, two paraneopteran, and six holometabolous insects) spanning winged insects to increase the statistical power of lineage specific tests for selection.

# RESULTS

## Phylogenetic Relationships

The species tree was inferred from a supermatrix including 1,178 single-copy orthologs (SCOs) and spanning an alignment length of 555,906 amino acid positions (partition coverage 100%, site-completeness score $C_a$ = 74.61%, see **Supplementary Material** and **Supplementary Figure S1**). Termites were monophyletic with *Cryptocercus* as sister group, consistent with earlier work (e.g., Lo et al., 2000; Klass and Meier, 2006; Inward et al., 2007; Legendre et al., 2008). Phylogenetic relationships within termites are largely consistent with Evangelista et al. (2019) and are statistically maximally supported (**Figure 1**). Consistent with earlier work (e.g., Legendre et al., 2008), neither wood-dwellers nor foragers constitute monophyletic groups, confirming that several independent switches in life type were included in our analyses. More details on phylogenetic analyses are provided in the **Supplementary Material**.

## Patterns of Selection on Termite Immune Genes

Between 13 and 78 SCOs of IGs per species were included in the analyses (**Table 1**). We found no evidence for positive selection on the IGs (**Table 2** and **Supplementary Table S2**).

Next, we tested the hypothesis that selection on the IGs of wood-dwelling species is relaxed compared to foragers. We found 47 cases of significantly relaxed selection across all termite species analyzed (**Table 1** and **Supplementary Table S3**, $P < 0.05$, FDR $< 0.2$). There was no evidence for a difference in the number of IGs under relaxed selection between the life types (generalized linear mixed effects model with binomial error distribution: $df = 7$, $z = 0.096$, $P = 0.92$). There was also no evidence for differences between species (generalized linear model assuming binomial error distribution: $df = 7$, $z = -1.75$–$0$, $P = 0.08$−$1$, ranges of $z$ and $P$ are for the different species). Because changes in the selection intensity on IGs could be obscured by genome-wide differences in selective constraint, it is important to test these hypotheses against the genomic background. To this end, we generated sets of background genes (BGs) that consisted of genes matching the GC-content and sequence length of each IG for each species (see section "Materials and Methods"). The number of IGs under relaxed selection did not differ significantly from that expected from the analysis of the BGs for any of the species [see 95% confidence intervals (CIs) for BG sets in **Table 1**]. In order to take genome-wide effects of selective constraint into account, when comparing selection intensity between wood-dwellers and foragers, (i) we calculated the ratio of genes under significantly relaxed selection between wood-dwellers and foragers for IGs and (ii) compared this ratio to that expected from BGs (see section "Materials and Methods"). If selection is relaxed specifically in the IGs of wood-dwellers relative to their genomic background, we expect the ratio of the number of significant genes under relaxed selection between wood-dwellers and foragers to be larger for the IGs than for the BGs. However, the ratio of the number of IGs under relaxed selection between wood-dwellers and foragers did not differ significantly from the expectation derived from BGs (**Figure 2A**), supporting the view that patterns of relaxed selection on IGs follow genome-wide trends in selective constraint.

Because we did not find any evidence for an increase in the number of IGs under significantly relaxed selection in wood-dwellers, we reasoned that a putative signal of relaxation of selective constraint might be more diffuse and only become visible as a general trend over all IGs investigated. In order to capture such more general trends, we assessed potential differences in $k$, a measure for the intensity of selection, for all IGs between life types. $k$ did not differ significantly between species (Kruskal–Wallis test: $df = 7$, $X^2 = 3.95$, $n = 322$, $P = 0.79$, for n per species see **Table 1**) nor was it lower for wood-dwellers (Mann–Whitney $U$-test, one-sided: $U = 11{,}690$, $n = 322$, $P = 0.41$). This indicated similar selection intensity on IGs for wood-dwellers and foragers. For the comparison of $k$ between life types it is, as above, important to take the selective constraint on the genomic background into account. $k$ for the IGs did not differ significantly from $k$ for sets of BGs for any of the species investigated (**Table 1**). Following the same rationale as above, we used the ratio of medians of $k$ between wood-dwellers and foragers as a test statistic. This ratio can be interpreted as the relative intensity of selection between wood-dwellers and foragers. We found that the relative intensity of selection between wood-dwellers and foragers on IGs matched that of the BG sets (**Figure 2B**), again suggesting that the IGs follow genome-wide trends of selective constraint.

Finally, we hypothesized that our results might be affected by the particular selection pressures that act on *Z. nevadensis,* which is a wood-dwelling species, but lives in dampwood nests. Nests in dampwood have a high microbial loads, as has been shown for *Z. angusticollis* (Rosengaus et al., 2003). Hence, selection would be expected to be stronger on *Z. nevadensis* IGs than on IGs in the other wood-dwellers, resulting in a smaller fraction of genes under significantly relaxed selection in *Z. nevadensis*. We found no evidence for this hypothesis (generalized linear model assuming binomial error distribution: $df = 3$, $z = 1.73$, $P = 0.084$). The overall intensity of selection on IGs ($k$) also did not differ significantly between *Z. nevadensis* and the other wood-dwellers (Mann–Whitney $U$-test, $U = 5{,}471$, $n = 215$, $P = 0.77$). Similarly, it could be argued that the assumption of relaxed selection only holds for the dry wood-dwellers *C. secundus* and *I. marginipennis*, assuming that only dry wood is a truly pathogen poor substrate. We could not find a difference in the number of genes under relaxed selection between the dry wood-dwellers and the other species (generalized linear model with binomial error distribution: $df = 7$, $z = 0.048$, $P = 0.96$) nor for the intensity of selection over all IGs as measured by $k$ (Mann–Whitney $U$-test: $U = 10{,}538$, $n = 322$, $P = 0.37$).

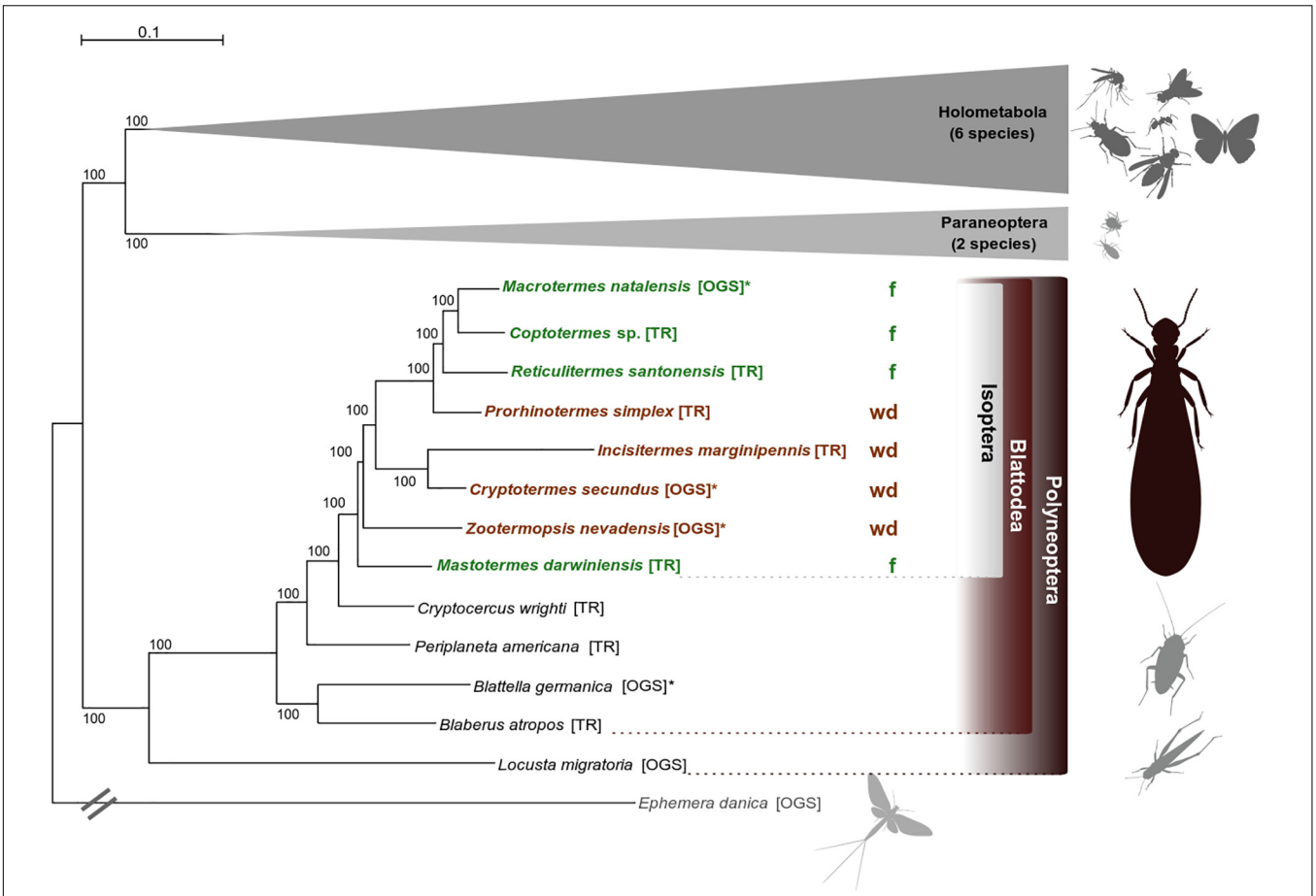


**FIGURE 1 |** Phylogenetic relationships. Best ML species tree (out of 50 trees) inferred from a super alignment of 1,178 single-copy orthologs (SCOs) with 555,906 amino acid positions (schematized for Holometabola and Paraneoptera, all ML trees had the same topology). Statistical bootstrap support was derived from 200 replicates. The tree was rooted with the mayfly *Ephemera danica* (the full tree is provided with the **Supplementary Files** on DRYAD). Color code: brown indicates wood-dwelling species (wd), green indicates foraging species (f). TR, derived from transcriptome assemblies (Misof et al., 2014; Evangelista et al., 2019); OGS, derived from official gene sets (**Supplementary Table S1**). The symbol "*" indicates reference species whose OGS was used to create the ortholog set. Pictograms were kindly provided by H. Pohl, Jena.

**TABLE 1 |** Results of RELAX analyses of changes in selection intensity on termite immune genes (IGs).

| Species | Life type | # Relaxed IGs | # Total IGs | Median # relaxed BGs (95% CI) | Median *k* IGs (95% CI) | Median *k* BGs (95% CI) |
|---|---|---|---|---|---|---|
| *Macrotermes natalensis** | Foraging | 10 | 53 | 12 (4.475–19) | 0.72 (0.16–43.10) | 0.74 (0.61–0.87) |
| *Coptotermes* sp. | Foraging | 1 | 16 | 3 (0–6) | 0.84 (0.13–31.80) | 0.68 (0.44–1.13) |
| *Reticulitermes santonensis* | Foraging | 1 | 13 | 1 (0–4) | 1.35 (0.23–48.15) | 0.73 (0.42–1.75) |
| *Mastotermes darwiniensis* | Foraging | 3 | 25 | 3 (0–7) | 0.69 (0.13–26.31) | 0.8 (0.63–1.13) |
| *Prorhinotermes simplex* | Wood-dwelling | 3 | 47 | 4 (1–11) | 0.76 (0.26–34.35) | 0.81 (0.69–1) |
| *Incisitermes marginipennis* | Wood-dwelling | 0 | 18 | 0 (0–3) | 0.81 (0–49.79) | 0.92 (0.53–1.28) |
| *Cryptotermes secundus** | Wood-dwelling | 13 | 72 | 13 (5–22) | 0.73 (0–45.85) | 0.77 (0.69–0.89) |
| *Zootermopsis nevadensis** | Wood-dwelling | 16 | 78 | 21 (12–27.525) | 0.74 (0.33–43.90) | 0.73 (0.66–0.81) |

*Genes that are under significantly relaxed selection were counted ($k < 1$, $P < 0.05$, FDR < 0.2) for columns containing #. BG, background single-copy ortholog. The symbol "*" indicates species with annotated genomes.*

To our surprise, we observed that median *k* for IGs was smaller than one for seven of the eight investigated termite species (**Table 1**), indicating an overall relaxation of selection (Mann–Whitney *U*-test: $U = 20{,}958$, $n = 322$, $P < 0.01$). This signal was not IG specific: *k* for the sets of BGs was also significantly smaller than one for all species ($P = 4 \times 10e{-}18$-$4.8 \times 10e{-}5$), suggesting genome-wide relaxation of selection on the termite lineages compared to the background branches of the phylogeny.

**TABLE 2 |** Summarized results of BUSTED analyses of single-copy IGs for each termite species tested against all other species in the alignment.

| | | | Foragers | | | | Wood-dwellers | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **ORTHOMCL ID[1] of single-copy immune gene (IG)** | **Annotation (*Z. nevadensis*, see Terrapon et al., 2014)** | **Pathway (*Z. nevadensis*, see Terrapon et al., 2014)** | **Csp** | **Mdar** | **Mnat** | **Rsan** | **Csec** | **Imar** | **Psim** | **Znev** |
| | | | ***P*-value/FDR** | ***P*-value/FDR** | ***P*-value/FDR** | ***P*-value/FDR** | ***P*-value/FDR** | ***P*-value/FDR** | ***P*-value/FDR** | ***P*-value/FDR** |
| 1867 | RB1-inducible coiled-coil 1 | Autophagy | NA | 0.5/0.5 | 0.5/0.5 | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 1974 | TEP2 | TEP | NA | 0.5/0.5 | 0.5/0.5 | NA | 0.4031383238/0.5 | NA | 0.3136310014/0.5 | 0.4978894366/0.5 |
| 1981 | JAK/hopscotch | JAK-STAT pathway | NA | NA | 0.5/0.5 | NA | NA | NA | NA | 0.3012329951/0.5 |
| 1985 | Coagulation factor XI | PO-related | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 1992 | TEP1 | TEP | NA | NA | 0.4783265125/0.5 | NA | 0.5/0.5 | NA | NA | 0.4730195429/0.5 |
| 2065 | prophenoloxidase | PO-related | NA | NA | NA | NA | 0.5/0.5 | NA | 0.4973137204/0.5 | 0.5/0.5 |
| 2188 | Cytokine receptor | JAK-STAT pathway | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 2287 | Spaetzle | TOLL pathway | NA | NA | 0.3560348813/0.5 | NA | 0.5/0.5 | NA | 0.5/0.5 | 0.4915286791/0.5 |
| 2385 | ATG3 (Autophagy-related protein 3) | Autophagy | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 |
| 2444 | serine protease inhibitor | Serpin | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | 0.5/0.5 | 0.5/0.5 |
| 2474 | cSP (serine protease stubble) | cSP | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 2712 | Peroxidase | Peroxidase | NA | 0.1904703203/0.5 | 0.5/0.5 | NA | 0.5/0.5 | NA | 0.5/0.5 | 0.5/0.5 |
| 2716 | Effector caspase | Apoptosis | NA | NA | NA | NA | 0.3484403198/0.5 | NA | 0.4891731281/0.5 | 0.4793197418/0.5 |
| 2739 | Galectin | Lectin | NA | NA | 0.5/0.5 | NA | NA | 0.5/0.5 | NA | NA |
| 2823 | CTL | C-Lectin | NA | 0.5/0.5 | 0.5/0.5 | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 2831 | Toll | Toll receptor | NA | NA | 0.4723275965/0.5 | NA | 0.5/0.5 | NA | 0.5/0.5 | 0.5/0.5 |
| 2908 | MAPKKK | IMD pathway | NA | NA | NA | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 3027 | serine protease inhibitor | Serpin | NA | 0.4258935235/0.5 | 0.4929521704/0.5 | NA | 0.3334998606/0.5 | NA | 0.2247329263/0.5 | 0.5/0.5 |

*(Continued)*

**TABLE 2** | Continued

| ORTHOMCL ID[1] of single-copy immune gene (IG) | Annotation (*Z. nevadensis*, see Terrapon et al., 2014) | Pathway (*Z. nevadensis*, see Terrapon et al., 2014) | Foragers | | | | Wood-dwellers | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Csp | Mdar | Mnat | Rsan | Csec | Imar | Psim | Znev |
| | | | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR |
| 3139 | Peroxidasin/Chorion peroxidase | Peroxidase | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 3207 | scavenger receptor class B | Scavenger Receptor B | 0.5/0.5 | NA | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | 0.5/0.5 |
| 3209 | cSP (serine protease stubble) | cSP | NA | NA | NA | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 3220 | TEP4 | TEP | NA | NA | NA | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 3273 | I-type Lysozyme | LYS | NA | NA | 0.4553265501/0.5 | NA | 0.1417919354/0.5 | 0.4780746285/0.5 | 0.3169259521/0.5 | 0.3828908153/0.5 |
| 3356 | ATG4 (Autophagy-related 4D) | Autophagy | NA | NA | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | 0.5/0.5 |
| 3387 | scavenger receptor class B | Scavenger Receptor B | NA | NA | 0.1465541142/0.5 | NA | 0.1500368111/0.5 | NA | 0.5/0.5 | 0.5/0.5 |
| 3389 | scavenger receptor class B | Scavenger Receptor B | NA | NA | NA | NA | 0.5/0.5 | NA | NA | 0.4989336553/0.5 |
| 3390 | scavenger receptor class B | Scavenger Receptor B | 0.3624292596/0.5 | NA | 0.5/0.5 | NA | NA | 0.3886840358/0.5 | 0.5/0.5 | 0.5/0.5 |
| 3640 | ULK3 | Autophagy | NA | NA | 0.4895575233/0.5 | NA | 0.4776215186/0.5 | NA | 0.5/0.5 | 0.5/0.5 |
| 3707 | PGRP | Pattern Recognition | NA | 0.3775306997/0.5 | 0.4998037831/0.5 | NA | 0.5/0.5 | NA | 0.5/0.5 | 0.3022896782/0.5 |
| 3708 | PGRP | Pattern Recognition | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | NA | 0.5/0.5 | NA | 0.5/0.5 | 0.5/0.5 |
| 3835 | scavenger receptor class B, croquemort type | Scavenger Receptor B | 0.5/0.5 | 0.4645645383/0.5 | 0.0718496487/0.5 | NA | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 |
| 3847 | serine protease inhibitor | Serpin | NA | NA | 0.5/0.5 | NA | NA | NA | 0.5/0.5 | 0.3222263845/0.5 |
| 4029 | serine protease inhibitor | Serpin | NA | NA | NA | NA | 0.3038620148/0.5 | NA | 0.5/0.5 | 0.5/0.5 |
| 4179 | NPC2-like | ML superfamily (The ML (MD-2-related lipid-recognition) domain) MD2-like receptors | 0.3121553945/0.5 | 0.5/0.5 | NA | 0.4133779858/0.5 | 0.5/0.5 | 0.5/0.5 | 0.1284915202/0.5 | 0.4943292826/0.5 |

*(Continued)*

**TABLE 2 |** Continued

| ORTHOMCL ID[1] of single-copy immune gene (IG) | Annotation (*Z. nevadensis*, see Terrapon et al., 2014) | Pathway (*Z. nevadensis*, see Terrapon et al., 2014) | Foragers | | | | Wood-dwellers | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Csp | Mdar | Mnat | Rsan | Csec | Imar | Psim | Znev |
| | | | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR |
| 4219 | Spaetzle-like | TOLL pathway | NA | NA | NA | 0.5/0.5 | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 4229 | TRAF | TOLL pathway | NA | NA | 0.5/0.5 | NA | 0.4463831699/0.5 | NA | NA | 0.5/0.5 |
| 4296 | Easter (Spaetzle-Processing enzyme) | TOLL pathway | NA | 0.5/0.5 | NA | NA | NA | NA | 0.2044616244/0.5 | NA |
| 4355 | Superoxide dismutase | SOD | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 |
| 4403 | C-type Lysozyme | LYS | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 4440 | ATG6 (Beclin) | Autophagy | NA | 0.5/0.5 | 0.5/0.5 | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 4442 | FAS-assocaited factor (TNFRSF6) | IMD pathway | NA | NA | NA | NA | 0.3984955304/0.5 | NA | 0.5/0.5 | 0.5/0.5 |
| 4516 | serine protease inhibitor | Serpin | 0.5/0.5 | 0.1292613242/0.5 | 0.1332373684/0.5 | 0.0532271128/0.5 | NA | NA | 0.5/0.5 | 0.5/0.5 |
| 4547 | ULK2 (unc-51-like kinase 2) | Autophagy | NA | NA | NA | NA | 0.4979050556/0.5 | NA | NA | 0.5/0.5 |
| 4632 | serine protease inhibitor | Serpin | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | 0.5/0.5 | 0.3504677063/0.5 |
| 4691 | ATG12 | Autophagy | 0.5/0.5 | NA | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 |
| 4764 | CTL | C-Lectin | NA | NA | NA | 0.3603908914/0.5 | NA | NA | 0.5/0.5 | 0.5/0.5 |
| 4766 | ATG4 (Autophagy-related 4B) | Autophagy | NA | 0.5/0.5 | NA | NA | 0.5/0.5 | 0.5/0.5 | NA | 0.5/0.5 |
| 4769 | CTL | C-Lectin | 0.4294501244/0.5 | 0.5/0.5 | NA | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.43474645/0.5 |
| 4883 | CTL (sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein) | C-Lectin | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | 0.5/0.5 | 0.4840795357/0.5 |
| 4915 | PGRP | Pattern Recognition | NA | NA | 0.3257325407/0.5 | NA | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 |
| 4933 | scavenger receptor class A-like | Scavenger Receptor A | NA | NA | NA | NA | 0.5/0.5 | NA | NA | 0.4939311011/0.5 |
| 5009 | Relish (NF-Kappa-B) | NF-K-B-related | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | 0.5/0.5 | NA |

*(Continued)*

**TABLE 2** | Continued

| ORTHOMCL ID[1] of single-copy immune gene (IG) | Annotation (*Z. nevadensis*, see Terrapon et al., 2014) | Pathway (*Z. nevadensis*, see Terrapon et al., 2014) | Foragers | | | | Wood-dwellers | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Csp | Mdar | Mnat | Rsan | Csec | Imar | Psim | Znev |
| | | | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR |
| 5380 | CTL (sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein) | C-Lectin | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | NA | 0.4202390948/0.5 |
| 5484 | serine protease inhibitor | Serpin | NA | NA | NA | 0.5/0.5 | 0.5/0.5 | NA | 0.5/0.5 | 0.1806195188/0.5 |
| 5640 | Tollip (Toll-interacting protein) | TOLL pathway | NA | NA | 0.2874485778/0.5 | NA | 0.5/0.5 | NA | 0.5/0.5 | 0.5/0.5 |
| 5668 | Mpk2 | NF-K-B-related | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 5849 | JNK-interacting SapK | TOLL pathway | NA | NA | NA | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 5878 | scavenger receptor class A-like | Scavenger Receptor A | NA | NA | 0.4921059824/0.5 | NA | 0.4501230548/0.5 | NA | NA | 0.5/0.5 |
| 6195 | ATG8 (Gabarap) | Autophagy | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 |
| 6214 | serine protease inhibitor | Serpin | 0.499244676/0.5 | 0.5/0.5 | NA | NA | 0.5/0.5 | NA | 0.4990348058/0.5 | 0.4763073094/0.5 |
| 6240 | Pelle | TOLL pathway | NA | 0.5/0.5 | NA | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 6273 | Leukocyte elastase inhibitor | Serpin | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | NA | 0.0911018183/0.5 |
| 6341 | IMD (immune deficiency) | IMD pathway | NA | NA | 0.4922438717/0.5 | NA | 0.5/0.5 | 0.5/0.5 | 0.1822033717/0.5 | 0.5/0.5 |
| 6356 | Peroxidasin/Chorion peroxidase | Peroxidase | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | 0.5/0.5 | 0.5/0.5 |
| 6454 | Peroxidasin/Chorion peroxidase | Peroxidase | NA | NA | 0.4453516888/0.5 | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 6467 | SRCR cysteine-rich | Scavenger Receptor A | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 6503 | NF-kappa-B-repressing factor | NF-K-B-related | 0.1736630914/0.5 | NA | 0.3322164668/0.5 | NA | 0.5/0.5 | 0.5/0.5 | 0.1942752065/0.5 | 0.4524943026/0.5 |
| 6509 | cSP (serine protease stubble) | cSP | NA | NA | 0.2815092467/0.5 | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 6924 | Peroxidase | Peroxidase | NA | NA | NA | NA | 0.5/0.5 | NA | 0.3508281805/0.5 | 0.5/0.5 |

*(Continued)*

**TABLE 2** | Continued

| ORTHOMCL ID[1] of single-copy immune gene (IG) | Annotation (*Z. nevadensis*, see Terrapon et al., 2014) | Pathway (*Z. nevadensis*, see Terrapon et al., 2014) | Foragers | | | | Wood-dwellers | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Csp | Mdar | Mnat | Rsan | Csec | Imar | Psim | Znev |
| | | | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR | *P*-value/FDR |
| 7074 | DCN1-like protein | DCN1-like protein | 0.5/0.5 | 0.5/0.5 | NA | 0.5/0.5 | 0.5/0.5 | 0.4730018494/0.5 | 0.5/0.5 | 0.2753454554/0.5 |
| 7076 | ATG16L1 | Autophagy | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 7078 | ATG16L1 (Autophagy-related protein 16-1) | Autophagy | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 |
| 7082 | ECSIT (signal intermediate in Toll pathway) | TOLL pathway | 0.5/0.5 | 0.4356314229/0.5 | 0.5/0.5 | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 7151 | serine protease inhibitor | Serpin | NA | 0.5/0.5 | 0.5/0.5 | NA | 0.3588950561/0.5 | NA | 0.5/0.5 | 0.4441004788/0.5 |
| 7193 | CTL (Macrophage mannose receptor 1) | C-Lectin | NA | NA | 0.5/0.5 | NA | 0.5/0.5 | NA | 0.5/0.5 | 0.5/0.5 |
| 7203 | TEP3 | TEP | NA | NA | 0.5/0.5 | NA | NA | NA | NA | 0.5/0.5 |
| 7226 | ATG10 (autophagy-related protein 10) | Autophagy | NA | NA | 0.4942581217/0.5 | 0.5/0.5 | 0.5/0.5 | 0.0593462005/0.5 | 0.4779133604/0.5 | 0.5/0.5 |
| 7306 | IG-domain containing | IG-domain containing | NA | NA | NA | NA | 0.5/0.5 | NA | NA | 0.5/0.5 |
| 7366 | Peroxidasin/Chorion peroxidase | Peroxidase | NA | NA | NA | NA | NA | NA | NA | 0.5/0.5 |
| 7372 | Kappa-B-ras (NF-kappa-B inhibitor alpha-interacting) | NF-K-B-related | NA | 0.5/0.5 | NA | NA | 0.5/0.5 | NA | 0.4722652742/0.5 | 0.5/0.5 |
| 7434 | cSP (serine protease stubble) | cSP | 0.5/0.5 | 0.4576422108/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.5/0.5 | 0.4231368404/0.5 | 0.5/0.5 |

[1]*Note that in* ***Supplementary Archive 1*** *(DYRAD) and in* ***Supplementary Tables 2–5*** *"ORTHOMCL0000" is part of the ID, e.g., "1867" in Table 2 is "ORTHOMCL00001867". Tests were conducted species-wise (one species against all remaining species per gene). Species shortcuts: Csec, Cryptotermes secundus; Csp, Coptotermes sp.; Imar, Incisitermes marginipennis; Mdar, Mastotermes darwiniensis; Mnat, Macrotermes natalensis; Psim, Prorhinotermes simplex; Rsan, Reticulitermes santonensis; Znev, Zootermopsis nevadensis; FDR, false discovery rate; NA, not applicable (for this species, the respective single-copy gene was not present in available genome/transcriptome data). Annotation and pathway are given following Terrapon et al. (2014).*

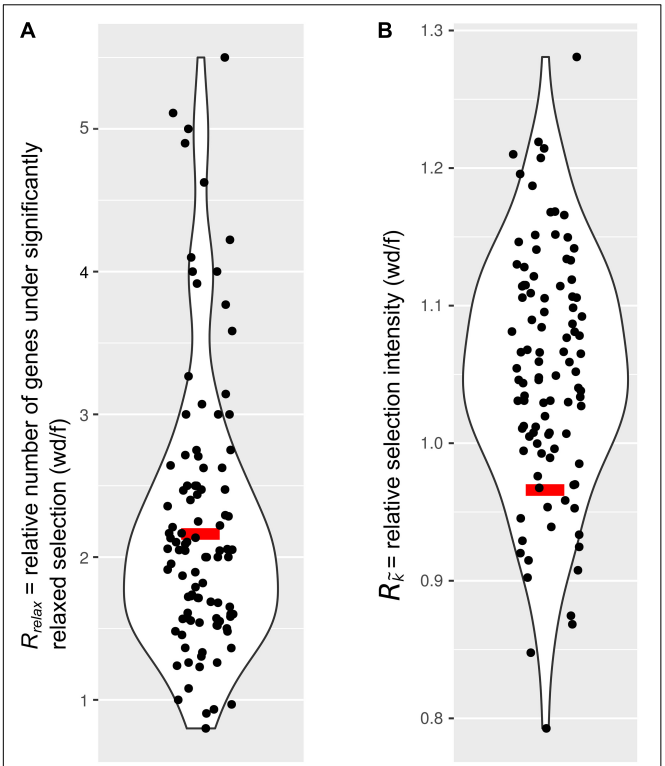


**FIGURE 2 |** Comparison of selection on immune genes to sets of genes that represent the genomic background. Relaxed selection on IGs does not differ from the genomic background. Red bar, immune genes (IGs); black dots, 100 sets of background genes (BGs). Curves represent smoothed point density. **(A)** The ratio of the number of genes under significantly relaxed selection between wood-dwellers and foragers ($R_{relax}$). **(B)** The relative intensity of selection ($R_k$) as measured by the ratio of median $k$ ($\tilde{k}$) between wood-dwellers and foragers.

## DISCUSSION

In this study we combined recent genomic and transcriptomic resources to test whether termite ecology, in particular exposure to pathogens, might affect the evolution of immune genes. Surprisingly, and in contrast to studies from *Drosophila* (Clark et al., 2007; Sackton et al., 2007; Hill et al., 2019), we could not find evidence for positive selection on the IGs for eight termite species. We extended our analyses, employing recently developed tests to explicitly assess relaxed selection (Wertheim et al., 2015), revealing 47 cases of significantly relaxed selection in IGs.

We expected that the intensity of selection would differ between termites of the wood-dwelling and foraging life types, as foraging termites are assumed to experience higher selection pressure from pathogens due to higher exposure. Contrary to our expectation, we did not detect an effect of life type on signs of selection for immune genes. Neither did wood-dwelling species differ from the soil foraging species, nor did the dampwood termite *Z. nevadensis* differ from the other wood-dwelling termites. A possible explanation may be that IGs that occur in multiple copies in at least one of the species that we analyzed contribute to adaptation to selection pressure from pathogens. Such multi-copy genes were excluded from our analyses. Furthermore, recent selective sweeps, as described for termicin in *Reticulitermes* (Bulmer et al., 2010), are difficult to detect with our methodology. For the detection of recent selective events, comprehensive polymorphism data would be required. Also, social mechanisms such as the exclusion of infested individuals and the impregnation of the nest walls with fungicidal compounds may protect efficiently against pathogens entering a colony or infecting individuals, thus buffering selection pressure on IGs (Traniello et al., 2002; Cremer et al., 2007; Bulmer et al., 2009; Rosengaus et al., 2011; Masri and Cremer, 2014). Another possible explanation for similar selection pressure on IGs between wood-dwelling and foraging species is that both harbor complex gut microbial communities that are essential for termite survival (Waidele et al., 2017), and perform, in principle, similar functions in lignocellulose digestion and nitrogen acquisition across several of the species that we analyzed (Waidele et al., 2019). It seems reasonable to assume that the immune system is likely to play a role in modulating these communities in wood-dwellers and foragers alike resulting in constant selection pressure. Our results support the results of a study in ants (Roux et al., 2014) that spanned a similar evolutionary time frame and number of focal species. These authors also found no evidence for a relaxation of selective constraint specific to IGs that could be related to social immunity. However, the authors found several instances of positively selected genes showing that it is in principle possible to detect positive selection with dn/ds based methods in a similar setup. Harrison et al. (2018) were able to pinpoint several selected codons using genome sequences from *Blattella germanica* and *C. secundus*, again spanning similar divergence times. In general, BUSTED is more powerful than other methods to detect positive selection because it has decent power to detect not only pervasive, but also episodic selection, while being fairly independent from evolutionary divergence times (Murrell et al., 2015).

We applied state-of-the-art methods (Pond et al., 2005; Murrell et al., 2015; Wertheim et al., 2015) and carefully controlled our study by analyzing BGs that matched IGs in GC-content and sequence length (see section "Materials and Methods"). We also combined, to our knowledge, the largest data set of termite genomic and transcriptomic resources for our study that has been used in that context so far. Nonetheless, our study might lack statistical power: First, we have relatively few species for life type comparisons (4 vs. 4). Second, for five of these species (*M. darwiniensis*, *I. marginipennis*, *R. santonensis*, *P. simulans*, and *Coptotermes* sp.) only transcriptome data are available. Naturally, the number of genes under significantly relaxed selection is lower for the species for which only transcriptome data are available, as fewer genes could be annotated and investigated in the transcriptome data (**Tables 1**, **3** and **Supplementary Table S4**). However, potential biases in data availability were taken into account in the procedure to sample sets of matching BGs. Note that we did not apply the group-based approach of the BUSTED and RELAX tests that might have increased power to detect selection or its relaxation. This was due to fragmented data coverage of the species representing

**TABLE 3 |** Number of analyzed IGs with HyPhy-BUSTED and RELAX and respective BG analyses with HyPhy-RELAX for each of the included termite species.

| Species | Life type | # analyzed IGs | # BG[1] analyses |
|---|---|---|---|
| *Macrotermes natalensis* | Foraging | 53 | 5,300 |
| *Coptotermes* sp. | Foraging | 16 | 1,700 |
| *Reticulitermes santonensis* | Foraging | 13 | 1,300 |
| *Mastotermes darwiniensis* | Foraging | 25 | 2,400 |
| *Prorhinotermes simplex* | Wood-dwelling | 47 | 4,700 |
| *Incisitermes marginipennis* | Wood-dwelling | 18 | 1,800 |
| *Cryptotermes secundus* | Wood-dwelling | 72 | 7,300 |
| *Zootermopsis nevadensis* | Wood-dwelling | 78 | 7,800 |

[1]*We refrained to run BUSTED on the background genes (BGs) because there was no evidence for positive selection on the IGs. Note that when running analyses with RELAX, few BG analyses failed due to numeric instability or convergence errors (from the error logfile from HyPhy-RELAX: "relax.K evaluated to a NaN; this can cause all kinds of odd behavior downstream"): one analysis failed for C. secundus, one for P. simplex, two for M. natalensis, and three for Z. nevadensis.*

both life types. This fragmented coverage for many genes would have led to only a limited gain in power. Furthermore, the careful selection of BGs would have become infeasible because too few BGs matched the species composition in the group-based tests on IGs. We are hopeful that the group-based approach will become more powerful in the future, when more termite genome data become available. Nonetheless, we were able to detect 47 instances of relaxed selection, showing that the RELAX approach can be powerful in a setup like ours. Because RELAX specifically tests for relaxed selection, while other studies often infer potentially relaxed selection indirectly by faster evolution in the absence of positive selection (Roux et al., 2014; Partha et al., 2017), we think it should be the preferred method.

When taking the genomic background and the sampling procedure into account (**Figure 2**), our data provide no evidence that selection intensity on SCO IGs differs between life types. Do our results imply that selection pressure on immune genes is the same between termite life types? No, we cannot conclude this as our study excluded IGs that are not SCOs. For example, several GNBPs were excluded from our study because they seemed to be present in multiple copies in at least one termite species that we analyzed (Znev_03257, Znev_03259). Different copies can be caste-specifically expressed in termites as shown for *Z. nevadensis* (Terrapon et al., 2014), and for *Reticulitermes speratus* (Mitaka et al., 2017), and they may be under positive selection as indicated by a study on GNBPs for several foraging *Nasutitermes* species (Bulmer and Crozier, 2006). Note that the original hypothesis for a difference in immune defense between wood-dwelling and foraging termites considered specifically GNBPs and AMPs (Korb et al., 2015). Other GNBPs were excluded because they were restricted to only some of the lineages that we based our ortholog set on (Znev_03260, Znev_02878, and Znev_00933). Thus, more studies are warranted that test selection on genes that are lineage-specific or might occur in multiple copies. However, orthology is difficult to infer for multi-copy genes making the restriction to SCOs a standard procedure (e.g., Dowling et al., 2016; Pauli et al., 2016; Mitterboeck et al., 2017; Ran et al., 2018; Brandt et al., 2019; Hill et al., 2019). Thus, we restricted our analysis to SCOs because orthology is a basic assumption of the current methods that identify selection in a powerful phylogenetic framework [e.g., codeML implemented in PAML, Yang, 2007; HyPhy applying BUSTED, see Murrell et al. (2015), and RELAX, see Wertheim et al. (2015)].

Applying state-of-the-art methods to a comprehensive termite data set, for which genome or transcriptome data are currently available, we found no evidence for differences in selection on immune genes that correlate with termite life type. Our results suggest that the putative evolutionary response to differences in expected pathogen exposure can not be found in single-copy immune genes. Interestingly, we detected a signal of genome-wide relaxation of selective constraint in termites. We speculate that this could be related to their social organization that might lead to smaller effective population size (Romiguier et al., 2014; Rubenstein et al., 2019) because only the kings and queens reproduce, and hence contribute to the effective population size. In smaller populations, natural selection becomes less effective at purging deleterious mutations as well as at driving advantageous mutations to fixation (Ohta, 1973). This is equivalent to a relaxation of selection in smaller populations. Thus, small effective population sizes compared to the other insects in our study could have manifested as the genome-wide signal of relaxed selection that we observed in termites.

# MATERIALS AND METHODS

A comprehensive diagram summarized major analysis steps in **Supplementary Figure S1**.

## Identifying Orthologous Sequence Groups of Protein-Coding Single-Copy Genes

As basis to identify SCOs, we designed an ortholog set from official gene sets (OGS) from available full (draft) genomes of four species: *C. secundus*, *M. natalensis, Z. nevadensis*, and *B. germanica* (**Supplementary Table S1**). The set of SCOs was inferred with the software OrthoFinder v.1.1.4 (Emms and Kelly, 2015) using default settings. As input, the OGS of respective species were downloaded from public databases as amino acid and nucleotide sequences. The OGS of *C. secundus* was kindly provided by the *C. secundus* consortium (via J. Korb) before it was published (Harrison et al., 2018). We only kept the longest isoform per orthologous group (OG). All OGs that included the amino acid Selenocysteine (U) were removed to avoid difficulties in downstream analyses as many software packages are not able to handle Selenocysteine. This was done using the package BioBundle [script isoformCleaner with boost 1.61.0 environment (Kemena, 2017, available from github[1])]. SCOs inferred with OrthoFinder, were summarized with custom-made Python scripts (kindly provided by A. Faddeeva and L. Wissler, available upon request). This resulted in a set of 5,382 SCOs across the four selected species.

[1]https://github.com/CarstenK/BioBundle

## Taxon Sampling

We included the four reference species that were used to create the ortholog set as well as genome and transcriptome data of 18 additional species in our analyses. Eight of the included species are termites: *Coptotermes* sp., *I. marginipennis*, *M. darwiniensis*, *P. simplex*, and *R. santonensis* (with published transcriptome data); *C. secundus*, *Z. nevadensis*, and *M. natalensis* (with published OGS), see Evangelista et al. (2019). Other included species were a representative of Cryptocercidae as it is supposed to be the sister group of termites (e.g., Lo et al., 2000; Inward et al., 2007), two other non-social cockroach species, and representatives from other polyneopteran, paraneopteran and holometabolous insects, and a mayfly as outgroup (Adams et al., 2000; Xia et al., 2004; Sinkins, 2007; Tribolium Genome Sequencing Consortium et al., 2008; Bonasio et al., 2010; International Aphid Genomics Consortium, 2010; Elsik et al., 2014; Misof et al., 2014; Poulsen et al., 2014; Terrapon et al., 2014; Wang et al., 2014; Mesquita et al., 2015; Pauli et al., 2016; Harrison et al., 2018; Evangelista et al., 2019; the full species list is provided in **Supplementary Table S1**). Access to transcriptome data (see **Figure 1**) was kindly granted by 1KITE before they were published, access to the OGS of the locust and the mayfly was granted by the i5K community.

## Assignment of Putative Orthologous Transcripts to the SCOs

The ortholog set was used as input for the assignment of putative SCOs (provided as **Supplementary Files** on DRYAD, doi: 10.5061/dryad.j6q573n98). Inference and assignment of putative orthologs from genome and transcriptome data of the 18 species that were not included for generating the ortholog set was performed with OrthoGraph v.0.6.1 (Petersen et al., 2017). OrthoGraph is recommended to infer orthologs from transcriptome data for which no OGS are available (see Petersen et al., 2017). OrthoGraph analyses resulted in 5,366 SCOs that were identified in at least one species that was not used as reference species to create the ortholog set.

## Multiple Sequence Alignments, Species Tree Inference and Testing for Selection

Individual SCOs were aligned at the amino acid level with MAFFT v7.310 using the L-INS-i algorithm (Katoh and Standley, 2013).

### Species Tree Inference

For inferring the species tree, we only kept those SCOs that were present in all 22 species. This resulted in 1,178 SCOs. Ambiguously aligned sections on the amino acid level were identified with Aliscore v2.2 (Misof and Misof, 2009; Kück et al., 2010) (settings: *-r* with all pairwise sequence comparisons, *-e* for gap-rich alignments, otherwise defaults) and masked with AliCUT v2.3 (Kück, 2011). Masked amino acid multiple sequence alignments (MSAs) were concatenated into a supermatrix (see also **Supplementary Figure S2**) with FASconCAT-G v.1.02 (Kück and Longo, 2014). We inferred phylogenetic relationships using a maximum-likelihood (ML) approach with IQTREE v1.5.4 (Nguyen et al., 2015; Chernomor et al., 2016). Statistical support was determined from 200 non-parametric, slow and thorough bootstrap replicates. We ensured bootstrap convergence with *a posteriori* bootstrap criteria (Pattengale et al., 2010) as implemented in RAxML (Stamatakis, 2014), v.8.2.11. The best ML tree, out of 50 inferred trees, which all showed an identical topology, was rooted with *Ephemera danica* using SeaView v.4.5.4 (Gouy et al., 2010; note that multiple tree viewers are not reliable, see Czech et al., 2017); trees were graphically edited with Inkscape (v.0.91)[2]. More details on the procedure of phylogenetic inference are provided in the **Supplementary Material**.

### Inferring Natural Selection

#### *Alignment processing and clean-up*

Methods to identify selection are sensitive to misalignments (Markova-Raina and Petrov, 2011; Privman et al., 2012). Therefore we performed extensive alignment clean-up. First, we identified and deleted badly aligned or gap-rich sequences on amino acid level with MaxAlign v1.1 (Gouveia-Oliveira et al., 2007). This procedure resulted in five SCOs with only one sequence which were excluded from further analyses. We subsequently compiled corresponding nucleotide (i.e., codon) MSAs with PAL2NAL (Suyama et al., 2006, v14.1, see Misof et al., 2014) using the 5,361 amino acid MSAs as blue-print. The nucleotide MSAs were then used for all following analyses. Second, we deleted all SCOs with less than four sequences (223 SCOs) leaving 5,138 SCOs. Third, we identified ambiguously aligned sections on amino acid level with Aliscore v2.2 (Misof and Misof, 2009; Kück et al., 2010) with the same settings as described for the species tree inference. Suggested sections were removed from the amino acid and correspondingly from the nucleotide MSAs with AliCUT v2.3 (Kück, 2011). Subsequent analyses were performed on the masked nucleotide MSAs. First, we classified 5,138 SCOs into 86 immune single-copy genes (IGs) and into the remaining 5,052 SCOs based on Supplementary Table S25 from Terrapon et al. (2014, for *Z. nevadensis*) and Korb et al. (2015, for *Z. nevadensis* and *M. natalensis*), see **Supplementary Table S5**. The 5,052 non-immune SCOs were used to generate gene sets from the genomic background, i.e., BGs that had similar GC-content and sequence length (see below) as the examined IGs. Note that from the 86 IGs (**Supplementary Table S5**) five IGs were excluded because there was no SCO fulfilling the criteria to serve as BG and these were not listed by Terrapon et al. (2014) or not reported by Korb et al. (2015). This left 81 IGs for analyses (detailed information are provided in the **Supplementary Material**).

To further reduce potential false positives that may originate from misalignments, we trimmed trailing ends of each MSA, i.e., each MSA started and ended with unambiguous nucleotides for all species. Because visual inspection of the trimmed MSAs still revealed putative misaligned nucleotides, we applied the GUIDe tree based AligNment ConfidencE approach (GUIDANCE) Guidance2 (Landan and Graur, 2008; Sela et al., 2015) version

[2]www.inkscape.org

2.02 using MAFFT as implemented alignment method on the trimmed MSAs (options: codon as sequence type, sequence cutoff = 0 and the default column cutoff = 0.93).

### *Inferring positive selection and selection intensity*

To test for evidence of positive selection we used BUSTED (Murrell et al., 2015) as implemented in the software package HyPhy (Pond et al., 2005). BUSTED uses a branch-site test for positive selection on entire genes in a foreground branch relative to the background branches in a phylogeny. A significant *P*-value means that at least one codon in the foreground branch has experienced at least an episode of positive selection. The high sensitivity of the method compared to tests from alternative packages (see e.g., Enard et al., 2016; Ebel et al., 2017; Venkat et al., 2018; Hill et al., 2019) and the option to define the foreground branches according to our research question made it perfectly suited for our study.

For inferring potential relaxation of selection, we used RELAX (Wertheim et al., 2015) as implemented in the software package HyPhy (Pond et al., 2005). RELAX has been designed to identify changes in the intensity of selection on a given protein-coding gene in a codon-based phylogenetic framework (see Wertheim et al., 2015). The basic expectation of RELAX is that under relaxed selection, the ω of sites under purifying and positive selection will move closer to neutrality. The change of ω for the selected sites relative to the background branches is quantified with the selection intensity parameter k, where

$$f(\omega, k) = \omega^k.$$

If parameter $k$ is significantly larger than one, selection has been intensified along the test branches. If $k$ is significantly smaller than one, selection has been relaxed.

We used BUSTED and RELAX as implemented in the software package HyPhy, version 2.4.0-alpha.2 (access: April, 2019). We performed BUSTED and RELAX for each gene in each termite species separately (focal species: foreground, remaining species in the alignment: background) and calculated false discovery rates (FDR) to correct for multiple testing. We then determined $k$ for each of the termite species relative to all other species in the tree. We chose this species-wise analysis setup because we wanted to take species level differences in potential pathogen exposure into consideration. For example, *Z. nevadensis* that resides in dampwood might differ in microbial exposure from *Cryptotermes* and *Incisitermes* that reside in dry wood, which in turn might affect selection pressure on IGs. Furthermore, an effective selection of BGs was only feasible in the species-wise framework (see section "Discussion"). Results from the species-wise analyses were summarized by life type after the BUSTED and RELAX analyses.

### *Comparison of IG selection parameter to the genomic background*

To test whether or not signals of selection were specific to IGs or the consequence of genome-wide trends, we generated sets of BGs. To this end, we searched the 5,052 non-immune SCOs for genes that closely matched the GC-content (±5%) and the sequence length (±5%). Following this procedure, we generated lists of matching BGs for each IG and each species. From these lists, we randomly sampled 100 gene sets such that there was a matching BG for each IG that was analyzed in the respective species (e.g., for *Z. nevadensis,* 78 IGs were analyzed, thus each of the 100 sets of BGs contained therefore 78 BGs, see also **Table 3**. Lists of analyses BG that are similar in GC-content and sequence length of the IGs are provided for each species as **Supplementary Files** on DRYAD). We performed BUSTED and RELAX analyses for each termite species on all IGs and RELAX on the species' respective BG set with default settings. The same cutoffs as for the IGs ($P < 0.05$, FDR $< 0.2$, $k < 1$) were applied to the BGs.

All analyses were performed on Linux Desktop PCs at the University of Freiburg, Germany and on the Linux HPC CSIRO Cluster Pearcey, Australia. Analyses results of all IGs are summarized in **Supplementary Tables S2, S3**; results of BGs are provided species-wise on DRYAD.

## Statistical Analyses

In order to assess potential differences in selection intensity on the IGs between species and life types, we summarized the RELAX results by counting the number of genes under significantly relaxed selection: genes with $k < 1$, $P < 0.05$, and FDR $< 0.2$ were considered. With our FDR cut-off, we followed the recommendation from Efron (2007) for genome-wide analyses. Potential differences were tested for statistical significance with generalized linear models with binomial error distribution using the functions glm and glmer from the lme4 R package [Bates et al., 2015, version 1.1-21 with R Core Team (2018), version 3.4.4]. The number of significant genes divided by the total number of genes analyzed was used as response variable. Species or life type were used as potential predictors. Varying sampling depths between species, as represented by the number of IGs analyzed per species, were taken into account as weights in the model. When comparing life types, species were treated as a random effect. See **Supplementary File** (RanalysisscriptfortermiteIGs.R on DRYAD, doi: 10.5061/dryad.j6q573n98) for a detailed R analysis script with all models, commands and functions used.

We also analyzed parameter $k$ to search for more diffuse trends in selection intensity that are distributed over the IGs so that individual IGs do not reach significance. According to its definition, $k$ should map linearly on a logarithmic scale. However, we found six strong outliers on the logarithmic scale that were more than three standard deviations away from the mean [$\log(k) < -9$, see **Supplementary Table S3**] that could make the analysis in a linear framework error prone. Visual inspection of the alignments underlying these extremely small values of $k$ did not reveal any obvious misalignments that would justify their exclusion. Therefore, potential differences in $k$ were assessed with non-parametric tests (Mann–Whitney *U*-test, Kruskal–Wallis test) that are robust to outliers.

Genome-wide trends in selection intensity can potentially obscure IG specific patterns or generate false positives. For example, changes in population size can affect the efficiency of both purifying and positive selection (Ohta, 1973) on a genome-wide scale. Population sizes might differ between

species and life types in our study depending on reproductive rates and degrees of sociality. Therefore, it is essential to put the results for IGs into the context of the genomic background. To this end, we generated expected values for the number of significant genes and for $k$ based on 100 sets of BGs (see above) per termite species, representing the genomic background. The median of $k$ and 95% CIs from the BG-based distributions for each species were calculated with R (version 3.4.4), using the median and quantile functions with standard settings. In order to compare differences between life types while taking the genomic background into account, we calculated (i) the ratio of the number of genes that were significantly relaxed between wood-dwellers and foragers

$$R_{relax} = \frac{\#\,genes_{wood\text{-}dwellers} \mid P < 0.05 \cap FDR < 0.2 \cap k < 1}{\#\,genes_{foragers} \mid P < 0.05 \cap FDR < 0.2 \cap k < 1}$$

and (ii) the ratio of median parameter $k$ ($R_{\tilde{k}}$, relative selection intensity):

$$R_{\tilde{k}} = \frac{\tilde{k}_{\text{wood-dwellers}}}{\tilde{k}_{\text{foragers}}}.$$

These ratios were calculated for the IGs and the BGs. Then the ratio of the IGs was compared to their expectation from the BGs. Significant shifts in selection intensity that are specific to IGs should lead to shifts of $R_{relax}$ and $R_{ki}$ only for IGs. Thus, if there were IG specific patterns of relaxed selection, the ratios $R_{relax}$ and $R_{ki}$ for the IGs should represent extremes of the distribution of sets of BGs. Therefore, we only considered a signal as significantly specific for IGs if our test-statistics of the IGs were outside of the 95% CI calculated for the BGs. However, this was not the case in our study.

## DATA AVAILABILITY STATEMENT

**Supplementary Data** (see **Supplementary Material**) used in this study can be found at the DRYAD digital repository, doi: 10.5061/dryad.j6q573n98.

## AUTHOR CONTRIBUTIONS

JK and FS conceived the study. KM, MS, and FS performed all analyses. FS, KM, and JK wrote the manuscript. All authors edited and approved the manuscript.

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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.2020.00026/full#supplementary-material

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# Comparing a Potential External Immune Defense Trait to Internal Immunity in Females of Wild Bumblebees

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External immune defense, such as antimicrobial secretions, is not generally viewed as part of the immune system. Nevertheless, it constitutes a first barrier to pathogens and manipulates microbial environments. Hygienic measures ranging from the protection of oneself or conspecifics, the nesting site, to stored food may be more efficient with secreted antimicrobials. However, the relationship between external immune defense and internal immunity, including potential life-history trade-offs, is not well-understood. As hymenopteran venom often contains antimicrobial peptides it could serve as an external immune defense. Assuming that antimicrobial venom is costly its production might be traded-off against the internal immune defense. Here we compared the antimicrobial activity of venom and hemolymph in fourteen different bumblebee species according to their life-history strategies and characteristics, i.e., overwintered queens, workers and young queens and cuckoo queens. We found no direct relation between antimicrobial activity of hemolymph and venom. Across all species, hemolymph mainly showed lysozyme-like activity (LLA) whereas venom mainly showed antimicrobial peptide (AMP) activity. While LLA activity in the hemolymph was similar among species and life-history strategies and characteristics, both factors significantly differed in venom AMP activity. Independent of body size or fat body content, young queens showed the highest venom AMP activity, followed by workers, overwintered queens, and cuckoo queens. Venom as a potential external immune trait seems not directly linked to internal immunity in bumblebees. However, the investment in external defense depends on the species and the life-history strategies and characteristics of an individual, such as social status or condition.

**Keywords: immune defense strategies, individual condition, immune traits, antibacterial secretions, density dependence, eusocial insects**

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## INTRODUCTION

Organisms are constantly exposed to parasites and opportunistic microbial pathogens. Due to this constant microbial selection pressure, organisms have evolved numerous defense mechanisms, most of which are part of their immune system. Physical barriers, such as the skin or the insect cuticle, form a first line of defense. However, once pathogens have overcome this barrier, a complex

interaction of humoral and cellular immune reactions exists to minimize the threat (Bulet et al., 1999; Lemaitre and Hoffmann, 2007). These immune responses can be constitutively expressed and/or induced upon recognition of a pathogen or of stress induced by pathogens (Schmid-Hempel, 2011). In insects, part of the constitutive immune effectors are peptides that circulate the hemolymph, for example lysozyme (Schmid-Hempel, 2011). Upon pathogen recognition, antimicrobial peptides (AMP) are induced and expressed in many different tissues (Schmid-Hempel, 2011). Both AMPs and lysozyme can be found in the fat body, in hemocytes (Cotter et al., 2004), in the midgut, in salivary glands (Hamilton et al., 2011), within reproductive organs (Samakovlis et al., 1991; Lung et al., 2001; Otti et al., 2009), and even on the cuticle (Ashida and Brey, 1995).

The maintenance and use of the immune system are costly and underly trade-offs with other life-history traits. Hosts should invest in immune defense mechanisms that reduce the risk of infection efficiently (Zuk and Stoehr, 2002; Moret, 2003). One option to minimize costs is to stop threats at the earliest moment possible by, for example, manipulating the microbial community in the immediate environment with externalized antimicrobial secretions (Otti et al., 2014). External immune defenses can be any heritable trait acting outside an organism, improving protection from pathogens or manipulating microbial composition in favor of the given organism and should therefore be seen as a part of an organisms' immune system (Otti et al., 2014). Similar to internal immune defense, allocation to external immune defense is predicted to depend on ecological characteristics, such as pathogen pressure, and life-history of an animal.

Antimicrobial secretions have been found in many different animal taxa, including insects. Such secretions occur in different glands of hymenopterans, e.g., in metapleural glands of ants (Tragust, 2016), in salivary glands of termites (Bulmer et al., 2009), and in the venom glands of ants (Tragust et al., 2013), bees (Kuhn-Nentwig, 2003), and wasps (Turillazzi et al., 2006). Both ants and honeybees have been shown to distribute antimicrobial secretions from their venom on their cuticle and nests to manipulate microbial communities in their environment. In this case, the venom serves as an external immune defense (reviewed in Tragust, 2016).

The connection between external and internal immune defenses and the role played by ecology and individual condition are not well-understood. Ecological and physiological aspects are known to influence internal immune defenses (Schmid-Hempel, 2005, 2011; Siva-Jothy et al., 2005; Adamo, 2009) and life-history theory implies that immune defense traits have costs and are traded-off against other fitness components (Sheldon and Verhulst, 1996; Moret and Schmid-Hempel, 2000). The same should apply to internal vs. external immune defense, also because the investment in immunity is selected to be optimal (Westra et al., 2015; Boots and Best, 2018). Baracchi and Turillazzi (2010) found venom components to vary with social status in honeybees, indicating that individuals with different life-history strategies or characteristics (i.e., different castes, eusocial, and parasitic life) might invest differently into external immune defenses. For example, during independent colony founding, queens of eusocial Hymenoptera need to care for the brood and keep potential pathogens at bay before the emergence of the first workers. This might require a larger investment into external immune defenses in comparison to a later life stage when queens are sheltered within the nest, cared for and groomed by workers. Then they may hardly ever have the need to use external immune defenses like venom, especially in comparison to a worker engaged in hygienic or defensive tasks in and around the nest.

Additionally, immune defenses may depend on the physiological condition of an individual (Schmid-Hempel, 2003; Cotter et al., 2019). Since resources are stored in the fat body, insects rely on their fat content to effectively use their immune defenses (Dolezal et al., 2019). The fat body, the production site of hemolymph proteins and several AMPs (Ferrandon et al., 2007; Dolezal et al., 2019), highlighting the importance of this organ as an important part of the immune system. Individuals with higher fat content relative to their body mass should be able to invest more in the production of antimicrobial peptides and have higher expression of immune defenses (Cotter et al., 2019).

At high population density, animals will generally experience a greater risk of pathogen exposure than at low density (Hochberg, 1991; Stow et al., 2007; Turnbull et al., 2011). Insects are known to be selected for higher immunity at higher densities, i.e., the expression of immune defenses is density-dependent. For example, several insect species show higher degrees of melanization of the cuticle, i.e., the insect skin is denser and thicker under high rearing densities than when raised alone (Reeson et al., 1998; Wilson et al., 2002). In social species, group size is obviously related to the density and the proximity of individuals within the nest (Naug and Camazine, 2002) and high relatedness among them (Baer and Schmid-Hempel, 1999) should make social insects highly vulnerable to pathogens. However, in social insects collectively performed immune defenses have evolved, such as allogrooming or the distribution of antimicrobial secretions among nestmates, that have been shown to effectively reduce pathogen pressure (Otti et al., 2014; Cremer et al., 2018). In commercial bumblebees, artificial group size manipulation led to higher innate immune gene expression in groups of workers relative to individually kept workers (Richter et al., 2012). Group size might, therefore, affect internal and external immune defenses.

In this study, we compared the internal immune defense with external immune defense traits in different bumblebee species (Hymenoptera, Apidae, *Bombus* spp.), by looking at antibacterial activity of hemolymph (internal) and venom (external) immunity. Bumblebees are very important as they provide pollinator services not only for our crops (Velthuis and van Doorn, 2006) but also in the wild, thereby maintaining plant diversity (Goulson et al., 2008). In Central and Northern Europe, bumblebees have an annual colony cycle with one generation per year, except for some short-lived species that can have two colony cycles, e.g., *Pyrobombus pratorum* (Sladen, 1912). Eusocial bumblebee colonies have a single reproductive queen (henceforth overwintered queens), non-reproductive female workers and, at the end of a colony cycle in autumn, produce new reproductive

females (henceforth young queens) and males. While males die after mating, the then fertilized young queens enter hibernation and found a new colony the following spring (Sladen, 1912). The appearance of sexual and non-sexual individuals performing different tasks can be related to different behaviors and morphologies within life-history strategies and characteristics (O'Donnell et al., 2000). Colony survival, colony growth and production of sexuals depend on the reproductive success of the founding queen and the effort of her workers (Oster and Wilson, 1978). This dependency on one reproductive individual requires special protection of the queens, as they are responsible for the fitness of the whole colony (Cremer et al., 2007).

Before and during the colony founding stage, queens should invest more into their immune defenses to overcome infections. Whereas, after the first workers emerge, those can take care of their queen's health. Some bumblebee species of the subgenus *Psithyrus* or cuckoo bumblebees have evolved brood parasitism (further referred to as cuckoo queens). Cuckoo queens have an annual life cycle as well. They generally emerge later from hibernation than their host species and then readily search for young host colonies. When cuckoo queens find a host colony, they kill the host queen and enslave the workers to care for their brood (Fisher, 1988). Cuckoo queens produce only reproductive offspring of which again only the females overwinter. Cuckoo queens have a thicker sting and enlarged venom glands (Richards, 1927; Fisher and Sampson, 1992) to improve the probability of a successful colony take over. Such a difference in the life-history strategy of cuckoo queens compared to eusocial queens might have implications for their investment in external and internal immune defense. We expect that cuckoo queens are less well-cared for by the enslaved workers of their host colony in comparison to their own queens. Workers do not benefit from an increase in the cuckoo queen's fitness. Therefore, cuckoo queens need to force workers to provide brood care. Due to the defensive role of venom toward enemies as well as pathogens, cuckoo queens may invest more into external immune defenses in comparison to host queens to compensate for the poor care behaviors. Similarly, constitutively expressed internal immune defenses should be stronger in cuckoo queens.

Here, we compared the internal and external immune defenses of different common bumblebee species in Germany by comparing lysozyme-like activity (LLA) and antimicrobial (AMP) activity of the hemolymph to the venom gland contents. We characterized the variation in immune defenses within and across life-history strategies and characteristics, i.e., cuckoo queens, overwintered queens, young queens, and workers. We predict that different life-history strategies and characteristics should lead to different immune defense strategies in hemolymph and venom. Since reproductive success is based on the success of the reproductive females, we assume overwintered queens and young queens show stronger immune defenses than workers. We also expect young queens to show stronger immune defenses than overwintered queens, as they should have more resources than queens just emerging from hibernation. Predicting the extent of external immune defense for cuckoo bumblebees is less obvious. On the one hand, they might show stronger immune defenses than overwintered queens, because they cannot rely on workers taking care of them. On the other hand, they might show weaker immune defenses, because workers are already protecting the nest and the cuckoo queen only has to fend for herself. Additionally, the strength of immune defense should be linked to individual condition, i.e., size and lipid content stored in the fat body. We assume that individuals from species with large colonies show stronger internal, but weaker external immune defense, than individuals from species with small colonies. This assumption is based on the idea that in large colonies (>150 individuals) the individual contribution to the protection of the group might be smaller than in small colonies (<120 individuals). Finally, we test whether internal and external immune defenses are traded-off against each other to optimize allocation costs.

## MATERIALS AND METHODS

From April to September 2016 we collected a total of 342 individuals from 14 different bumblebee species (**Table S1**) in the region of Bayreuth (Upper Franconia, Bavaria, Germany). From nine eusocial bumblebee species we collected 78 overwintered queens between April and June, 59 young queens between July and September and 164 workers between April and September. In addition, we collected 41 cuckoo queens from five social parasite species of eusocial bumblebees between April and June (**Table S1**). The collection of samples was conducted with the permission of the government of Upper Franconia (Obere Naturschutzbehörde Oberfranken, Permit reference number 55.1-8646-1-7-24). All individuals were identified to the species-level using the identification key published by Gokcezade et al. (2010).

### Hemolymph and Venom Collection

To minimize stress on collected individuals we processed all bumblebees within a maximum of 3 h after field collection. This immediate processing should also give a relatively accurate snapshot of the state of individual immunity and general individual physiology in the field. Once in the laboratory, feces samples were taken (Otti and Schmid-Hempel, 2008) (see Characterization of individual condition) and individuals were immobilized by chilling them on ice for 30 min. Then we collected hemolymph and venom. Hemolymph was collected by inserting a sterilized glass capillary pulled to a fine point (inside diameter 0.58 mm, GB100F-10, Science Products GmbH, Hofheim, Germany) between the sternites of the third and fourth abdominal segment. From the same individual we then collected venom, by first removing the venom gland from the abdomen by pulling the stinger. We used a sterilized glass capillary to remove the venom from the gland. All capillaries were calibrated beforehand to measure the total amount of venom. Hemolymph and venom samples were divided into two parts. One part was used for a lysozyme assay while the other part was used for a zone of inhibition assay to measure antimicrobial peptide activity.

### Characterization of Internal and External Immune Defenses

To compare internal and external immune defense across species and female group (i.e., cuckoo, overwintered, and young queens

and workers) we investigated a constitutive and an induced immune trait in all individuals. For this we characterized lysozyme-like activity (LLA) and antimicrobial peptide (AMP) activity of the hemolymph and venom, using a lysozyme assay (Otti et al., 2009) and a zone of inhibition assay (Haine et al., 2008), respectively. LLA is often constitutively expressed in insects and has a relatively broad range activity against bacteria (Nayduch and Joyner, 2013). AMP activity is normally induced (Broderick et al., 2009), but still present at rather low levels in non-challenged individuals. However, in many species AMPs are constitutively expressed in venom and antimicrobial secretions (Otti et al., 2014). AMPs are more specific in their activity against bacteria and are among the most potent antimicrobial agents in nature (Broderick et al., 2009).

### Lysozyme Assay

We prepared 24-well culture plate lids (Nunclon, D7039, Sigma-Aldrich) with 10 ml of bacterial agar [500 mg agar, 50 mg streptomycin sulfate, 1 ml Triton-X, 50 ml $dH_2O$, 250 mg lyophilized *Micrococcus lysodeikticus* (ATCC No. 4698, M0508, Sigma-Aldrich)]. In a flask the mixture was heated to ∼100°C until all components had fully dissolved. Streptomycin sulfate (S6501, Sigma-Aldrich; 0.1 mg/mL) was added to prevent microbial contamination of the plates. We punched 24 equidistant holes (1.5 mm in diameter) into each agar plate using sterile pipette tips. We randomly distributed hemolymph and venom samples of different individuals across plates. From each individual we introduced 1 µl of hemolymph into a hole. For the venom, due to body size differences we could consistently harvest 0.5 µl of venom from queens and 0.25 µl from workers to transfer into the holes. LLA plates were incubated for 48 h at 30°C in an LTE® Raven incubator (Greenfield, Oldham, United Kingdom).

### Zone of Inhibition Assay (ZIA)

To measure AMP activity, we followed the zone of inhibition assay protocol developed by Haine et al. (2008). For this the gram-positive bacteria *Arthrobacter globiformis* (ATCC no. 8010) was cultivated from a glycerol stock, stored at −80°C, on LB-agar (10 g tryptone, 5 g yeast, 10 g NaCl, 15 g agar, 1 l $H_2O$) and incubated at 30°C for 24 h in an LTE® Raven incubator (Greenfield, Oldham, United Kingdom). After incubation, one single colony was picked and transferred into 5ml sterile LB broth (10 g tryptone, 5 g yeast, 10 g NaCl, 1 l $H_2O$) to prepare a liquid culture, which was incubated at 30°C for 24 h in a shaking incubator (LTE® Raven incubator, Greenfield, Oldham, United Kingdom) at 150 rpm. 0.1 ml of the liquid culture was transferred into 50 ml of 1% sterile agar at 44°C. From this, assay plates were prepared by pouring 5ml of bacteria-containing agar into a 90mm petri dish. Nine equidistant holes (1.5 mm in diameter) were punched in the agar using sterile pipette tips. One hole was left empty to serve as a sterile control. We randomly distributed hemolymph and venom samples of different individuals across plates. As for LLA we put 1 µl hemolymph from all individuals and 0.5 µl of venom from queens and 0.25 µl from workers into the holes. ZIA plates were incubated for 24 h at 30°C.

### Calculation of LLA and AMP Activity

After incubation, each plate was photographed using a Gel iX Imager (INTAS Science Imaging Instruments GmbH, Göttingen, Germany) with matching software INTAS GDS (INTAS Science Imaging Instruments GmbH, Göttingen, Germany). For each inhibition zone on a given plate the diameter was measured twice using ImageJ (version 1.50i; Schneider et al., 2012). The zone area from the lysozyme assay was then converted to units of lysozyme using a standard curve (**Figure S9** and **Supplementary Information**) to make LLA comparable to other studies. For AMP activity the zone areas in $mm^2$ were used as a dependent variable.

### Colony Size and Immune Defense

In addition, we extracted mean colony sizes and size ranges of the sampled bumblebee species from the literature (**Table S1**) to investigate a potential link between immune function and sociality or group size as was shown for other bee species by Stow et al. (2007).

## Characterization of Individual Condition

As we expect individual condition to influence the investment in immune defenses, be it internal or external, we checked for infections in the feces (Otti and Schmid-Hempel, 2008), measured body size and fat body content of all sampled individuals. Immediately after bringing individuals into the laboratory, feces were screened for different parasites (e.g., *Nosema sp., trypanosome parasites*) under a light microscope. To our astonishment no visible infection with trypanosomes (*Crithidia bombi*, *C. expoeki* or *Lotmaria sp.*) could be detected, because for example *Crithidia bombi* often shows quite high prevalence during the summer months (Popp et al., 2012). Approximately 1.5% (5 out of 342; 1 worker, 4 young queens) of the individuals showed spores of microsporidia (*Nosema sp.*). Roughly 23% (77 out of 342) of the individuals had phoretic mites (14% in cuckoo queens (6 out of 41), 34% in overwintered queens (27 out 78), 27% in young queens (16 out of 59) and 17% in workers (28 out of 164). All individuals were assessed for internal and external immune defense.

After dissection, the wings from each bumblebee were removed and the length of the radial cell of the right forewing was measured to the nearest 0.001 mm using ImageJ. The length of the radial cell of the right forewing is a surrogate for body size because this measure is independent of body mass (Medler, 1962; Owen, 1988, 1989). Next, the fat body content of individuals was measured using the lipid extraction protocol developed by Bazazi et al. (2016). First, each bumblebee was placed in a single 15 ml reaction tube and dried at 70°C for 5 days in an UFE 600 compartment drier (memmert, Schwabach, Germany). Then, dry bumblebees were weighed to the nearest 0.01 mg using an OHAUS Explorer balance (OHAUS Europe GmbH, Greifensee, Switzerland). After measuring initial dry body weight, 5 ml of chloroform was added to each reaction tube. To wash out all lipids the chloroform was replaced three times every 24 h. After 72 h the chloroform was removed, and bumblebees were dried for 5 days and weighed to the nearest 0.01 mg to get a measure for dry weight after fat

body extraction. The fat body content of each individual was calculated from the difference between initial dry weight and dry weight after fat body extraction. Finally, we calculated the proportion of fat body as a function of initial dry body weight, i.e., fat content relative to dry body weight, as an estimate of individual condition.

## Statistical Analysis

The data was analyzed using the statistical platform R version 3.6.1 (R Core Team, 2019). First, with a principal component analysis we investigated if the four female groups formed clusters (see **Supplementary Information**). The female groups clustered into three groups. Workers were well-separated from overwintered and cuckoo queens and overlapped to some extent with young queens (**Figure S2**). The young queens also overlapped with the overwintered and cuckoo queens. Finally, the overwintered and cuckoo queens formed one group in the PCA (**Figure S2**). Due to the clustering of the different eusocial female groups and the extremely different life-history strategy of cuckoo queens, we decided to investigate significant differences in the measured traits between the four female groups. Because parasitic species contained only one female group and eusocial species three, we analyzed the effects of female group and species separately. Therefore, in the first set of models, we used the fixed factors female group and immune defense, i.e., internal vs. external, and their interaction term with species as a random effect. In the second set of models, we used species and immune defense and their interaction term as fixed effects with female group as a random effect. Using the R packages *car* (Fox and Weisberg, 2011), *nlme* (Pinheiro et al., 2019) and *multcomp* (Hothorn et al., 2008), we analyzed the presence-absence of internal and external expression of an immune defense separately for LLA and AMP activity. We fitted linear mixed-effects models (LME) with a binary response variable for the presence or absence of LLA and AMP activity, respectively. Because body size significantly differed between female groups (LME: $X^2 = 167.09$, df = 3, $p < 0.001$, all pairwise Tukey comparisons: $p < 0.05$) and species (LME: $X^2 = 429.48$, df = 13, $p < 0.001$) (**Figure S8**), we fitted radial cell length as a covariate to control for body size. To account for this procedure in the statistical analysis of immune activity, we represent both immune measures relative to radial cell length on the y-axis in our figures. To analyze the effect of condition on the probability to show an immune defense, we fitted body fat content relative to dry body weight as a covariate. As fixed factors, we first fitted female group and immune defense, i.e., internal vs. external, and their interaction term and in a second series of models, species and immune defense. As random effects, we fitted individual nested within female group nested within species to account for the hierarchical nature of the data and variation between species in the models with the female group as a fixed factor. In the models with species as a fixed factor, we nested individual within female group nested within species as a random effect.

For the analysis of internal and external immune expression, we first removed 26 individuals from nine different species, because they expressed neither LLA nor AMP activity, neither internally nor externally (cuckoo queen: 1 individual, overwintered queen: 11, young queen: 2, worker: 12). Using the R packages *glmmTMB* (Brooks et al., 2017), *DHARMa* (Hartig, 2019), and *multcomp* (Hothorn et al., 2008), we then fitted linear mixed-effects models (LME) with LLA and AMP as response variables to investigate effects of body size, condition, internal vs. external defense, female group and species. The package *glmmTMB* allowed us to account for zero inflation in the expression of LLA and AMP activity in the LMEs. As fixed factors we first fitted female group and immune defense, i.e., internal vs. external, and their interaction term and in a second series of models we fitted species and immune defense as fixed factors. We again fitted radial cell length as a covariate to control for body size effects. To analyze the effect of condition on immune defense we fitted body fat content relative to dry body weight as a covariate. As random effects we fitted individual nested within female group nested within species to account for the hierarchical nature of the data and for variation between species in the models with the female group as a fixed factor. In the models with species as a fixed factor we nested individual within female group and species as a random effect. If female group or species showed a significant effect, we ran multiple comparisons to identify differences among immune defense mechanisms adjusting *p*-values according to Westfall (Bretz et al., 2010).

To investigate associations between traits we accounted for phylogenetic relatedness of the different bumblebee species by running phylogenetic comparative analysis with phylogenetic generalized least-squares (PGLS) using the R packages *Rphylip* (Felsenstein, 2005; Revell and Chamberlain, 2014) and *caper* (Orme et al., 2018). First, we constructed a phylogenetic tree using four DNA sequences from the literature with the software Geneious (see **Supplementary Information**, **Table S2**). Second, we analyzed the strength of the antimicrobial activity of individuals in relation to colony size in the eusocial species. For this we calculated the mean external and internal LLA and AMP activity, i.e., mean units of lysozyme per µl and zone area in $mm^2$, respectively, for each female group and species. We used PGLS to relate each immune defense mechanism to mean colony size of the sampled bumblebee species (extracted from literature; **Table S1**).

Third, we investigated a general link in the expression of internal and external immune defense with condition we additionally tested for associations between external LLA and AMP activity, between internal LLA and external AMP, as well as all between four immune defense traits and fat content relative to dry body weight fitting PGLS.

Total fat content was significantly positively correlated with initial dry body weight (PGLS: estimate = 0.071, SE = 0.013, $R^2 = 0.72$, t = 5.620, $p < 0.0001$) and body size (PGLS: estimate = 0.041, SE = 0.006, $R^2 = 0.79$, t = 6.749, $p < 0.0001$). However, body condition (fat content relative to dry body weight) did not correlate with body size (PGLS: estimate = −0.042, SE = 0.046, $R^2 = 0.07$, t = −0.915, $p = 0.38$). Initial dry body weight was significantly positively correlated with body size (PGLS: estimate = 0.547, SE = 0.030, $R^2 = 0.96$, t = 18.017, $p < 0.0001$).

# RESULTS

## Presence-Absence External vs. Internal Immune Activity

### Female Groups

Overall, the three different types of queens and workers significantly affected the proportion of individuals showing internal or external LLA (LME: $X^2 = 13.304$, df = 3, $p < 0.01$) (**Figure 1A**), but not AMP activity (LME: $X^2 = 2.760$, df = 3, $p = 0.43$) (**Figure 1B**). Young queens and workers showed the highest proportions of LLA expression and young queens had a significantly higher proportion of individuals expressing LLA than overwintered queens (Tukey comparison: $p < 0.01$).

### Species

Overall, we found that species significantly varied in the proportion of individuals expressing LLA (LME: $X^2 = 31.740$, df = 13, $p < 0.01$) (**Figures 2A,B**) and AMP activity (LME: $X^2 = 59.715$, df = 13, $p < 0.001$) (**Figures 2C,D**). Two species comparisons showed significantly different proportions of individuals expressing LLA (*B. pratorum* vs. *B. lucorum* and *B. pratorum* vs. *B. sylvarum*: Tukey comparisons: $p < 0.05$; all other Tukey comparisons $p > 0.05$). For LLA the proportion of individuals expressing internal or external immunity depended on the species (LME: $X^2 = 27.961$, df = 13, $p < 0.01$), whereas for AMP activity it did not matter (LME: $X^2 = 14.566$, df = 13, $p = 0.34$).

### External vs. Internal Immune Defense

Significantly fewer individuals expressed external (40 ± 24%) than internal LLA (67 ± 8%, mean ± sd) (LME: $X^2 = 63.545$, df = 1, $p < 0.001$) (**Figure 1A**), whereas significantly more individuals expressed external (65 ± 12%) than internal AMP activity (39 ± 5%) (LME: $X^2 = 76.623$, df = 1, $p < 0.001$) (**Figure 1B**). Further, the probability of showing internal or external LLA depended on the female group (LME: $X^2 = 31.340$, df = 3, $p < 0.001$). Cuckoo and overwintered queens showed a bigger difference in the proportion of external vs. internal expression of LLA (25 ± 19 vs. 73 ± 18% and 19 ± 15 vs. 67 ± 28%, respectively) than young queens and workers (73 ± 20 vs. 57 ± 33% and 45 ± 19 vs. 73 ± 9%, respectively) (**Figure 1A**). Interestingly, only young queens showed a higher proportion of external than internal LLA (**Figure 1A**). The proportion of individuals expressing external or internal AMP activity did not depend on the female group (LME: $X^2 = 3.590$, df = 3, $p = 0.31$). For AMP activity, young queens and workers showed a bigger difference between external and internal immune defense expression (82 ± 16 vs. 41 ± 24% and 67 ± 20 vs. 44 ± 12%, respectively) than cuckoo and overwintered queens (54 ± 34 vs. 31 ± 27% and 60 ± 32 vs. 39 ± 38%, respectively) (**Figure 1B**). Approximately 8% (26 out of 342) of the sampled bumblebees showed no measurable LLA and AMP activity in both internal and external immune defense.

### Effects of Body Size and Condition

Neither body size (LME: LLA: $X^2 = 2.254$, df = 1, $p = 0.13$; AMP activity: $X^2 = 0.07$, df = 1, $p = 0.79$) nor fat content relative to dry body weight (LME: LLA: $X^2 = 1.242$, df = 1, $p = 0.27$; AMP activity: $X^2 = 1.527$, df = 1, $p = 0.22$) affected the probability of constitutively expressing an immune defense.

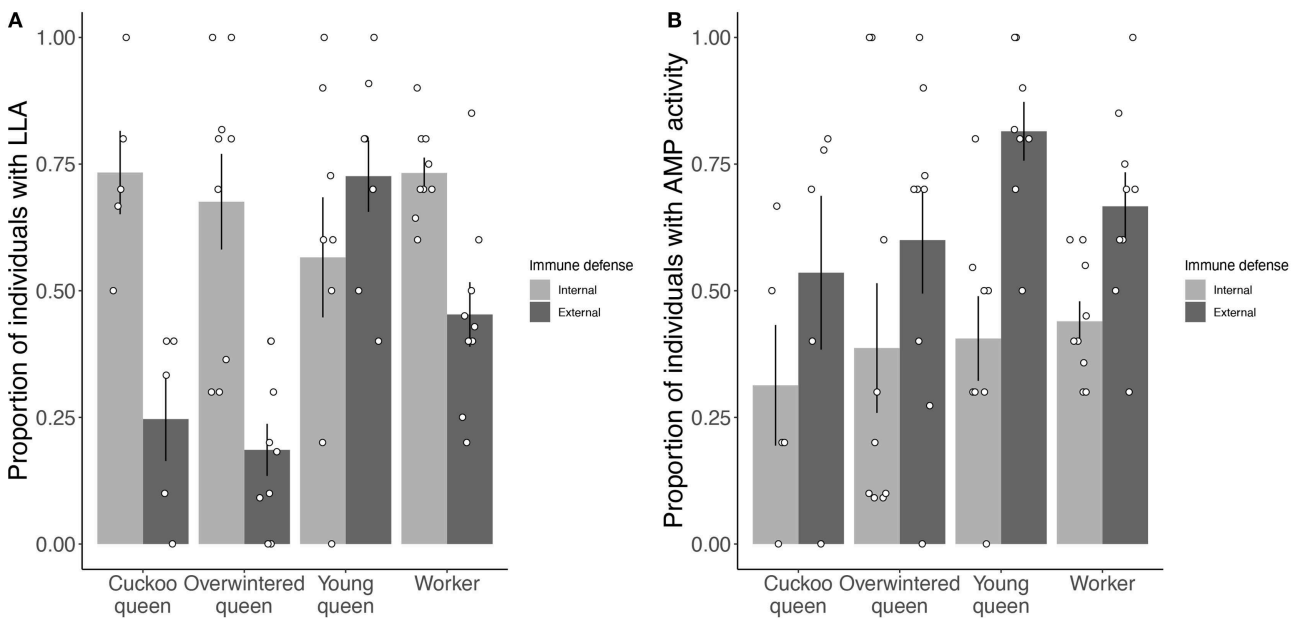


**FIGURE 1 |** Proportion of individuals showing constitutive expression of **(A)** LLA and **(B)** AMP activity for both internal (hemolymph) and external (venom) immune defense for each female group. Positive tests for internal immune defense are shown in light gray and for external immune defense in dark gray. Bars give mean proportions with error bars representing one standard error and black circles give the proportion for each species in a female group.

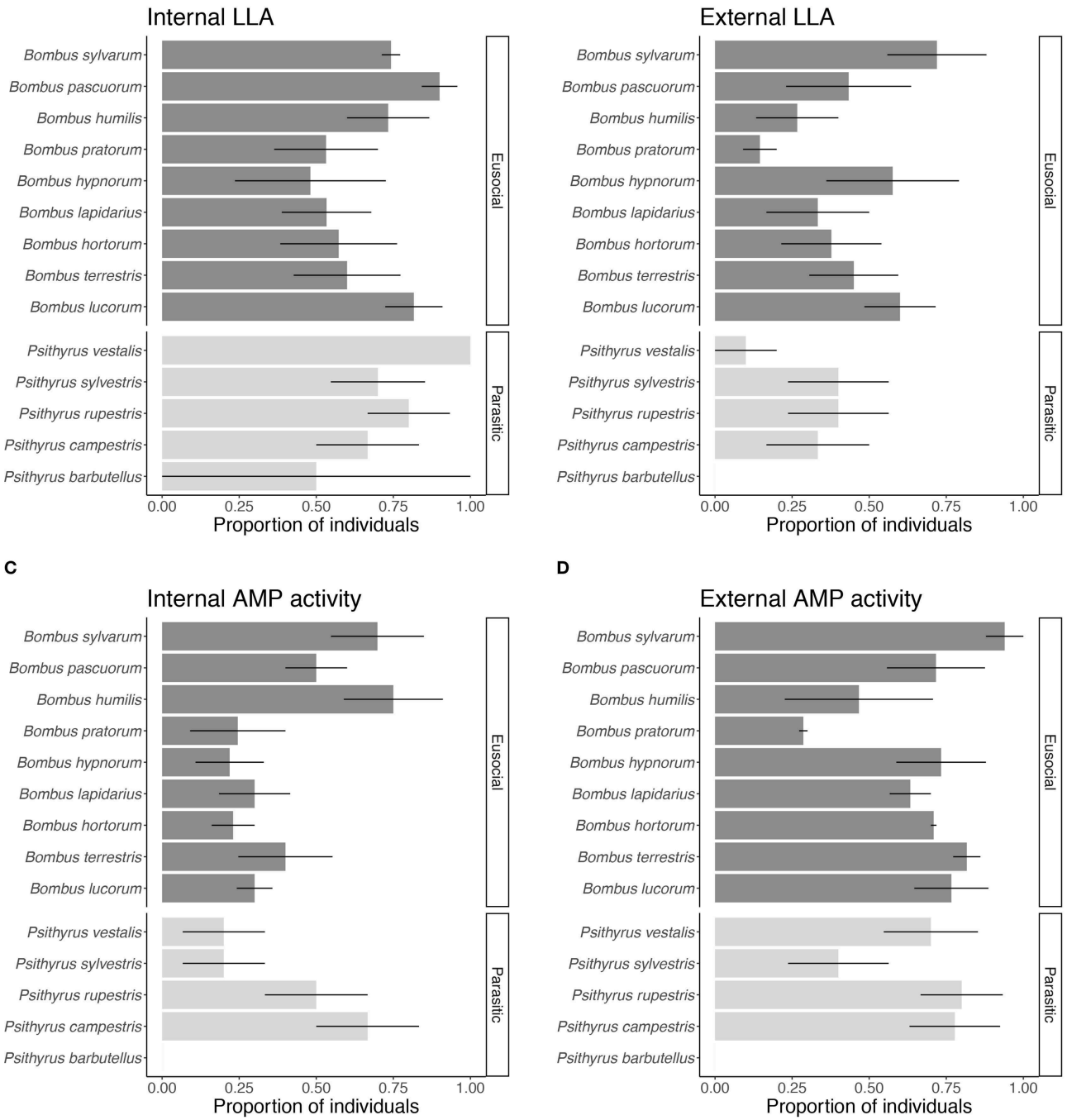


**FIGURE 2 |** Proportion of individuals showing constitutive expression of **(A)** internal and **(B)** external LLA and **(C)** internal and **(D)** external AMP activity for each species. Positive tests for parasitic species are shown in light gray and for eusocial species in dark gray. Bars give mean proportions with error bars representing one standard error.

## Strength of Constitutive External vs. Internal Immune Activity

### Female Group

Overall, the different types of queens and workers significantly affected LLA (LME: $X^2 = 14.160$, df $= 3$, $p < 0.01$) (**Figure 3A**) and AMP activity (LME: $X^2 = 108.886$, df $= 3$, $p < 0.001$) (**Figure 3B**). Young queens showed the highest LLA followed by workers overwintered queens and cuckoo queens (Tukey comparison: young vs. overwintered queens: $p < 0.05$ and young queens vs. workers: $p < 0.01$ (**Figure 3B**). All female types

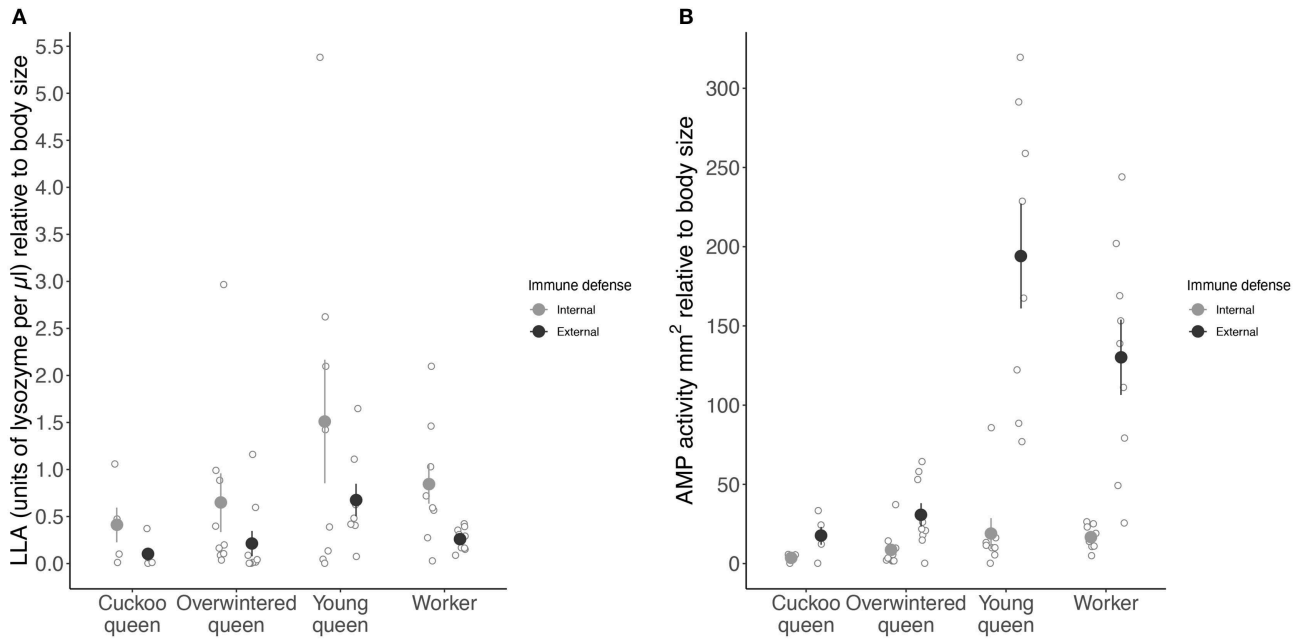


**FIGURE 3 |** Mean constitutive expression of **(A)** LLA as units of lysozyme per μl and **(B)** AMP activity as zone area in $mm^2$ relative to body size (immune activity divided by radial cell length in mm) for both internal (hemolymph) and external (venom) immune defense for each female group. Internal immune defense is given as filled light gray circles and external immune defense as dark gray circles. Error bars represent one standard error and open light gray circles show immune activity for each species in a female group.

differed from each other in their expression of AMP activity (cuckoo vs. overwintered queens: Tukey comparison: $p < 0.05$, all other pairwise Tukey comparisons: $p < 0.001$).

### Species

Overall, LLA showed a similar pattern across species (LME: $X^2 = 5.585$, df = 13, $p = 0.96$) (**Figure 4A**), whereas AMP activity significantly varied between species (LME: $X^2 = 52.194$, df = 13, $p < 0.001$) (**Figure 4B** and **Table S5**).

### External vs. Internal Immune Defense

External LLA was significantly lower than internal LLA (LME: $X^2 = 16.470$, df = 1, $p < 0.001$) (**Figure 3A**), whereas external AMP activity was significantly higher than internal AMP activity (LME: $X^2 = 344.375$, df = 1, $p < 0.001$) (**Figure 3B**). Further, the strength of external and internal AMP activity depended on the female group (LME: $X^2 = 8.409$, df = 3, $p < 0.05$).

Young queens expressed roughly 10 times higher and workers eight times higher external than internal AMP activity, whereas cuckoo and overwintered queens showed only five and four times higher external than internal AMP activity, respectively (**Figure 3B**). Young queens showed the highest external LLA (194.14 ± 93.60 $mm^2$ relative body size) followed by workers (130.21 ± 71.30 $mm^2$ relative body size), overwintered queens (30.73 ± 22.20 $mm^2$ relative body size) with cuckoo queens having the lowest external LLA (17.50 ± 12.60 $mm^2$ relative body size) (**Figure 3A**). However, internal LLA followed a similar pattern, indicating that the difference between external and internal LLA is not related to female group (LME: $X^2 = 1.536$, df = 3, $p = 0.67$) (**Figure 3A**).

### Effects of Body Size and Condition

Neither body size (LME: LLA: $X^2 = 1.028$, df = 1, $p = 0.31$; AMP activity: $X^2 = 0.196$, df = 1, $p = 0.66$) nor fat content relative to dry body weight (LME: LLA: $X^2 = 0.001$, df = 1, $p = 0.98$; AMP activity: $X^2 = 1.731$, df = 1, $p = 0.19$) (**Figure S9**) affected the expression of an immune defense.

### Colony Size and Immune Defense

Mean colony size was neither correlated with LLA (Pearson's product-moment correlation: external: $R^2 = -0.33$, $z = -1.660$, $p = 0.11$; internal: $R^2 = -0.10$, $z = -0.51$, $p = 0.62$) nor AMP activity (Pearson's product-moment correlation: external: $R^2 = -0.08$, $z = -0.39$, $p = 0.70$; internal: $R^2 = -0.22$, $z = -1.086$, $p = 0.29$) (**Figure S8**).

Internal immune defense is given as filled light gray circles and external immune defense as dark gray circles. Error bars represent one standard error and open light gray circles show individual immune trait expression.

## Associations of Internal and External Immune Defense With Body Condition

Overall, we found no association between external and internal immune defenses neither for LLA (PGLS: estimate = 0.709, SE = 0.834, $R^2 = 0.06$, t = 0.850, $p = 0.41$) (**Figure S9A**) nor for AMP activity (PGLS: estimate = 0.048, SE = 0.031, $R^2 = 0.16$, t = 1.522, $p = 0.41$) (**Table S3**, **Figure S9B**). There was also

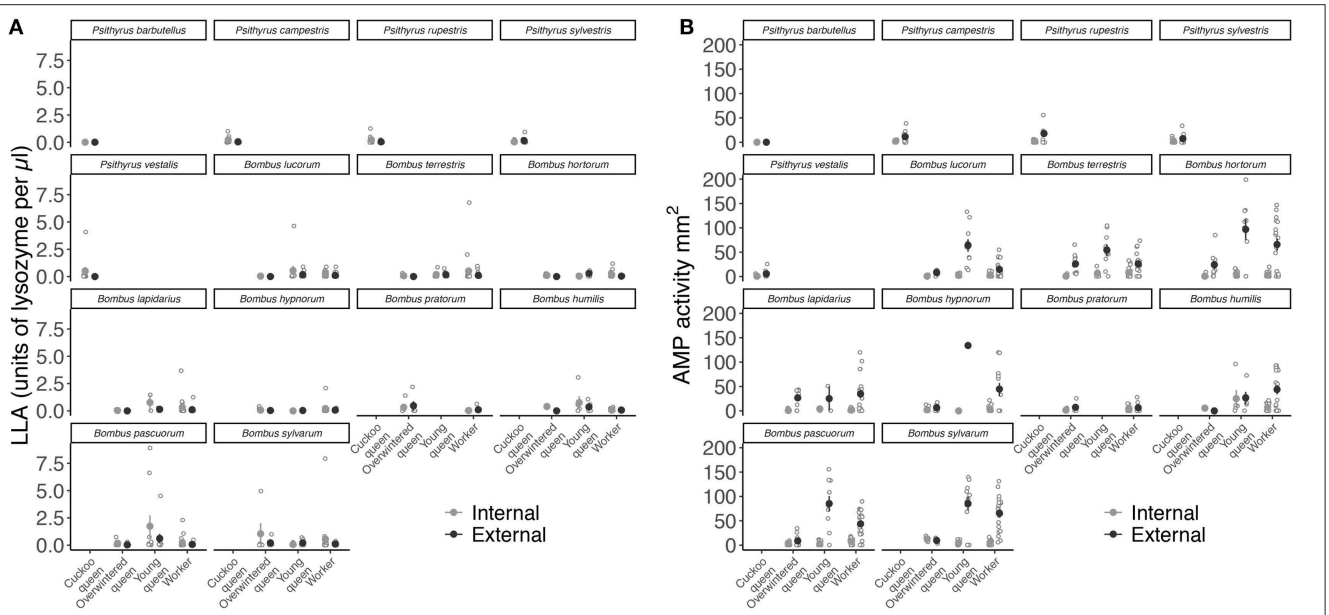


**FIGURE 4 |** Mean constitutive expression of **(A)** LLA as units of lysozyme per µl and **(B)** AMP activity as zone area in $mm^2$ relative to body size (immune activity divided by radial cell length in mm) for both internal (hemolymph) and external (venom) immune defense for each female group and species.

no link between external AMP activity and internal LLA (PGLS: estimate = 0.002, SE = 0.002, $R^2 = 0.09$, t = 1.091, $p = 0.30$). Finally, none of the four immune defense measures was related to fat content relative to body weight (PGLS: external LLA: estimate = −0.001, SE = 0.001, $R^2 = 0.32$, t = −1.813, $p = 0.11$; internal LLA: estimate = 0.001, SE = 0.001, $R^2 = 0.03$, t = 0.494, $p = 0.64$; external AMP: estimate = −0.027, SE = 0.079, $R^2 = 0.02$, t = −0.345, $p = 0.74$; internal AMP: estimate = −0.010, SE = 0.012, $R^2 = 0.09$, t = −0.819, $p = 0.44$) (**Table S3**, **Figure S9**).

# DISCUSSION

We could show that the constitutive expression of LLA was more prominent internally than externally and constitutive AMP activity was the main component of a potential external immune defense. The presence of an external immune defense and its degree of expression was highest in young queens and workers, indicating differences in the investment in internal and external immunity among the tested female groups. Also, the difference between internal and external immunity was largest in young queens and workers, indicating a greater importance of external immune defenses in those female groups. While species showed very similar patterns of external and internal LLA and internal AMP activity, external AMP activity differed between species. The drivers of such variation remain unknown, but we speculate that this might be related to the ecology of the species, i.e., variation in pathogen prevalence due to different nest sites (Fussell and Corbet, 1992) or pathogen prevalence in combination with population density and genetics (Whitehorn et al., 2011). We could not find any relationship between internal and external immune defense, which might be due to the fact that we measured constitutive expression of immune defense. Further, fat content relative to dry weight seems not to be related to the constitutive expression of immune defenses and neither does body size. We think that an investigation of induced internal immune defenses might add more information on the connection between external and internal immune defenses, and also the importance of fat reserves.

## Variation in External and Internal Immune Defenses Among Female Groups

Cuckoo queens and overwintered queens less often showed constitutive expression of external immunity and its activity was also rather low in comparison to young queens and workers. These findings might be explained by the fact that cuckoo and overwintered queens have spent considerable resources to survive the winter and then in spring need to invest into reproduction, which is traded-off against immune defense (Sheldon and Verhulst, 1996; Råberg et al., 1998; Schwenke et al., 2016). Bumblebee queens lose over 25% of their body mass over hibernation (Brown et al., 2003). In comparison to overwintered queens, young queens and workers might have more resources to spend on external immune defenses as they do either not immediately reproduce or do not reproduce at all. In addition, it may be more important for individual workers to be able to defend the colony against enemies and pathogens than just itself. The reduction in immunity of overwintered queens might partly represent an age effect. Immunity decreases with age in several insect species, including bumblebees (Doums et al., 2002; Whitehorn et al., 2011), honeybees (Laughton et al., 2011), stingless bees (Ravaiano et al., 2018), mosquitoes (Hillyer et al., 2005), and crickets (Adamo et al., 2001). Probably also related to age, in the honeybee *Apis mellifera*, the composition of venom between queens and workers differs (Baracchi and Turillazzi,

2010). Baracchi and Turillazzi (2010) also found differences in the peptide composition of honeybee venom, depending on the task of an individual in the colony, i.e., queens, nursing, and foraging workers. For example, the venom of honeybee queens contains a smaller number of different AMPs than the venom of workers (Baracchi and Turillazzi, 2010). Whether such differences in the composition of venom might affect the variation in external immune defense across the female groups of the different bumblebee species would need to be investigated further.

Young queens had an almost 10 times higher external AMP activity than overwintered queens. In contrast, honeybees reduce immune gene expression over winter (Steinmann et al., 2015). However, honeybees overwinter in colonies and do not hibernate like bumblebees. Probably in preparation for hibernation young queens invest in external immunity similar to the paper wasp *Polistes dominulus* where females spread their venom within hibernation sites to protect themselves from bacteria during hibernation (Turillazzi et al., 2006). An increased antibacterial activity of the venom might be useful for a better protection. However, we are not aware of any accounts of bumblebee queens using their venom in such a way.

Workers showed stronger external immune defense than overwintered queens. In addition to the effects mentioned above reducing the immunity of an overwintered queen, it could well be that workers increase external immunity to protect the brood and the nest site and to be prepared for the encounter with pathogens when foraging. Higher workload in workers, however, leads to a reduced encapsulation response (König and Schmid-Hempel, 1995; Doums and Schmid-Hempel, 2000).

Interestingly, cuckoo queens had the lowest external as well as the internal immune defense of all female groups. Cuckoo queens might not have to invest in immunity as much as overwintered queens, who might need to keep their brood protected from microbes and pathogens during colony foundation. The basis for a potentially lower immune investment in the parasitic compared to the eusocial life-history strategy might be investigated further by monitoring the immunity of cuckoo and overwintered queens over time. We expect the investment in the external immune defense of cuckoo queens not to change once a nest has been taken over. Mainly, because the usurped nest should already contain workers that most likely already protect the nest site. However, overwintered queens have to establish a new colony. Similar to ant queens, we expect bumblebee queens to upregulate external immune defenses once a nest site has been identified to provide protection for the first brood (Tragust et al., under review).

## Variation in External and Internal Immune Defense Across Species

Species differed in the presence of potential external immunity but varied mainly in the expression of external AMP activity. The pattern for external and internal LLA expression was similar across species and for the differences between female groups within species. For internal LLA, we expected this because LLA is part of the constitutive immune defense and has a rather broad range. The extent of LLA was comparable to LLA in hemolymph of honeybees, the only other hymenopteran where LLA has been measured so far (Dickel et al., 2018).

External AMP activity expression varied across species. We found most differences in external AMP activity among the eusocial species and between eusocial and parasitic species. Cuckoo species showed almost the same extent of external and internal immune defense. Therefore, the investment in external immune defenses seems more based on life-history strategies and characteristics than on species. Even though species differed overall in their AMP activity, the pattern between the female groups was relatively similar in each species. Young queens and workers had a higher potential external immune defense than cuckoo and overwintered queens. Only in one of the nine eusocial species, i.e., *B. lapidarius*, all female groups had similar external AMP activity. We take this as an indication that life-history strategy and characteristics have a greater impact on the expression of external immunity than ecological or genetic differences between species.

## Effect of Fat Reserves, Body Size, and Colony Size on External and Internal Immune Defenses

Neither fat reserves nor body size were correlated to immunity or had any effect on it. The differences between female groups or species do not seem to be explained by these two covariates. Similarly, body size had no effect on encapsulation response in *B. terrestris* (Schmid-Hempel and Schmid-Hempel, 1998) and melanization through the phenoloxidase pathway in the dung fly *Scathophaga stercoraria* (Hosken, 2001). One interpretation of this finding would be that organisms can compensate for the extra demand by increasing the intake of resources (Schmid-Hempel, 2003). Higher fat reserves were not associated with higher internal and potential external immune defense across the different life-history strategies and characteristics. However, all species showed a similar proportion of fat content, even across life-history strategies and characteristics. Although somehow surprising this finding might suggest that the costs of constitutively expressed immunity are maintenance costs. Such costs would only be visible if variation in the selection for the maintenance of an immune defense varied between species. Therefore, further studies should experimentally test the condition-dependence of external immunity by manipulating diet and/or by immune challenge similar to Moret and Schmid-Hempel (2000).

Even though cuckoo species showed lower immunity than eusocial species, colony size across eusocial species was neither correlated with internal nor with potential external immune defenses. Baracchi et al. (2012) suggested a threshold for a degree of sociality or a sufficient number of individuals in a society to be reached before an efficient collective immunity serves as a mechanism of disease resistance. Bumblebee societies might have already reached such a threshold. It would be interesting to study the colony size continuum in connection to immunity across a larger range of taxa, including ants, wasps, bees, and probably termites.

## Conclusion

In conclusion, internal immunity was similar across life-history strategies and characteristics and species while potential external immune defense varied across the life-history strategies,

characteristics and species. In general, the venom could serve as a potential external immune defense, but whether or not bumblebees use it, will have to be investigated further. The detection of antimicrobial components in the nest material or hibernation sites, on eggs or brood, or the body surface of bumblebees, as for other insects (e.g., ants, bees, and wasps), could provide evidence for the use of venom as external immune defense. Venom might not be the only component of external immune defense in bumblebees. Therefore, future work will need to incorporate other exocrine glands, such as the mandibular, salivary or Dufour's gland (Ayasse and Jarau, 2014) as these could also contain antimicrobial properties. We found no general link between individual condition and immune defenses. Further studies might find such links by altering either immune defense traits or resource availability.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## AUTHOR CONTRIBUTIONS

GB, HF, and OO conceived the idea and designed the study. GB carried out the study. GB and OO performed the statistical analysis. GB, HF, and OO interpreted the results and wrote the manuscript. All authors read and approved of the final manuscript.

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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.2020.00062/full#supplementary-material

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# Balancing Life History Investment Decisions in Founding Ant Queens

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Reproduction is a very critical step in the life of an organism. Females must balance their investment in different life-history traits while reproducing. During the process of colony founding in social organisms, such as ants or bees, a trade-off between reproduction and immunity might be very stringent, because queens might be constrained to invest into immune protection of themselves and their developing offspring until the first workers emerge. Here we investigate how different levels of microbial pressure affect colony founding success of *Lasius niger* ant queens and whether investment into immune defense traits comes at a substantial cost to the queens. In a first experiment mated queens were exposed to four different environments: sterile housing, autoclaved soil, untreated soil and soil containing two opportunistic pathogens. In this experiment, we investigated an immediate cost, i.e., the success of producing the first brood, and a potential delayed cost, i.e., queen survival and colony founding success after hibernation. For the latter, we removed the first brood after hibernation to reveal hidden costs via the application of an additional stressor. We found that irrespective of the microbial environment all queens successfully managed to start a colony, with queens in the soil treatments showing a higher worker production than the queens in the sterile environment. This suggests that either soil components or soil microbes benefit colony growth. After hibernation queens in microbe soil showed significantly lower survival and could not replace a lost brood. In a second experiment, we investigated whether external immune defense in the form of formic acid use can explain part of the costs imposed on queens. We found that queens used formic acid to sanitize their new nest suggesting that queens founding a colony under high microbial pressure are forced to pay a substantial cost by investing in both reproduction and immunity simultaneously. Our results suggest that early, simultaneous investment in reproduction and immunity can allow colony growth under microbial pressure but may be costly in terms of resistance to later challenges. Ant queens may thus be trading off insurance against future challenges for increased pathogen immunity.

**Keywords: external immune system, eusociality, colony founding costs, pathogen pressure, antimicrobial secretion, antimicrobial venom, life-history trade off**

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## INTRODUCTION

Reproduction is one of the most critical steps in the life of an organism (Harshman and Zera, 2007). Especially females have to balance their investment in different life-history traits while reproducing (Zuk and Stoehr, 2002). For example, depending on the degree of microbial pressure in the environment, in addition to reproduction, an individual has to invest in immunity (Schwenke et al., 2016). To ensure not only its own survival but also the one of its offspring it will have to invest in internal and external immune traits (Otti et al., 2014). In social organisms, such as ants, wasps from temperate regions or bumblebees, this trade-off might be very steep during the process of colony founding and might, thinking in terms of a superorganism, rather represent an investment into growth than reproduction *per se* (Hölldobler and Wilson, 2008). In ants, starting a colony comes with great challenges (Wheeler, 1910; Hölldobler and Wilson, 1990; Schmid-Hempel, 1998). Before founding a colony, virgin queens leave their nest in search for a mating partner. After successful mating, they bite off their wings and search for a suitable nest site to start laying the first eggs (Hölldobler and Wilson, 1990). Over 95% of queens are estimated to die during this process (Baer et al., 2006; Cole, 2009; Marti et al., 2015), i.e., colony founding events have a very high failure rate (Wilson, 1971). Microbes have been suggested as a major driver of this mortality. For example, in leaf cutter ants 74% of queen deaths during colony founding were associated with pathogens (Baer et al., 2006). Leaving the well-protected maternal colony increases the probability of encountering novel pathogens in the environment. In addition to facing a high microbial pressure herself (Rosengaus and Traniello, 1993; Schmid-Hempel, 2005; Cronin et al., 2013; Gálvez and Chapuisat, 2014; Cole et al., 2018), a recently mated queen founding a colony needs to protect also her offspring. Protection through immunity can occur via activation of internal immune defenses (Cremer et al., 2007; Otti et al., 2014), improving the chances of the queen's survival, or by application of external immune defenses (Otti et al., 2014). The use of formic acid is an example to reduce the microbial pressure in the nest (Tragust, 2016; Baracchi and Tragust, 2017). Obviously, this will come at a cost adding to the common costs of producing the first brood, which depends exclusively on parental care (Andersson, 1984; Cole et al., 2018). As queens rely exclusively on their own energy reserves during colony foundation (Hölldobler and Wilson, 1990; Cronin et al., 2013; Norman et al., 2016) queens experience energetic stress (Camargo et al., 2011; Gálvez and Chapuisat, 2014). This stress might constrain the investment in immune defenses and result in a trade-off between colony growth (reproduction) and immunity (Calleri et al., 2007; Schwenke et al., 2016; Cole et al., 2018) as an increase in energy expenditure during the founding can strongly affect survival (Camargo et al., 2011). Two strategies might thus be pursued by colony founding queens to optimize the use of resources imposed by the constraints of this growth-immunity trade-off: (1) investment in immune defense of herself and/or her offspring or (2) investment in colony growth either producing high numbers of offspring (Schwenke et al., 2016) and/or speed up its development (Bordoni et al., 2017). With the latter strategy, queens might benefit from later protection against pathogens, because workers will not only forage for food, but they will also provide protection for the colony (Cremer et al., 2007; Otti et al., 2014; Tragust, 2016; Baracchi and Tragust, 2017). We assume both strategies are highly dependent on the microbial pressure in and around the nest site of a new colony and the queen's current condition (Harshman and Zera, 2007; Schwenke et al., 2016). In founding queens, the production of a first brood is of paramount importance. Consequently, environmental stressors such as the presence of microbial pathogens might not only lead to short- but also long-term fitness effects. Costs induced by stressors might only show months later (Bordoni et al., 2017) or might be alleviated in the long run, as lost resources during colony founding might be compensated for by the colony as a whole later in life. To reveal potential hidden costs of long-term effects not only the direct effects of microbial pressure on the queen should be investigated. If the cost is context-dependent (Moret and Schmid-Hempel, 2000) an additional stressor, such as loss of brood, might uncover a potential hidden cost of microbial pressure.

Here we investigated how microbial pressure affects colony founding success of *Lasius niger* ant queens and to reveal potential hidden costs we exposed the queens to an additional stressor, i.e., the loss of the first brood, after founding. In a first experiment, we measured colony founding success in four different environments, ranging from a sterile housing to soil containing a bacterial and fungal pathogen. After collection in the field, we recorded queen survival and the production of workers. We assumed that queens exposed to microbe-enriched soil will have to invest more in their first brood than the queens in the other treatments. To reveal such a cost of investment we removed the complete offspring and transferred all queens to a sterile environment to minimize differences between treatments after hibernation (Bordoni et al., 2017). Then, we assessed their ability to start a second brood without food and any help by workers. We also assumed that investment into immune defense traits comes at a cost to the queens during colony founding. In a second experiment, we therefore investigated whether the application of formic acid to the environment as an external immune defense trait explains part of the costs imposed on queens during the first round of colony foundation.

## MATERIALS AND METHODS

### Ant Queen Collection and Housing

In July 2015 we collected 200 *Lasius niger* wing-less queens after their mating flight on the campus of the University of Bayreuth and in Bindlach, a suburb of Bayreuth. These queens were randomly assigned to the following three soil treatments and a control treatment ($N = 50$ queens per treatment): untreated soil, autoclaved soil, autoclaved soil with added microbes (henceforth called microbe-enriched soil) and a sterile control without any soil (for details see "Preparation of Soil Treatments"). We used 15 ml Falcon tubes as nest for the queens. First, 5 ml autoclaved $H_2O$ was added to each tube and then a cotton ball placed into the tube to guarantee a certain degree of humidity. Then

1.5 g from one of the three soil treatments were added onto the cotton ball, whereas the sterile control remained without any soil. Finally, an ant queen was placed into the tube and the tube was closed with the lid.

## Preparation of Soil Treatments

The soil for the three treatments was collected in the same area on the campus of the University of Bayreuth as the ant queens. Then we split up the soil in two parts. One part was left as it was collected, i.e., served as the untreated soil treatment. Fifty 15 ml tubes were provided with untreated soil. The other part was autoclaved at 125°C for 20 min to kill the microbes present in the soil (Trevors, 1996). Once the soil had cooled down it was added to a hundred 15 ml tubes. Fifty tubes were left without further processing and to the other fifty tubes with autoclaved soil 1 ml of a mix of two opportunistic pathogens, one fungus (*Metarhizium anisopliae*, isolate KVL 03-143, obtained from the Faculty of Life Science, University of Copenhagen, Denmark) and one bacterium (*Serratia marcescens*, strain DSM12481, DSMZ Braunschweig, Germany), was added. *Metarhizium* fungi frequently occur in soil and are responsible for natural infections of ants (Hughes et al., 2004; Reber and Chapuisat, 2012). *Serratia marcescens* is pathogenic in a range of insects (Grimont and Grimont, 2006) and can cause immune system activation in the ant *Camponotus floridanus* (Ratzka et al., 2011). *M. anisopliae* spores were harvested from a malt-extract agar plate, their viability checked to be above 90% by scoring approximately 500 spores for germination after 18 h of incubation on an agar plate. Then spores were mixed to a concentration of $\sim 2 \times 10^8$ spores/ml. *S. marcescens* was produced by plating out from a glycerol stock on LB agar from which a single colony was picked to prepare an overnight culture in LB broth. 5 ml of the overnight culture (concentration $\sim 10^9$ bacteria/ml) was mixed together with 5 ml of the *M. anisopliae* solution. To this we added 1,000 ml autoclaved tap water resulting in a bacteria concentration of $5 \times 10^6$ bacteria/ml and a fungal spore concentration of $1 \times 10^6$ spores/ml. This third soil treatment represents the microbe-enriched soil. The two other soil treatments received 1 ml of autoclaved $H_2O$ to account for the added liquid in the microbe-enriched soil treatment.

## Founding Experiment

After housing the queens in the respective treatments ($N = 50$ queens per treatment), we started the founding experiment under constant darkness in a climate chamber at 22°C and 70% humidity. We checked for queen survival (checks done at 2 days and 1 month after the start of the experiment) and 4 months later (83 days) we put all tubes into hibernation by placing them into a refrigerator at 4°C. After 5 months of hibernation (day 221–225, for treatments autoclaved soil, untreated soil, microbe-enriched soil and sterile control without any soil respectively) all tubes were opened, the number of workers produced was counted and all offspring and dead queens were removed. Live queens (untreated soil: 26, autoclaved soil: 33, microbe-enriched soil: 19, sterile control: 27) were put into a fresh, sterile 15 ml Falcon tube provided with 5 ml autoclaved $H_2O$ blocked off with a cotton ball without any soil like the sterile control treatment before hibernation. They were then forced to produce a second brood to measure a potential hidden or delayed cost of colony founding. As mentioned above all brood was removed beforehand, and no food was provided. The tubes were again kept under constant darkness in climate chamber at 15°C and 70% humidity until the April 04, 2016 (day 257 of the experiment). For the queens surviving until the April 04, 2016 (untreated soil: 21, autoclaved soil: 21, microbe-enriched soil: 12, sterile control: 24), we raised the temperature to 22°C to initiate brood production and checked queen survival and brood production over the next 3 months.

## Formic Acid Use in Different Colony Founding Environments

In July 2016 we collected 200 *Lasius niger* wing-less queens after their mating flight on the campus of the University of Bayreuth to measure the use of formic acid under different environmental challenges. For this we collected soil from the premises of the University of Bayreuth and prepared three different soil supernatants as environmental challenges for the colony founding period. The soil was split in two parts of 500 ml and each part was filled into a one liter Duran glass bottle to which tap water was added up to the 1 l mark of the bottle. The mix was left on the bench top at room temperature for 48 h and occasionally shaken. After this it was left to settle for another 48 h. Then one mix was autoclaved at 125°C for 20 min to kill the microbes (Trevors, 1996). The other mix was left untreated. From both mixes we then took the supernatant, which yielded in 400 ml each. The supernatant of the autoclaved mix was split again into two parts. 200 ml were left as they were and we added the same mix at the same concentration of two opportunistic pathogens as in the founding experiment, i.e., *M. anisopliae* and *S. marcescens*, to the other 200 ml. We will refer to these three supernatants in the following as untreated soil, autoclaved soil and microbe-enriched soil.

To measure the use of formic acid during the colony founding stage we prepared 6-well microtiter plates (Cellstar 657185, Greiner Bio-One, Germany) as follows. First, we padded five wells of a plate with cotton wool on the side and placed a blue litmus paper (34 × 10 mm, 37135, Fluka, Germany) along the bottom center into each well. Blue litmus paper turns red under acidic conditions below pH = 4.5 (**Supplementary Figure S1**, and **Supplementary Material**). The cotton wool in the wells was then soaked with 3 ml of either of the four treatment solutions in a random fashion to four wells on a plate, i.e., sterile control, untreated soil, autoclaved soil, and microbe-enriched soil. For the sterile control we used autoclaved tap water. To the fifth well with cotton wool we added 3 ml autoclaved tap water as a control for humidity effects on the litmus paper. Into the sixth well we put a blue litmus paper without anything else as a reference. No discoloration of the litmus paper was seen in both of these control wells. To minimize the handling effect and to avoid excessive formic acid use while placing the queens into the wells, they were immobilized on ice for 30 min. Then we placed queens individually into a well ($N = 200$) and put them into a

climate chamber at 22°C and 70% humidity and kept them under constant darkness.

We checked every week for queen survival, brood production and brood development until the first workers hatched. We also took a picture (Olympus Pen F) of the blue litmus paper every week until the end of the experiment. Once a week we also added 1 ml of autoclaved tap water to the cotton wool.

## Statistical Analyses

All statistical analyses were performed using R 3.6.1 (R Core Team, 2019). Survival data was analyzed with a Cox proportional hazard regression (COXPH) with environmental challenge (sterile control, autoclaved soil, untreated soil, or microbe-enriched soil) as predictor (package "survival", Therneau and Grambsch, 2000). The proportion of queens producing workers and the number of workers produced in the founding experiment (before hibernation) was analyzed in two separate models (generalized linear model, GLM, with binomial errors for the proportional data – package *lme4*, Bates et al., 2015 – and zero-inflated generalized linear model for the count data – package glmmTMB, Brooks et al., 2017) with environmental challenge as a predictor. The proportion of queens producing brood after hibernation was similarly analyzed with environmental challenge as a predictor in a GLM with binomial errors. Only queens surviving until the initiation of brood production in the founding experiment after hibernation were used for this analysis and separate models were constructed for the different brood types (eggs, larvae, pupae, and workers). The proportion of queens externalizing formic acid during colony foundation was analyzed in a generalized liner mixed model (GLMER, package *lme4*, Bates et al., 2015) with binomial errors, environmental challenge, time in weeks and their interaction as fixed predictors and a random effect with random slopes for queen and random intercepts for time in weeks accounting for the repeated measure of formic acid use over time for each queen. Only data from the first 4 weeks was analyzed as upon week four workers started to eclose. These workers can externalize their own formic acid and thus bias the results of formic acid use of queens in the following weeks. To assess significance of predictors in all analyses models were compared to null (intercept only) or reduced models (for those with multiple predictors) using Likelihood Ratio (LRT) or Chi-square tests. Pairwise comparisons between factor levels of a significant predictor were performed using pairwise post-hoc tests adjusting the family-wise error rate according to the method of Westfall (package *multcomp*, Bretz et al., 2010). Model assumptions of all (zero-inflated) generalized linear and mixed models were checked using model diagnostic tests (overdispersion and zero-inflation) and plots (qq-plot and residual vs. predicted plot) (package DHARMa, Hartig, 2019).

# RESULTS

Before hibernation until day 35 of the founding experiment, mortality among queens was under 20% for all environmental challenges (sterile control: 10%; autoclaved soil: 4%; untreated soil: 6%; microbe-enriched soil: 16%). Mortality however, rose to a maximum of 62% for founding queens in microbe-enriched soil until the end of hibernation on day 221–225 (sterile control: 46%; autoclaved soil: 34%; untreated soil: 46%) and thereafter. Overall the environmental challenge of microbe-enriched soil led to a significantly lower survival of founding queens in the founding experiment, while the other environmental challenges did not differ between each other (**Figure 1** COXPH, overall LR-test, $\chi^2 = 9.687$, $df = 3$, $P = 0.021$, post-hoc Tukey comparisons: microbe-enriched soil vs. all other treatments: $P < 0.048$, all other comparisons: ns). This indicates a survival cost of queens founding a new colony in microbe-enriched soil.

Before hibernation until day 83 the proportion of queens producing workers was lowest in the sterile control treatment (66%), followed by the microbe-enriched soil (70%), untreated soil (76%) and autoclaved soil (84%) treatment with no significant differences between treatments (**Figure 2A**, GLM, overall $\chi^2$-test, Deviance = -4.958, $df = 3$, $P = 0.175$). A similar pattern was observed for the number of workers produced, but with queens in the sterile control treatment producing a significantly lower number of workers than queens in the untreated and the microbe-enriched soil treatment (**Figure 2B**, glmmTMB, overall $\chi^2$-test, $\chi^2 = 8.164$, $P = 0.043$, post-hoc Tukey comparisons: sterile control vs. untreated soil and microbe-enriched soil: $P = 0.049$, all other comparisons: $P > 0.059$). After hibernation, the proportion of surviving queens (untreated soil: 21, autoclaved soil: 21, microbe-enriched soil: 12, sterile control: 24) producing brood successively declined with advancing brood development (**Figure 3**) but was not significantly affected by environmental challenge (eggs: GLM, overall $\chi^2$-test, Deviance = -1.775, $df = 3$, $P = 0.9811$; larvae: GLM, overall $\chi^2$-test, Deviance = -0.076, df = 3, $P = 0.995$; pupae: GLM, overall $\chi^2$-test, Deviance = -7.484, $df = 3$, $P = 0.058$; worker: GLM, overall $\chi^2$-test, Deviance = -4.694, $df = 3$, $P = 0.196$).

The use of formic acid during colony founding was not significantly influenced by environmental challenge (**Figure 4**; GLMER; interaction treatment × time: $\chi^2 = 3.551$, $df = 3$, $P = 0.314$; time: $\chi^2 = 0.089$, $df = 1$, $P = 0.765$; treatment $\chi^2 = 2.598$, $df = 3$, $P = 0.458$). However, the use of formic acid followed a conspicuous pattern over time. Formic acid use peaked, except for the sterile control, in week two (sterile control: 10%; autoclaved soil: 7%; untreated soil: 9%; microbe-enriched soil: 19%) and declined in the weeks thereafter. This pattern was most pronounced for queens founding in microbe-enriched soil and appeared to be generally linked to brood development, specifically the appearance of larvae.

# DISCUSSION

In this study, we investigated how microbial pathogen pressure in the environment affects colony founding success of *Lasius niger* ant queens. We tested whether the need to simultaneously invest into immune defense and the production of the first cohort of offspring comes at a substantial cost to the queens and whether the use of formic acid as external immune defense trait explains part of the costs imposed on queens. Irrespective of the microbial pathogen pressure in the environment, queens showed a high

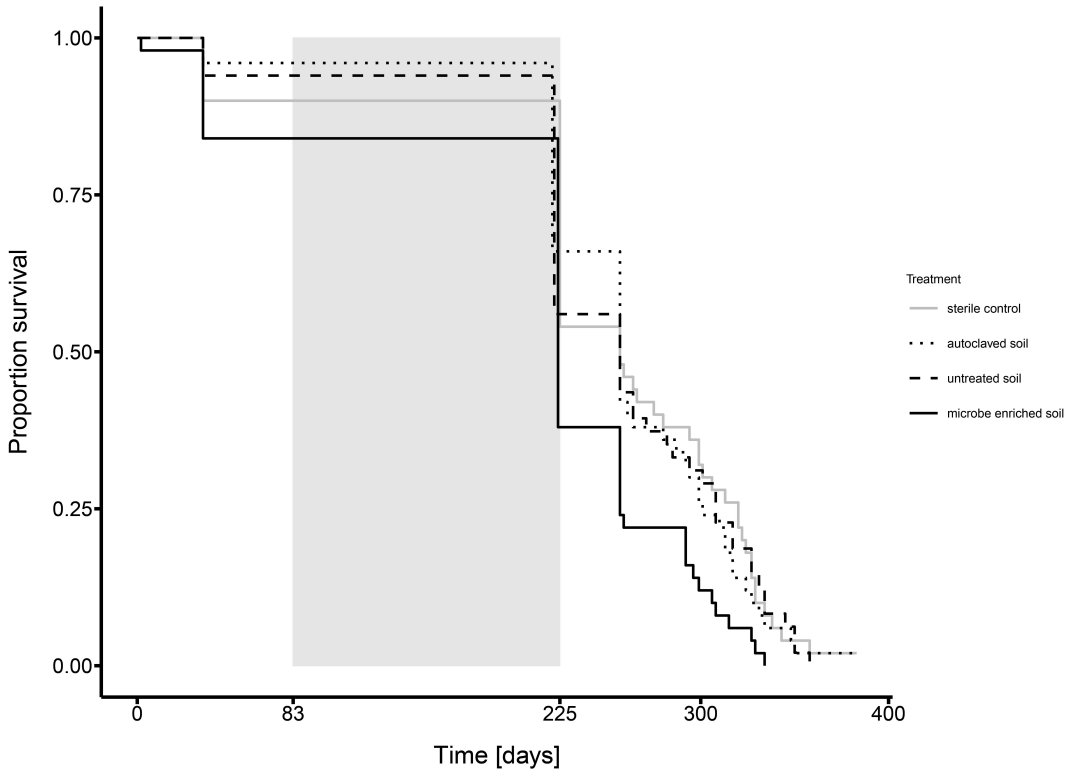


**FIGURE 1 |** Survival of queens founding a colony under different environmental conditions over the complete experimental period (gray solid line: sterile control, dotted line: autoclaved soil, dashed line: untreated soil, solid black line: microbe-enriched soil), i.e., from the start of the founding experiment until the end of the experiment, with the time of hibernation (day 83 to 221–225 depending upon treatment, see materials and methods) indicated by the gray rectangle.

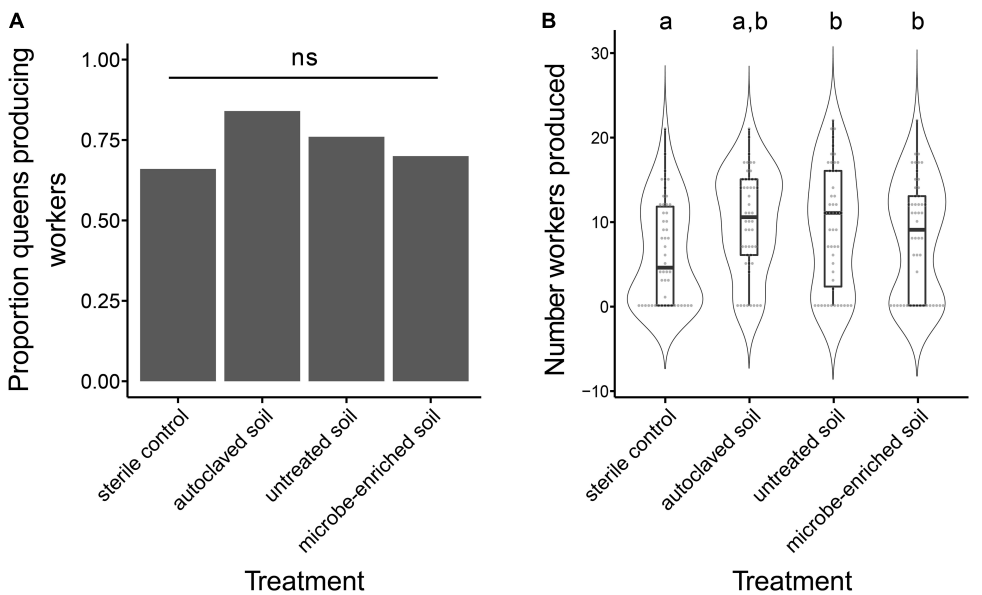


**FIGURE 2 |** The proportion of queens producing workers **(A)** and the number of workers produced **(B)** before hibernation for the different environmental treatments (sterile control, autoclaved soil, untreated soil, and microbe-enriched soil) in the founding experiment. Bars represent the proportion of queens producing workers while boxplots show the median number of workers, as well as the upper and lower quartiles, with whiskers encompassing 1.5 times the interquartile range. Violins around the boxplots show the probability density of the data and gray points show the distribution into discrete bins. Small letters indicate statistically significant different groups at $\alpha = 0.05$, while ns indicates non-significant groups.

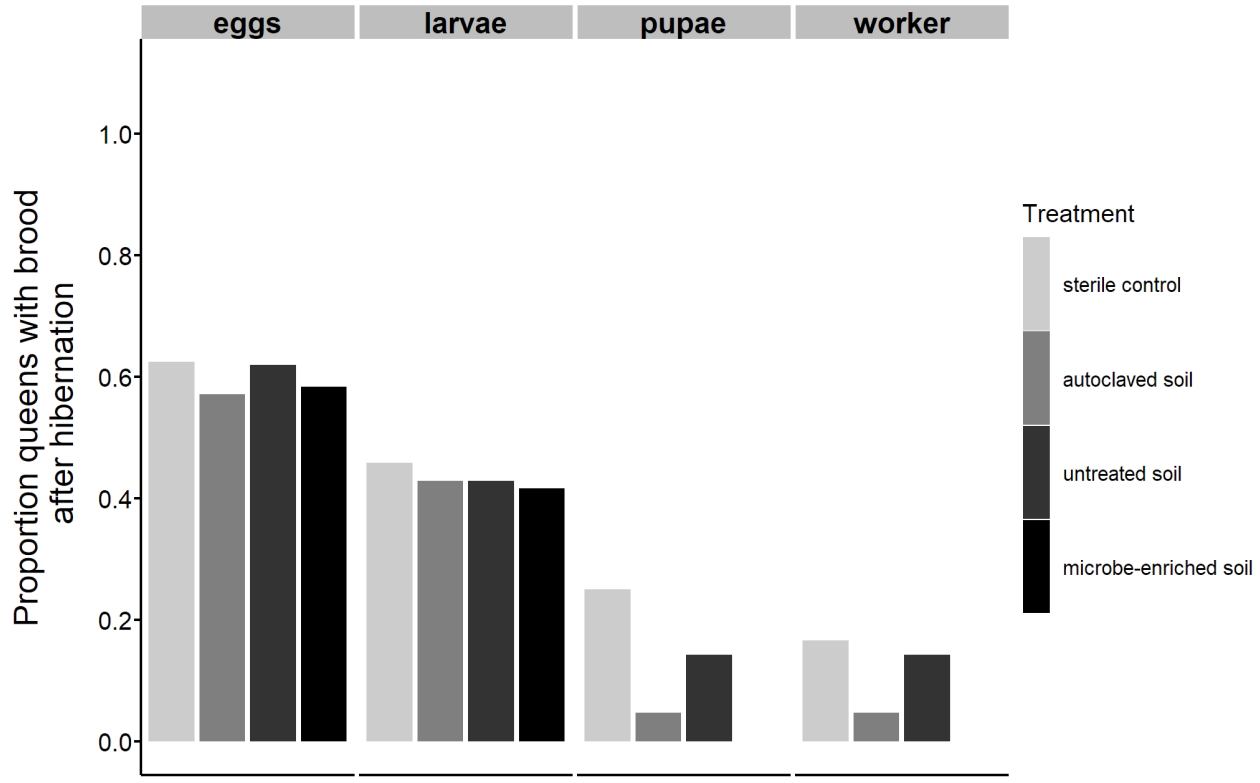


**FIGURE 3 |** The proportion of queens producing brood after hibernation in the founding experiment. Bars represent the proportion of queens producing eggs, larvae, pupae, and workers under different environmental conditions (sterile control, autoclaved soil, untreated soil, and microbe-enriched soil). Only queens surviving hibernation are taken into account.

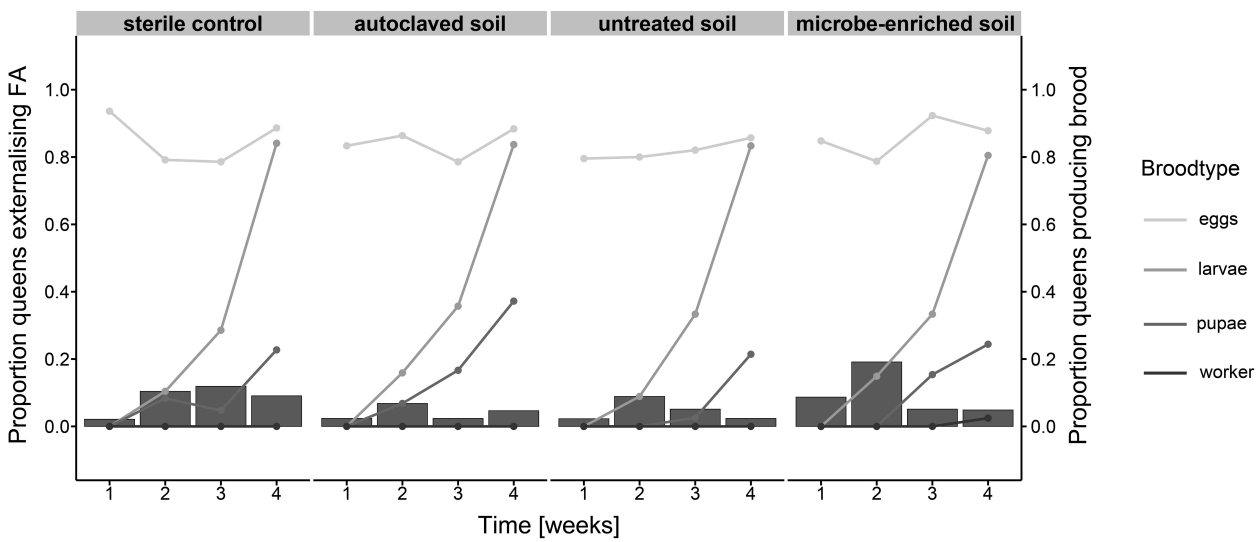


**FIGURE 4 |** The proportion of queens externalizing formic acid (FA) in their environment (bars) and producing brood (points with connecting lines with gray shades from light gray to black for eggs, larvae, pupae, and workers respectively) over the course of 4 weeks under different environmental conditions (sterile control, autoclaved soil, untreated soil, and microbe-enriched soil).

success rate in founding a new colony, indicated by the high proportion of queens producing workers. While no significant differences were found in the proportion of queens producing workers, the number of workers produced was significantly lower in the sterile control treatment than the other treatments. In all experimental environments, 2–19% of queens (depending on week) invested in formic acid as an external immune defense to sanitize their nest. Together, we take this as evidence that no trade-off between colony growth (reproduction) and immunity (Calleri et al., 2007; Schwenke et al., 2016; Cole et al., 2018) is present in *L. niger* founding queens. Queens invested in both, brood production and nest sanitation. This "mixed" strategy comes at a significant cost to queens under microbial pressure. We found that queens in microbe-enriched soil experienced a significantly lower survival during and after hibernation when forced to produce a second batch of brood. Other long-term or potentially hidden costs induced by the loss of the first batch of brood after hibernation could not be detected in queens surviving hibernation, because the proportion of queens producing the second batch of brood did not differ between environmental treatments. However, as our experimental design did not include incipient colonies without a brood loss after hibernation, we are unable to disentangle the relative contributions of microbial challenge and brood loss on survival patterns, respectively. Thus, we cannot completely rule out the existence of hidden costs induced by the loss of the first batch of brood after hibernation.

The mixed strategy of investing in both colony production and immunity represents an alternative in addition to prioritize either colony growth or immunity (Morris, 1987; Schwenke et al., 2016; Duffield et al., 2017). Mixing the investment in reproduction and immunity was recently also found in termites (Cole et al., 2018). There, microbial stress reduced the survival of termite kings and queens, the likelihood of oviposition and total egg number. The onset of oviposition or egg quality did not change in the face of disease, indicating that termite queens choose to maintain offspring quality over quantity (Cole et al., 2018). Similar to termite kings and queens, we suggest that *L. niger* queens maintain a high reproductive output and a high external immune investment in the face of microbial pressure in the environment. They do this despite the potential costs of lower survival. The strategy of *L. niger* queens makes intuitive sense, because once a colony with the first cohort of workers is established, workers will forage for food alleviating the energetic costs of reproduction and immunity on queens (Hölldobler and Wilson, 1990). In addition, once a colony is established the cost of external immune defense via the use of formic acid is partitioned up among workers, alleviating the cost of external immune defense in microbe-rich environments.

It is likely that queens in microbe-enriched soil must invest heavily in their own protection during colony foundation. Thus, further studies might still uncover a trade-off between colony growth and immunity investigating the investment in internal immune system activation of the queen in a similar experiment. Another possibility is that this trade-off is not as bilateral as it seems. The evidence suggests that the investment in colony growth, immunity and in the insurance against unexpected challenges, i.e., the loss of the first brood, are traded off against each other. Queens invested equally in growth regardless of pathogen level. However, the queens surviving an immune challenge seem to have fewer resources left over as an insurance and are thus unprotected from an unexpected failure, while some unchallenged queens could produce a second brood. Ant queens may thus be trading off insurance against later challenges for increased pathogen immunity. But, as previously mentioned, future studies will have to disentangle the relative contribution of stressors, i.e., microbial challenge and loss of the first brood, to the costs imposed on founding queens.

Interestingly, our experiments revealed that queens in the sterile environment treatment showed the lowest probability of producing workers and also produced significantly fewer workers than queens in untreated and microbe-enriched soil. This might indicate a general positive effect of soil presence. It has been proposed that the spinning of a cocoon by ant larvae requires small particulate matter (Wheeler, 1910). Therefore, small particulate soil material might have been an underestimated beneficial factor in our experimental design. Alternatively or complementary to this, microbes naturally occurring in the soil might provide an as yet unknown and undescribed benefit to founding queens and the first cohort of brood. Ants entertain a variety of interactions with microbes spanning the continuum of symbiotic, mutualistic and parasitic interactions (Chomicki and Renner, 2017; Russell et al., 2017), which are embedded in a wider microbial community including the microbial community of an individual but also free-living microbial communities in the environment of an individual (Dittmer et al., 2016; Adair and Douglas, 2017; Brinker et al., 2019a). Therefore, the right microbial environment might be very important to ants. Indeed it has been found that ants often influence microbial communities surrounding them, causing a microbial shift between nest soil and soil adjacent to their nest (Brinker et al., 2019b and references therein). The importance of the microbial community in the environment might also explain the use of formic acid by queens in all our experimental environments, as it might not only function to sanitize the nest but might represent an external immune defense trait as originally defined, i.e., a trait acting outside an organism improving protection from pathogens or manipulating the composition of the microbial community in favor of the organism (Otti et al., 2014).

Over the first 4 weeks of colony founding, we also found that the changes in the use of external immune defense between the treatments showed a conspicuous pattern. The use of formic acid increased until week two (week three for the sterile control), followed by a decline over several weeks. Queens in the microbe-enriched soil treatment showed the largest increase and decrease in the use of formic acid, suggesting they were very limited in their use of external immune defense. Also, week two approximately coincides with the appearance of the first larvae. This pattern might indicate an adaptive use of formic acid as external immune defense according to brood developmental stage. It has recently been argued that pupae in cocoons might be less susceptible to the negative effects of formic acid (Pull et al., 2018). An adaptive use of formic acid as external immune defense according to brood developmental stage would therefore make sense. We would argue that the observed pattern indicates that

the environment and the development status of the colony can both define the investment in external immune defense.

However, other mechanisms might also be at play here. Challenged by microbial pressure during colony founding, queens could benefit from immune priming their worker offspring (Moret and Schmid-Hempel, 2000; Sadd et al., 2005). Ant colonies normally stay in the same location over the years. Therefore, they are likely to repeatedly encounter the same or similar pathogens and trans-generational immune priming (Gálvez and Chapuisat, 2014; Roth et al., 2018) could be a beneficial strategy to assure the successful establishment of a strong and healthy colony. Indeed, several studies on colony founding, migration and nest building have shown a high preference of pathogen rich nesting sites compared to uninfected sites (founding: Brütsch et al., 2014, immigration: Pontieri et al., 2014, nest-structure: Leclerc et al., 2018), though the evidence for the existence of transgenerational immune priming in ants is currently mixed (Bordoni et al., 2018; Fuchs et al., 2018).

In our experimental setup, costs incurred by queens were discovered under microbial pressure and by enforcing an additional stressor, i.e., the removal of the first brood. This raises the rather interesting question whether in the absence of the additional stressor colonies could have fully recovered (Bordoni et al., 2017) or if the microbial pressure at the start of the colony cycle would have led to a shorter colony lifespan. It could well be that once foraging workers are present in the colony, resource costs paid early in life can be compensated by the work force. However, successful colony founding does also depend on the quality of workers. Under microbial stress queens might produce workers of low quality (Smith and Fretwell, 1974; Negroni et al., 2016), which would only delay the crash of a colony. More studies are needed, investigating the general quality of workers (e.g., body size, fat content or foraging efficiency) and in more detail the immune potential and internal immune system activation of the queen. Because our results suggest that early, simultaneous investment in reproduction and immunity can allow growth under a microbial challenge but may be costly in terms of resistance to later challenges, only long-term studies of colony development will be able to reveal the long-term/total costs of an early life investment in multiple life history traits.

## DATA AVAILABILITY STATEMENT

Data underlying the study are included in the **Supplementary Material**.

## AUTHOR CONTRIBUTIONS

ST, PB, and OO conceived the study, designed and performed the analysis, and wrote the manuscript. ST, PB, NR, and OO supervised and participated in data collection. NR contributed substantially to revisions. All authors read and approved of the final manuscript.

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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.2020.00076/full#supplementary-material

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# Inhibition of a Secreted Immune Molecule Interferes With Termite Social Immunity

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Social immune behaviors are described in a great variety of insect societies and their role in preventing emerging infectious diseases has become a major topic in insect research. The social immune system consists of multiple layers, ranging from the synthesis of external immune molecules to the coordination of individual behaviors into sophisticated collective defensive tasks. But our understanding of how complex group-level behavioral defenses are orchestrated has remained limited. We sought to address this gap in knowledge by investigating the relationship between the external activity of an important immune effector molecule in termites, Gram negative binding protein 2 (GNBP-2) and collective grooming and cannibalism. We reasoned that as an external enzyme capable of degrading entomopathogenic fungi, GNBP-2 can facilitate the spread of pathogenic molecules in the colony, and thus serve to trigger collective defenses in a manner analogous to pathogen-associated molecular signatures (PAMPs) of the individual immune system. To test whether GNBP-2 could play a role in regulating social immune behavior, we experimentally inhibited its fungicidal activity using the glycomimetic molecule, D-d-gluconolactone (GDL) and recorded collective behavioral responses to an infected nestmate. Contrary to expectations, GNBP-2 inhibition did not influence the rate or intensity of grooming of either control or fungus-infected nestmates. By contrast, we found that the probability of being harmed through defensive cannibalistic behaviors was significantly reduced by the inhibition of GNBP-2. Our findings indicate that the regulation of collective immune behaviors may depend in part on the external secretion of an enzyme originating from the individual immune system, but that other cues are also necessary.

**Keywords: termite, social immunity, cannibalism, hygienic behavior, GNBP-2, GDL, Metarhizium, entomopathogen**

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## INTRODUCTION

The evolutionary and ecological success of social insects can in large part be attributed to the evolution of division of labor. However, sociality also poses specific disadvantages, including increased exposure of colonies to infectious diseases (Richard, 1974; Cremer et al., 2018). The apparent disease susceptibilities associated with social live have imposed significant selection

pressures on social insects to regulate the emergence and spread of disease (Schmid-Hempel, 1995; Cremer et al., 2007; Meunier, 2015). This may help to explain why epizootics that can kill entire social insect colonies are in fact quite rare (Chouvenc and Su, 2012; Schmid-Hempel, 2017). Social insect individuals are able to limit infection using their individual immune systems (Cotter and Kilner, 2010; Meunier, 2015) but they have also evolved a variety of collective disease defenses to mitigate the occurrence and dissemination of infectious diseases (Cremer et al., 2007; Wilson-Rich et al., 2009) including both behavioral and physiological adaptations (Cremer et al., 2018; Bulmer et al., 2019; Liu et al., 2019). Social actions resulting in the control or elimination of infections are examples of "social immunity." Social immunity combines defenses exhibited by the host with defenses that can be generated by surrounding relatives (Van Meyel et al., 2018). Social immunity has been termed a key property of social system evolution (Cremer et al., 2018), although a unique link between social immunity and true sociality has recently been questioned (Van Meyel et al., 2018).

Despite growing interest in the study of social immunity, we remain far from understanding how collective defensive behaviors are regulated. This is partly because social immunity represents a "distributed organ" that is comprised of a diverse array of defensive traits. For example, externally-secreted molecules derived from the individual immune system, such as toxins, acids and peptides often operate in conjunction with collective behavioral responses to protect groups against infection (Hamilton et al., 2011; Otti et al., 2014), with such molecules likely serving a critical role as a primary barrier to infection (Zasloff, 2002). In ants, termites as well as other social insect groups, behavioral defenses can be supplemented with the secretion and spread of antimicrobial substances onto body surfaces, where they function as a potent external disinfectants (Hamilton et al., 2011; López-Riquelme and Fanjul-Moles, 2013; Otti et al., 2014; He et al., 2018; Pull et al., 2018). Termites in particular can deploy a wide repertoire of social immune responses including alarm behaviors, avoidance, prophylactic, or antimicrobial secretions, burial of dead bodies, necrophagy, mutual grooming, and cannibalism (Rosengaus et al., 1998, 1999, 2011; Yanagawa and Shimizu, 2007; Chouvenc et al., 2008; Chouvenc and Su, 2010; He et al., 2018; Bulmer et al., 2019). Antimicrobial secretions in termites are produced by sternal as well as head glands, and can include antimicrobial compounds found in rectal fluids and feces (Rosengaus et al., 2011; Bulmer et al., 2019).

Termites therefore represent an excellent eusocial model for studying the evolution and function of animal immunity and sociality. However, understanding when and why different collective defenses are deployed in response to an infectious disease threat remains a significant challenge to research. We recently showed that termites can employ a range of collective responses depending on the individual's progression along the stepwise-infection process (Davis et al., 2018). But we do not understand the underlying mechanism(s) responsible for regulating the point at which these different responses are deployed. Here, we chose to examine whether immune components synthesized and secreted by individuals could play an important role in regulating group-level behavioral responses to disease. Specifically, we focus on the role of the Gram-negative bacteria binding protein 2 (GNBP-2), which alongside the peptide Termicin, has received particular attention in termite immunity research (Lamberty et al., 2001; Yuki et al., 2008; Bulmer et al., 2009). Termicins are a class of antimicrobial peptides (AMPs) with strong antifungal activity, while GNBP-2 belongs to a class of bifunctional pattern recognition receptors (PRRs) that can recognize lipopolysaccharide (LPS) of Gram-negative bacteria and β-1,3-glucans of fungal cell walls (Bulmer et al., 2009; Hamilton and Bulmer, 2012). These proteins were first described in *Nasutitermes* (Bulmer et al., 2009) and later in *Reticulitermes* (Hamilton et al., 2011; Hamilton and Bulmer, 2012). The β-1,3-glucanase activity of termite GNBP-2 can protect termites against lethal infection by damaging conidia cell walls and thereby inhibiting germination (Rosengaus et al., 2014). GNBP-2 has been found on the insect cuticle after allogrooming as well as in nest materials, where it is likely to provide protection against generalist pathogenic fungi found in the colony environment (Bulmer et al., 2009; Hamilton et al., 2011; Hamilton and Bulmer, 2012). GNBP-2 mediated release of digested β-1,3-glucans or other fungal cell components into the nest environment could help to prevent the spread of infection by eliciting an immune response in (and thereby priming the immune defenses of) uninfected nearby termites (Bulmer et al., 2009; Hamilton and Bulmer, 2012). In addition to putative roles in termite external immunity, GNBP-2 is known to occur in the termite alimentary canal where it may act as an internal disinfectant and serve to regulate gut symbiosis during digestion (Yuki et al., 2008).

While inactivation of GNBP-2 results in suppressed immune defenses at the individual level, its involvement in collective behavior is unknown. We hypothesize that by facilitating the degradation and spread of fungal cell wall components, GNBP-2 could act as a signal amplifier within the colony, serving to recruit nestmates to the source of infection, and therefore acting as an important molecular cue for collective defensive behavior. We test whether GNBP-2 can act as a molecular mediator of collective defense behavior by experimentally inhibiting the β-1,3-glucanase activity with D-d-gluconolactone (GDL) (Bulmer et al., 2009; Hamilton et al., 2011) and recording collective behavioral responses to nestmates infected with the fungal entomopathogen *Metarhizium anisopliae*. Entomopathogenic fungi such as *Metarhizium* have been important microorganisms in the study of insect social immunity (Rosengaus et al., 1998; Yanagawa et al., 2008; Konrad et al., 2012, 2018; Chouvenc and Su, 2012). These pathogens infect insects via cuticular penetration, leading to host death and the subsequent production of a large number of infectious spores (Vega et al., 2012; Mora et al., 2017). As facultative pathogens, they are widespread in the environment surrounding insect colonies (Cremer et al., 2018). We selected *M. anisopliae* for use in our experiments as it is a natural pathogen of termites including *Reticulitermes flavipes* (Zoberi, 1995; Dong et al., 2007) and has served as an effective model entomopathogen in the study of virulence and termite immune defense (Chouvenc et al., 2009; Chouvenc and Su, 2010; Hamilton and Bulmer, 2012; Davis et al., 2018).

# METHODS

## Insect Hosts

Three *R. flavipes* colonies were used in experiments: colonies 11 + 13, 10, and X. Pieces of wood containing dense aggregations of termites belonging to these colonies were collected from the field. Colonies 11 + 13 and 10 were collected in Île d'Oléron, France, in 1999 and 1994 respectively and maintained in a dark room at 26°C, 84% humidity. Colony X was collected in 2015 in Soulac-sur-Mer, France. It was maintained in a dark room at 28°C, 83% humidity. Primary reproductives of *R. flavipes* can live to 18 years in the wild and up to 25 years in captivity (Lainé and Wright, 2003). Furthermore, secondary reproductives, which can breed amongst themselves, frequently replace primary reproductives in both native and invasive populations of this species, meaning that high levels of inbreeding are not uncommon in *R. flavipes* (Vargo and Husseneder, 2009). Colonies were kept in separate sheet metal tanks as described by Becker (1969) and had access to wood as well as sufficient damp soil to burrow. Cardboard baits were used to extract termites from their parent colonies according to Tracy (2003). After collection, we maintained termites derived from the same colony inside plastic boxes containing cellulose pads (Pall Corporation, Port Washington, United States) that had been moistened with tap water. Collected termites were kept at the same temperature as the parent colony until they were used for the experiment.

## Preparation of Petri Dish Nests

The Petri dish nest was built as described elsewhere (Davis et al., 2018) to house experimental mini-colonies of *R. flavipes*. The petri dish experimental nest (94 × 16 mm) contained two cellulose pads (45.5 mm diameter, 0.9 mm thick) (Pall) which were placed on top of the two thin filter paper disc Whatman No. 5 (47 mm diameter, 0.2 mm thick). A standard microscope slide made of glass (76 × 26 mm) was then placed on top of all the filter papers. In every Petri dish, we introduced 49 healthy termites (not including the focal individual): 48 medium-to-large workers (3–5 mm body length) and one soldier. Experimental nests were sealed with parafilm to maintain a high level of humidity within petri dishes, and left in a dark room at 27°C and 70% humidity for 15 days to enable the termites to establish tunnels under the glass. To ensure a clear view into the nest a cotton swab was used to remove debris from the top surface of the glass 24 h prior to the observation experiment.

## Fungal Conidia Preparation

Preparation of *M. anisopliae* conidia for use in experiments was done following Davis et al. (2018). Briefly, *M. anisopliae* DSM 1490 was maintained on potato dextrose agar (PDA) at 25°C in the dark. The conidia used in experiments were derived from a plate that had undergone a single passage from the frozen stock. Conidia from 15 days old cultures were harvested by scraping off the conidia with a sterile cotton swab moistened with sterile 0.05% Tween 80 and suspending them in sterile 0.05% Tween 80 solution. The suspension was vortexed for 30 s, then filtered through a piece of sterile miracloth (Merck KGaA, Darmstadt, D). Filtering removes hyphae and large clumps of conidia from the suspension. The filtered conidia were centrifuged for 10 min at 5,000 *g* at 4°C and the pellet was resuspended and washed three times with sterile 0.05% Tween 80, with repeated centrifugation (10 min at 5,000 *g* at 4°C) between each washing step. Conidia concentration was estimated in a BLAUBRAND Thoma counting chamber (depth 0.1 mm; BRAND, Wertheim, Germany) and the conidia suspension was adjusted to a final concentration of $1 \times 10^8$ conidia/mL and stored at 4°C until use. Conidia viability following lab culturing was evaluated by streaking with 10 μL of the same $1 \times 10^8$ conidia/mL suspension and incubating at 25°C in the dark. After 21 h of inoculation, at least 300 conidia per plate were evaluated for germination. A conidium was considered germinated if the length of the germ tube was at least half the diameter of the conidium. The germination rate was > 95% for all experiments.

## Infection With Conidia or 0.05% Tween 80

We marked focal termites with Nile blue dye in order to differentiate them from colony nestmates. Nile blue dyeing was carried out following a rapid method for marking termites as described previously (Davis et al., 2018), adapted from Evans (2000). Termite workers were dipped into 2 mL microcentrifuge tubes and a sufficient quantity of 0.025% Nile blue (diluted in distilled water) was added to ensure they were completely covered. Focal termites were gently mixed for 1 min, then tipped out onto a dry Whatman No. 1 filter paper disc (90 mm diameter, 0.18 mm thick). Termites were transferred to small plastic containers, one per colony, each containing cellulose pads moistened with tap water, once they had recovered sufficiently to be able to walk. The plastic containers containing the focal termites were closed with a red tight-fitting lid to prevent desiccation and were left overnight in a dark room at 27°C and 70% humidity. Nile blue-marked termites were immersed in $1 \times 10^8$ conidia/mL suspension for 10 s and then allowed to dry onto a Whatman No. 1 filter paper disc. Infected termites were transferred individually into separate small (35 mm) Petri dishes, each containing a cellulose pad moistened with 1 mL tap water. Control termites were handled similarly but using a conidia-free solution sterile 0.05% Tween 80. The infected and control termites were incubated for 12 h at 25°C before use in the behavioral experiment. This incubation time point was chosen based on a previous study that explored termite collective behavioral responses to termites at different stages of infection (Davis et al., 2018). At 12 h post-infection, the authors recorded significantly elevated levels of allogrooming performed by nestmates, followed by a gradual transition to cannibalism, as the infected termites began to show visible signs of sickness. The 12-h incubation time point therefore represents an optimal stage during *M. anisopliae* infection to measure the impact of treatment on two essential nestmate behaviors (i.e., allogrooming and cannibalism).

## Inhibition of GNBP-2

D-d-gluconolactone (GDL) was used to block the activity of termite gram-negative binding protein (GNBP-2). GDL is a simple, non-toxic and naturally occurring derivative of glucose.

It was prepared to a final working solution of 300 mM GDL and 100 mM sodium acetate (NaOAc), pH 5.0 (Bulmer et al., 2009; Hamilton et al., 2011; Hamilton and Bulmer, 2012). An equivalent control solution containing only 100 mM sodium acetate (NaOAc), pH 5.0 was prepared. GDL or control solution were applied directly in the cellulose pad food source of the nest, with which colony nestmates had direct contact.

## Experimental Design

Briefly, the *R. flavipes* mini-colonies were divided into control and GDL treatments after the 15-day colony establishment period had elapsed. Twenty-four hours prior to the introduction of the focal termites into mini-colonies, the paper food source inside every petri dish nest was moistened with 900 μl of the GDL or control solution. Focal termites were comprised of either control (treated with 0.05% Tween 80) or infected (treated with $1 \times 10^8$ conidia/mL) individuals. Treatments are categorized from here on as follows: GDL+/ Ma−, GDL−/ Ma−, GDL+/Ma+, GDL−/Ma+. For each of the treatments containing *M. anisopliae* there were 15 replicates (five per colony for three colonies) and nine replicates of the control treatments (three per colony for three colonies). We recorded behavioral responses of the experimental colonies to individuals treated with a lethal dose of the entomopathogenic fungus *M. anisopliae* or a Tween 80 control solution. Infected and control termites were added individually to the Petri dish nests and then resealed with parafilm. This took ∼9 min, and the observation period began immediately after the last nest dish was sealed.

## Behavioral Recording

We adopted the scan sampling method used in Davis et al. (2018). This form of instantaneous sampling allows for screening of multiple individuals (Altmann, 1974) and was used to observe the interactions between the focal termite and its nestmates. We recorded behavioral states at a single time-point during each scan of a focal termite in each experimental colony. Treatments were blinded and petri-dish locations were randomized prior to scanning. Scans typically took <1 min during which the location of the focal termite was identified and the observed behavioral state was immediately recorded. Where relevant, the number of groomers was quantified. A Samsung S7 smartphone was used as a digital voice recorder. Scans were performed every 5 min for a total of 3 h. All observations were made at 27°C, 70% humidity under bright, constant overhead light. Experimental colonies were allowed to acclimatize to light for a period of 15 h prior to introduction of focal termites. Behaviors were classified into categories that are relevant to social immunity, and which are visually distinguishable and non-overlapping. As in Davis et al. (2018), we divided these behaviors into five different states:

**Groomed by n:** Focal termite is being groomed by n nestmates with no evidence of biting.

**Cannibalism:** Focal termite is being bitten by one or more nestmates and/or focal termite body is no longer intact.

**Buried:** Focal termite has had pieces of paper or feces placed on top of it. Although increasingly difficult to assess, the termite may still be alive.

**Not visible:** Focal termite is in a part of the nest where it cannot be observed.

**Other:** Focal termite is alive, intact, and unburied, but nestmates are not interacting with it. This reflects behavioral states unrelated to social immunity.

## Statistical Analysis

All statistical analyses were performed using R version 3.6.0.

## Grooming

Grooming amount(number of grooming states/total observed states) was analyzed by fitting a generalized linear mixed model using the glmer function in the R package lme4 v1.1-21 (Bates et al., 2015), employing a binomial error structure to account for proportion data (Crawley, 2014). The model was composed of an interaction between GDL and presence of *M. anisopliae* as a fixed effect, in addition to amount of cannibalism and colony. Petri dish nest ID was used as a random effect. The anova function was used to remove fixed effect parameters that did not lead to a significant alteration in deviance, as well as to perform likelihood ratio test comparisons. The final model was tested for overdispersion in the package blmeco v1.4 using the dispersion gmer function. We carried out *post hoc* pairwise comparisons using the glht function from the multcomp package v1.4-10 with Tukey correction (Korner-Nievergelt et al., 2015a,b).

Grooming intensity was analyzed with glmer to fit a generalized linear mixed model with a Poisson error structure, using total number of groomers in each experimental nest as the response variable. As before, the model was composed of an interaction between GDL and *M. anisopliae* presence as a fixed effect, in addition to amount of cannibalism and colony. Petri dish nest ID was used as a random effect. We logged the number of grooming states, and treated these as an offset to control for the fact that each observed grooming state increased the number of groomers by at least one. As before, we used anova to compare models. Again, *post hoc* pairwise comparisons were performed using glht with Tukey correction.

## Cannibalism

To analyze whether GDL had an impact on time spent cannibalizing (number of cannibalism states/total observed states), we fitted a zero-inflated generalized linear mixed model using the glmmTMB function in the package glmmTMB v1.0.0 (Brooks et al., 2017) employing a binomial error structure to account for proportion data. We restricted our model to GDL+/Ma+ and GDL−/Ma+, owing to insufficient data ($N = 1$ observation of cannibalism) in experimental colonies exposed to control-treated focal individuals (GDL+/Ma− and GDL−/Ma−) and subsequent model convergence issues. The conditional component of the model contained GDL as a fixed effect, in addition to amount of grooming and colony. As before, petri dish nest ID was used as a random effect. The zero-inflation component of the model contained GDL as a fixed effect. Again, we used the anova function to inspect fixed effects, as well as to perform likelihood ratio test comparisons. Although GDL did not

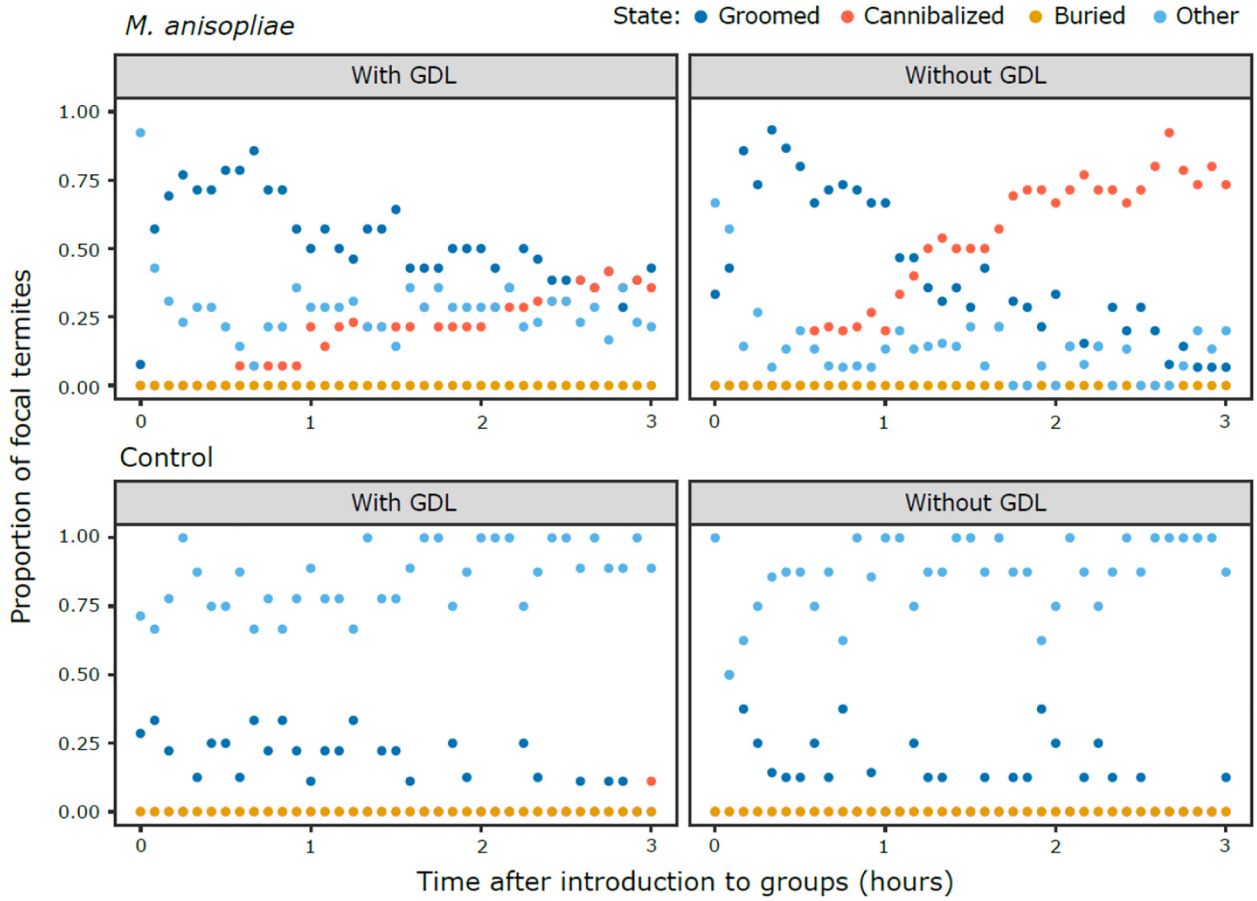


**FIGURE 1 |** Patterns of behavior over the 3 h scan period, showing *M. anisopliae* **(top panels)** and control treatments **(bottom panels)** in groups of nestmates exposed to GDL **(left panels)** or a control solution **(right panels)**. The proportion of focal termites that are observed in a given state at each scan (conducted every 5 min) are represented by a single point. The points overlap when more than one state was present at the same proportion across experimental colonies of a given treatment.

significantly improve the model when it was included as a factor in the conditional component of the model, its inclusion did slightly improve the distribution of residuals, and so was retained in the final model.

## RESULTS

Following the exposure of focal termites to a control Tween 80 solution (Ma−) or *M. anisopliae* (Ma+) and isolating them for 12 h, treated termites were introduced individually to experimental nests that had been exposed to GDL or a control NaOAc solution. Behavioral patterns (**Figure 1**) in the *M. anisopliae* absent groups (GDL−/ Ma−, GDL+/Ma−) were similar regardless of GDL treatment, in that they consisted mostly of behavioral states in the "other" category (states unrelated to social immunity), with low levels of grooming, one incident of cannibalism, and no observations of burial. Behavioral patterns in the GDL−/Ma+ groups after focal termites were introduced into the experimental colonies were characterized by a concentrated phase of grooming. Cannibalism began shortly thereafter, and almost completely replaced grooming before the end of the observation period. The GDL+/Ma+ groups were also typified by initially high levels of grooming, but the intensity of grooming slowly decreased over the course of the observation period. Although cannibalism was also observed this was largely at a lower level than in the GDL−/Ma+ groups and predominantly in the final hour of the scan. We did not observe burial in any of the treatments.

## GROOMING

The amount of grooming was significantly higher in all *M. anisopliae* treatments compared with the controls (*M. anisopliae* treatments vs. corresponding controls: GDL−/Ma+ vs. GDL−/ Ma−, $z = 7.399$, $P < 0.001$; GDL−/Ma+ vs. GDL+/ Ma−, $z = 6.861$, $P < 0.001$; GDL+/Ma+ vs. GDL+/ Ma−, $z = 7.255$, $P < 0.001$; GDL+/Ma+ vs. GDL−/ Ma−, $z = 7.801$, $P < 0.001$) (**Figure 2** and **Supplementary Table S1**). The controls (GDL−/ Ma−, GDL+/Ma−) were not significantly different from each other

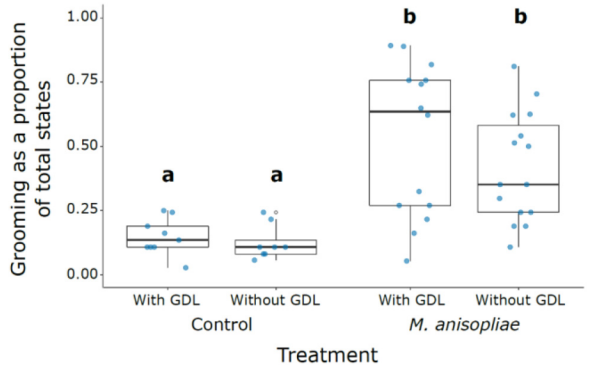


**FIGURE 2 |** Grooming as a proportion of total states across treatments. Different letters indicate treatments that were significantly different following *post hoc* comparison. First and third quartiles are indicated by the lower and upper hinges. Whiskers extend to the smallest/largest value when no smaller/greater than 1.5 times the inter-quartile range from the hinge.

and there was no significant effect of GDL treatment on proportion of grooming states. Low levels of grooming in Ma+ treatments were significantly linked with a high proportion of cannibalism states ($z$ = -5.807, $P < 0.001$) (**Supplementary Figures S1, S2**). The negative relationship between cannibalism and grooming in Ma + treatments may explain the trend towards an increased proportion of grooming in GDL-treated experimental colonies (**Figure 2**). We also found the amount of grooming to significantly depend on colony source, with colony X displaying higher grooming amounts than either of the other two colonies (Colony X vs. 10. $z$ = 2.902, $P < 0.011$; Colony X vs. 13 + 11, $z$ = 2.526, $P$ = 0.031; Colony 13 + 11 vs. 10, $z$ = 0.254, $P$ = 0.965) (**Supplementary Figure S1**). Intensity of grooming (number of groomers) was also significantly higher in all *M. anisopliae* treatments over the controls (*M. anisopliae* treatments vs. corresponding controls: GDL−/Ma+ vs. GDL−/Ma−. $z$ = 3.603. $P < 0.002$; GDL−/Ma+ vs. GDL+/Ma−. $z$ = 3.213, $P < 0.007$; GDL+/Ma+ vs. GDL+/ Ma−, $z$ = 3.676, $P < 0.002$; GDL+/Ma+ vs. GDL−/ Ma−, $z$ = 4.015, $P < 0.001$) (**Supplementary Figure S3** and **Supplementary Table S2**). The controls (GDL−/ Ma−, GDL+/Ma−) were not significantly different from each other and there was no significant effect of GDL treatment on number of groomers. Grooming intensity and number of groomers increased sharply in both *M. anisopliae* treatments following the introduction of focal termites, particularly in the GDL−/Ma+ treatment (**Figure 3**). Ma + groups lacking GDL also exhibited a sharper decline in both intensity and number of groomers over the course of the observation period, as grooming states were gradually replaced with cannibalism (**Figure 1**). In contrast to amount of grooming, high numbers of groomers in Ma + treatments were significantly associated with a high proportion of cannibalism states ($z$ = 2.651, $P$ = 0.008). Higher numbers of groomers were also observed in colony X compared with the remaining two colonies (Colony X vs. 10, $z$ = 2.547, $P$ = 0. 0291; Colony X vs. 13 + 11, $z$ = 3.222, $P$ = 0.004; Colony 13 + 11 vs. 10, z = -0.788, $P$ = 0.71024) (**Supplementary Figures S4, S5**).

## Cannibalism

The probability of being harmed during the observation period following exposure to *M. anisopliae* was significantly reduced by the inhibition of GNBP-2 (zero inflation term, GDL− vs. GDL+, $z$ = 2.218, $P$ = 0.027) (**Figure 4** and **Supplementary Table S3**). Furthermore, amount of cannibalism was negatively associated with amount of grooming ($z$ = -9.053, $P < 0.001$). Cannibalism also varied significantly by colony, with colony 13 + 11 displaying lower amounts of cannibalism than either colonies 10 and X (Colony X vs. 10, $z$ = -0.164, $P$ = 0.985; Colony X vs. 13 + 11, $z$ = 2.744, $P$ = 0.017; Colony 13 + 11 vs. 10, $z$ = -3.041, $P$ = 0.007) (**Supplementary Figures S6, S7**).

# DISCUSSION

GDL treatment resulted in suppression of pathogen-induced cannibalistic behavior. But contrary to our expectations, the amount and intensity of grooming was not influenced by the application of GDL. This indicates that GNBP-2 glucanase activity can stimulate the transition from intense grooming to cannibalism but appears not to play a major role in the initial stimulation of grooming or in acting to recruit more groomers. Yanagawa et al. (2011) found that the filtrate from a suspension of *M. anisopliae* conidia was enough to induce grooming in *Coptotermes formosanus*, suggesting that grooming can be induced by the presence of fungal pathogen-associated molecular signatures (PAMPs). Interestingly, the same study did not detect any evidence of enhanced cannibalism, indicating that these are behaviors induced by signals released after infection. Davis et al. (2018) confirmed this suspicion by showing that defensive cannibalism only takes place once an infection has yielded an explicit sickness response in the termite host. In the same study, grooming was found to increase after conidia had begun to germinate, becoming even more elevated once hosts began to display signs of sickness. Similarly, in a study on ants, Pull et al. (2018) showed that pupae-derived chemical cues are used by workers to target infected pupae for destruction with poison spraying. These findings suggest that fungal PAMPs in combination with host-derived stimuli drive both grooming and destructive disinfection behaviors in social insects, as well as regulating the transition between these states. The data from the current study suggest that while GNBP-2 is unlikely to be the main mechanism by which termites detect fungal PAMPs, its activity can nonetheless influence collective behavior once the host has become sick, potentially via the release of fungal PAMPs from damaged host cuticle. It is possible that GNBP-2 inhibition does not strongly discourage grooming because termites could employ a variety of host and/or pathogen-derived signals, involving behavioral, chemical or even oscillatory cues (e.g., body vibrations) to initiate collective defense tasks (Rosengaus et al., 1999; Wilson-Rich et al., 2007; Zhukovskaya et al., 2013; Davis et al., 2018; Bulmer et al., 2019). In this scenario, although GNBP-2 activity may itself accelerate the transition from a caring to a killing response, it represents just one component of a complex repertoire of social immune mechanisms that termites could use to regulate infectious threats exposed to the colony. Given

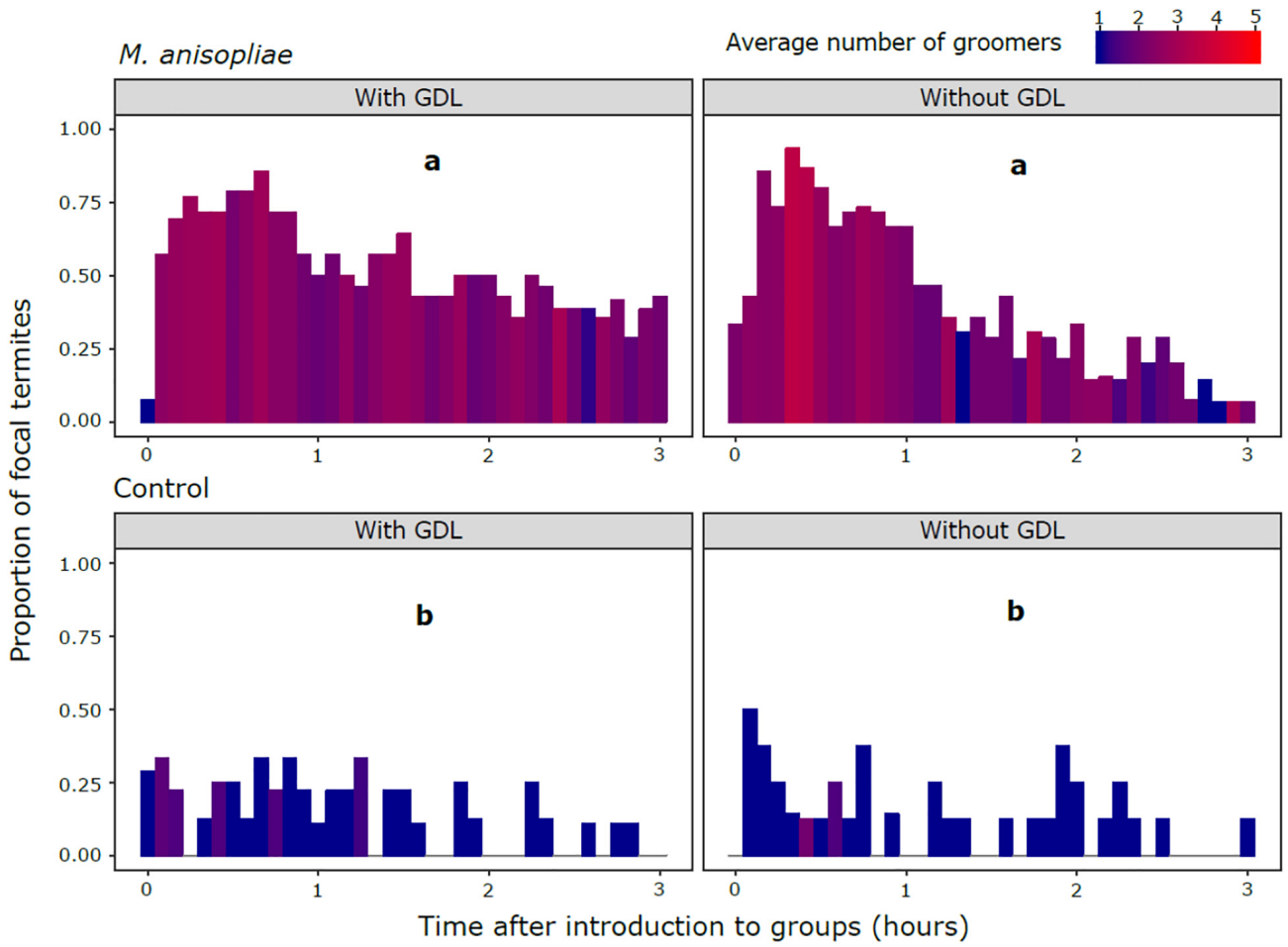


**FIGURE 3 |** Proportion of focal termites observed being groomed by nestmates in each scan. *M. anisopliae* **(top panels)** and control treatments **(bottom panels)** in groups of nestmates exposed to GDL **(left panels)** or a control solution **(right panels)**. Different letters indicate treatments in which overall number of groomers were significantly different following *post hoc* comparisons, after accounting for number of grooming states. Color of the fill represents the average number of groomers at each time point.

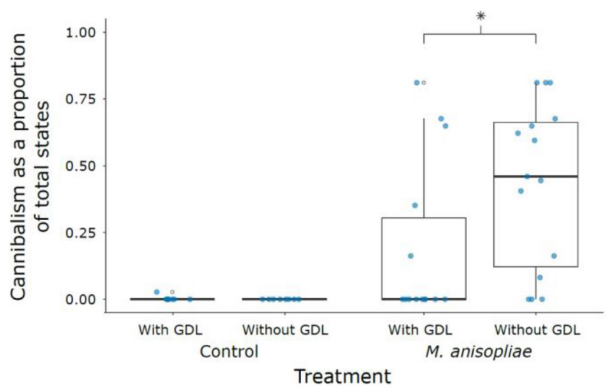


**FIGURE 4 |** Cannibalism as a proportion of total states across treatments. Absence of cannibalism in *M. anisopliae* groups depends significantly on GDL treatment (indicated by an asterisk). First and third quartiles are indicated by the lower and upper hinges. Whiskers extend to the smallest/largest value when no smaller/greater than 1.5 times the inter-quartile range from the hinge.

the additional function of GNBP-2 as an internal disinfectant and putative regulator of gut symbiosis, the observed behavioral shift could represent an individual feedback response linked to disrupted digestion, rather than as a regulator of social immunity. However, it is also conceivable that GNBP-2 could fulfill both functions simultaneously.

While many studies underline the importance of collective defenses in preventing pathogen infection in termite colonies (Rosengaus et al., 1998; Traniello et al., 2002; Yanagawa and Shimizu, 2007; Zhukovskaya et al., 2013) this is to our knowledge the first to show a link between an immune molecule and collective behavioral defense. Social immune behaviors are described in several insect societies and their role in preventing emerging infectious diseases is now an established field of research (Cremer et al., 2007, 2018; Cotter and Kilner, 2010; Meunier, 2015; Kennedy et al., 2017). In addition to representing an effective model for social immunity research, our study highlights the importance of termites as a key comparative lineage to the social Hymenoptera, particularly ants, which have

been a favored model for investigations into social immunity (Hughes et al., 2002; Baer et al., 2005; Ugelvig et al., 2010; Reber et al., 2011; Walker and Hughes, 2011; Pull et al., 2018). It would be particularly interesting to understand whether convergent social immune mechanisms have evolved in independent eusocial and superorganismal hymenopteran lineages. An expectation might be that externally secreted antimicrobial compounds or immune molecules can also influence collective hygienic behaviors in such groups, in addition to acting as straightforward external disinfectants.

## CONCLUSION

In recent years, researchers have been trying to understand the relationships between the different layers of the social immune system: from internal physiological defenses, to the secretion of antimicrobial compounds, and culminating in the careful coordination of collective defensive behaviors. These studies are focused mainly on their evolution (Harpur and Zayed, 2013; Otti et al., 2014; Meunier, 2015; Cremer et al., 2018; Van Meyel et al., 2018) or in understanding resource allocation among the different levels of immunity to discover possible trade-offs (Armitage and Boomsma, 2010; Cotter et al., 2013; Rosengaus et al., 2013; Harpur et al., 2014; Gao and Thompson, 2015). Our aim in this study was to experimentally test the functional relationship between these different immune layers, with the specific goal of exploring whether the "care or kill" collective defense response of a termite could be influenced by the inhibition of the fungicidal immune enzyme, GNBP-2. Although GNBP-2 represents just one piece of a larger puzzle, our findings indicate that different components of the social immune system may interact with one another. Our study describes how the orchestration of group-level hygienic behaviors could rely at least in part on relatively simple cues mediated by externally secreted molecules from the individual immune system.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

DM and SM conceived the study. ME-M and HD carried out the experiments. ME-M, HD, and DM analyzed the data. ME-M and DM wrote the manuscript. All authors were involved in editing the manuscript.

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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.2020.00075/full#supplementary-material

**DATA SHEET S1 |** Raw data.

**DATA SHEET S2 |** R code.

**PRESENTATION S1 |** Supplementary Information (**Supplementary Figures S1–S7** and **Supplementary Tables S1–S3**).

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# How Do Leaf-Cutting Ants Recognize Antagonistic Microbes in Their Fungal Crops?

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Leaf-cutting ants employ diverse behavioral strategies for promoting the growth of fungal cultivars in a structure known as fungus garden. As a nutritionally rich resource for the ants, the fungal crop is threatened by microbial antagonists and pathogens. Strategies for protecting the garden against harmful microbes have been described in detail, although the process of microbial threat recognition is not fully understood. Here, we review the literature on leaf-cutting ants' social immunity traits, in search of possibilities by which workers recognize harmful microbes in their system. Based on current data, we suggest mechanisms regarding (1) chemical recognition, where discrimination could be related to chemical cues from the antagonistic microbe or semiochemicals released by the fungus garden during harmful interactions, or (2) through associative learning when workers would connect the microbe cues with a damage in the fungus garden, developing a "colony-level memory" toward this threat. We also discuss evidence supporting ant–fungus communication as key for maintaining the health of the fungus garden, as well as experimental setups for future evaluation of threat detection and recognition by leaf-cutting ants.

**Keywords: attine ants, social immunity, behavioral immunity, communication, diseases**

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## INTRODUCTION

Social behavior evolved in several lineages of insects, ranging from diverse to complex levels of organization (Toth and Rehan, 2017). Across this continuum, some social insects achieved a major transition point of no return, where queen and workers are a lifetime morphological differentiated caste in a superorganismal level of hierarchy (Wheeler, 1911; Boomsma and Gawne, 2018). Social evolution is influenced by several environmental factors, including interactions between insect societies and microbes (Boomsma et al., 2005; Biedermann and Rohlfs, 2017; Toth and Rehan, 2017). By living in dense aggregations of genetically similar individuals, social insects have increased risks of infectious diseases spreading in their colonies (Schmid-Hempel, 1998, 2017; Naug and Camazine, 2002; Cremer et al., 2007, 2018; Rosengaus et al., 2011; Boomsma et al., 2014; Loreto et al., 2014; Stroeymeyt et al., 2014; Meunier, 2015; Cremer, 2019). Selective forces between social insects and pathogenic organisms have modulated defensive strategies, adaptations in physiological traits, behavior, and social organization (Pie et al., 2004; Fernández-Marín et al., 2006; Cremer et al., 2007, 2018; Ugelvig and Cremer, 2007; Yanagawa and Shimizu, 2007; Stow and Beattie, 2008;

Walker and Hughes, 2009; Wilson-Rich et al., 2009; Yanagawa et al., 2011, 2012; Konrad et al., 2012, 2018; Kamhi and Traniello, 2013; Stroeymeyt et al., 2014; Liu et al., 2015, 2019; Quevillon et al., 2015; Malagocka et al., 2019), such as communication (Rosengaus et al., 1999) and caste specialization (Hughes et al., 2003; Brown et al., 2006; Griffiths and Hughes, 2010; Abramowski et al., 2011). Because these defensive adaptations involve the cooperation of the individuals for a colony-level response, they are collectively described as *social immunity* (Cremer et al., 2007). In this context, group members collaborate to avoid, control, or eliminate pathogens, thus acting as parts of an immune system (Cremer and Sixt, 2009; Cremer, 2019).

Social traits are also strongly influenced by interactions between social insects and beneficial microbes, either for defensive or nutritional symbiosis (Biedermann and Rohlfs, 2017). Fungal cultivation by social insects is a remarkable example of an insect–microbe association impacting social behavior (Mueller et al., 2005). The fungus-growing lifestyle independently evolved in the ants of the subtribe Attina (Hymenoptera: Formicidae: Myrmicinae; Mueller et al., 2001), termites in the subfamily Macrotermitinae (Isoptera: Termitidae; Aanen et al., 2002), and the subsocial beetles in the subfamilies Scolytinae and Platypodinae (Coleoptera: Curculionidae; Farrell et al., 2001; Hulcr and Stelinski, 2017). Because of their dependence on the fungal crop for nutritional resources, fungus-growing insects present a series of adaptations for fungal cultivation, maintenance, propagation, and protection (Mueller et al., 2005). Defending the fungal cultivar through chemical and behavioral responses is fundamental to the evolutionary success of the insect–fungal symbiosis, as the crop is a nutritionally valuable resource susceptible to microbial competitors and pathogens (Bass and Cherret, 1996; Currie et al., 1999a; Mueller et al., 2005; Morelos-Juárez et al., 2010; Visser et al., 2011; Um et al., 2013; Beemelmaans et al., 2017; Biedermann and Rohlfs, 2017). Thus, traits of social immunity in fungus-growing systems could have evolved targeting both insect hosts and the fungal crops.

The complex microbial environment of leaf-cutting ants, the most derived clade in the subtribe Attina, provides an interesting perspective for investigating how the responses to both harmful and beneficial microbes could have influenced ants' social immunity (Biedermann and Rohlfs, 2017). Leaf-cutting ants have an obligate association with the basidiomycete species *Leucoagaricus gongylophorus* (Leucocoprini: Agaricales: Agaricaceae), on which all larvae and most of the adult ants feed (Mueller et al., 2005; Schultz and Brady, 2008; De Fine Licht et al., 2013). The maintenance of fungus gardens involves continuous substrate incorporation, which depends on specific behaviors for foraging and processing fresh leaves and flowers (Quinlan and Cherrett, 1977; Hölldobler and Wilson, 1990; Diniz and Bueno, 2010). However, foraging activities bring into the fungus garden several microorganisms along with the plant biomass (Fisher et al., 1996; Rodrigues et al., 2008; Van Bael et al., 2009). Also, mated queens may bring microorganisms during colony foundation on their integuments and in the piece of fungus gardens they carry (Poulsen et al., 2005; Pagnocca et al., 2012; Andersen et al., 2013, 2015; Meirelles et al., 2016). Regardless how they are introduced, once inside the colony, microbes may engage in distinct interactions with the fungal crop, as antagonists (Currie et al., 1999a; Rodrigues et al., 2008) or as mutualists (Poulsen et al., 2005). Ant workers can detect intruders and employ diverse physiological and behavioral strategies to protect the fungal crop (Currie and Stuart, 2001; Poulsen et al., 2002; Fernández-Marín et al., 2006; Abramowski et al., 2011; Gerstner et al., 2011; Rocha et al., 2014, 2017; Tranter et al., 2015; Nilssøn-Moller et al., 2018). It is reasonable to consider that ants may recognize and discriminate beneficial microorganisms from those detrimental to the fungus garden. However, the mechanisms by which leaf-cutting ants carry out these processes are poorly understood.

Here we review the literature for investigating the influence of the leaf-cutting ants' microbial environment on their hygienic behavior. We first present the microbial environment where leaf-cutting ants live and the social immunity traits that evolved to protect the fungal culture and the ants from pathogens in general. Then, we propose two mechanisms by which ants could recognize distinct microbes and apply such defenses: (1) by responding to chemicals or semiochemicals released by microbes and the fungus crop indicating the presence of harmful interactions and (2) by associative learning and memorization derived from recurrent infection events. Through these scenarios, we aim to discuss the potential contribution of the fungal crop to the leaf-cutting ant's social immunity.

## THE MICROBIAL ENVIRONMENT OF LEAF-CUTTING ANTS

All ant lineages in the subtribe Attina cultivate fungus for food, although both the fungal symbiont and the strategies for cultivation vary throughout these fungus-growing systems (Mueller et al., 1998, 2017; Schultz and Brady, 2008; Diniz and Bueno, 2010; Henrik et al., 2014). Attine ants in the genera *Atta* and *Acromyrmex* practice higher leaf-cutting fungiculture cultivating *L. gongylophorus*, a truly domesticated fungal symbiont that seems unable to support a free-living existence (Schultz and Brady, 2008; De Fine Licht et al., 2013; Nygaard et al., 2016; Mueller et al., 2017). The fungal crop is vertically transmitted when the foundress ant queen leaves her original colony carrying a mycelium pellet inside the infrabuccal pocket, which forms the initial crop inoculum (Mueller et al., 2001). The fungal symbiont evolved several adaptations to the symbiotic lifestyle, including swollen hyphal tips (i.e., gongylidia) that provide carbohydrates, amino acids, and enzymes to the ants (Quinlan and Cherrett, 1979; Schultz and Brady, 2008; Mikheyev et al., 2010; De Fine Licht et al., 2013). Leaf-cutting ants nourish the crop using fresh leaves as substrate, ultimately creating a structure known as the fungus garden (**Figure 1**), which is kept within underground chambers for most attine ant species (Hölldobler and Wilson, 1990; Mueller et al., 2001). The lignocellulolytic capacity of the fungus garden has been fundamental for supporting the mutualism (De Fine Licht et al., 2013; Khadempour et al., 2016; Vigueras et al., 2017), allowing the enzymatic conversion of massive amounts of fresh leaves

into nutrients available to the queen, larvae, and most of the ant workers (Hölldobler and Wilson, 1990; Costa et al., 2008; De Fine Licht et al., 2013).

Fungus gardens are nutritionally rich environments (Martin et al., 1969; Huang et al., 2014), harboring a wide diversity of microorganisms including bacteria, yeasts, and filamentous fungi (Currie, 2001a; Rodrigues et al., 2005, 2008, 2011; Sen et al., 2009; Scott et al., 2010; Suen et al., 2010; Aylward et al., 2013). These microbes may access the fungus garden in different ways, such as via the foraged plant material and from the belowground surroundings. Endophytic fungi (fungi for which part of their life cycle takes place within plant tissue) were thought to interact with the fungus gardens as neutral transients (Poulsen and Currie, 2006). However, some authors suggest that these fungi are potential antagonists (nutritional competitors or pathogens) of the fungal crops (Van Bael et al., 2009, 2012; Mighell and Van Bael, 2016). Besides the presence of endophytes, soil-borne fungi in the genera *Fusarium*, *Syncephalastrum*, *Trichoderma*, and *Cunninghamella* were isolated from fungus gardens of *Atta sexdens* and *Acromyrmex* species (Rodrigues et al., 2005, 2008). Bacteria and yeasts also contribute to the complex and diverse microbiota of the fungus gardens (Craven et al., 1970; Carreiro et al., 1997, 2004; Rodrigues et al., 2009; Scott et al., 2010; Kellner et al., 2015).

Although the functional capacity is undefined for most of the microorganisms found in the ant fungus-growing system, some microbes are considered symbionts (Currie et al., 1999a; Pinto-Tomás et al., 2009; Sen et al., 2009; Suen et al., 2010; Aylward et al., 2013). For instance, fungi in the genus *Escovopsis* (Ascomycota: Hypocreales) are considered specialized antagonists of the fungus garden (Currie et al., 1999a) and are reported to negatively impact colony health (Currie, 2001b). Tripartite coevolution between the ants, the cultivated fungi and *Escovopsis* species are supported by patterns of phylogenetic congruence (Currie et al., 2003; Gerardo et al., 2006b). Thus, harmful potential of *Escovopsis* possibly has regulated the leaf-cutting ants' defenses on an evolutionary scale. Indeed, ant workers employ physiological and behavioral strategies when the garden is infected by *Escovopsis* conidia (Currie and Stuart, 2001; Abramowski et al., 2011; Nilssøn-Moller et al., 2018).

Actinobacteria in the genus *Pseudonocardia* and in other genera play a role in the attine ants' defensive strategies (Currie et al., 1999b; Mueller et al., 2008; Poulsen et al., 2010; Li et al., 2018). Antimicrobial compounds produced by Actinobacteria protect workers and the fungus garden from infection and dispersal of pathogenic microbes, including *Escovopsis* (Currie et al., 1999b, 2003; Oh et al., 2009; Sen et al., 2009; Mattoso et al., 2012). For several attine ant species, these bacteria are maintained in cuticular structures (e.g., tubercles, tubercles within crypts) on the ant's exoskeleton, nourished by glandular secretions (Currie et al., 2006). Cuticular structures that house Actinobacteria and where these bacteria are located on the ant integument vary per ant genus (Li et al., 2018). Evidence supports the association between Actinobacteria and attine ants evolved close to the origin of fungus-farming by ants, even though this mutualistic symbiosis has been lost multiple times over the evolutionary time (Currie et al., 2006; Li et al., 2018). While *Acromyrmex* species host abundant *Pseudonocardia* layers on their integuments, these bacteria are found in low frequency (or even absent) on the integument of *Atta* species (Currie et al., 2006; Marsh et al., 2013; Li et al., 2018). This could suggest that *Atta* species have replaced the use of Actinobacteria defenses by alternative mechanisms, including the application of glandular chemical compounds and intricate behavioral strategies to physically remove pathogens (Currie and Stuart, 2001; Fernández-Marín et al., 2009; Yek et al., 2012). However, the coevolution of *Pseudonocardia* with the ants and *Escovopsis* is debated, and our knowledge is still limited on how the diversity of these bacteria is distributed on individual ants as well as within colonies (Mueller et al., 2008, 2010; Andersen et al., 2013).

## DEFENSIVE STRATEGIES IN LEAF-CUTTING ANT SOCIETIES

Managing disease outbreaks is a central aspect of the ant–fungal symbiosis (Currie and Stuart, 2001; Hart et al., 2002). Besides the antimicrobial compounds produced by Actinobacteria (Currie et al., 1999b; Oh et al., 2009; Sen et al., 2009), the fungal crop potentially controls pathogen growth. The fungal cultivar of *Apterostigma auriculatus* was reported to inhibit the *in vitro* growth of *Escovopsis* (Gerardo et al., 2006a), and the fungal cultivar of *Atta colombica* was able to inhibit the growth of several endophytic fungi, including *Glomerella cingulata* (Van Bael et al., 2009). This inhibition could involve compounds with antimicrobial properties, as observed for the cultivar of *Cyphomyrmex* ants, which produces lepiochlorin (Hervey and Nair, 1979) and diketopiperazines (Wang et al., 1999). An additional defensive barrier could be constituted by cultivar-secreted laccases (De Fine Licht et al., 2013), detoxifying secondary metabolites produced by antimicrobial-producing antagonists (Divya and Sadasivan, 2016).

Beyond antimicrobial barriers from the fungus garden and associated symbionts, multiple hygienic behaviors represent a key part of attine ants' social immunity for avoiding the spread of diseases in the colony (Currie and Stuart, 2001; Fernández-Marín et al., 2013). Ant workers monitor the foraged substrate, the fungus garden, brood, and nestmates for disease traits, employing diverse strategies to deal with infections (Currie and Stuart, 2001; Poulsen et al., 2002; Fernández-Marín et al., 2006, 2013; Little et al., 2006; Rocha et al., 2014). Some of these strategies are hygienic behaviors commonly performed by insects, such as grooming contaminated body areas. Ants employ grooming by rubbing one or more legs at different parts of their bodies, thus targeting themselves (self-grooming). Besides, social insects can groom each other (allogrooming) removing contaminants from body areas difficult to access by self-grooming (Schmid-Hempel, 1998; Morelos-Juárez et al., 2010; Fernández-Marín et al., 2013; Zhukovskaya et al., 2013) or from the immature castes. The grooming behavior, common to nestmates inside the colony, may be more frequent for those ants returning from foraging (Richard and Errard, 2009; Morelos-Juárez et al., 2010). For instance, *Acromyrmex subterraneus* foragers spend more time on self-grooming than non-foragers, presumably due to their recurrent

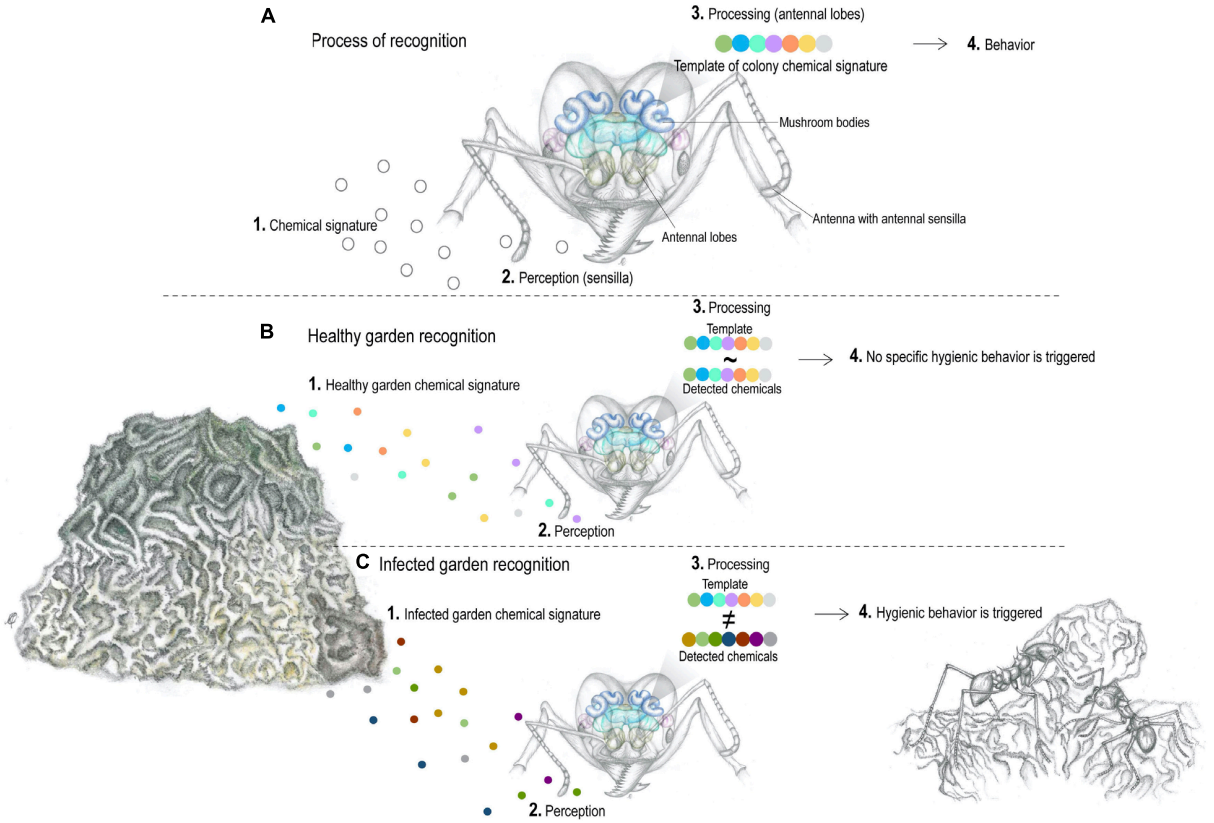


**FIGURE 1 |** Proposed mechanism for detecting harmful interactions with microbes. **(A)** In a generalized process of recognition, (1) the chemical signature of the detected organism (that could include the garden, nestmates, non-nestmates, larvae, and microbes) is (2) perceived by workers' antennal sensilla. (3) In the antennal lobes, the detected label is processed by comparison to the template of colony odor, a neural pattern that workers have stored (as memory) in the mushroom bodies. Depending on the label-template differences, (4) hygienic behaviors may or may not be triggered. **(B)** (1) A healthy garden chemical signature is (2) perceived by the antennal sensilla and (3) processed as similar to the memorized template, (4) not triggering hygienic behaviors. **(C)** (1) Harmful interactions may result in a particular chemical signature, (2) which is perceived by the antennal sensilla and (3) processed in the antennal lobe as different from the template, (4) triggering specific hygienic behavior for removing the pathogen and/or the infected area. Pencil drawings by Mariana O. Barcoto: ant head was adapted from a photograph by Casey Richart (http://bit.ly/2OZ6Oyf); ant brain was adapted from Mizunami et al. (2010); composition of ant head and brain was inspired on Bos and d'Ettorre (2012); and the two ants on the fungus garden were adapted from a photograph by Don Parsons (http://bit.ly/3bKgAOi).

contact with microbial contaminants (Richard and Errard, 2009). Spatial avoidance of both contaminated environments and sick nestmates may also reduce the risk of infection (Stroeymeyt et al., 2014; Quevillon et al., 2015; Tranter et al., 2015). Microbial infections are additionally controlled through antimicrobial secretions from workers' metapleural glands (Fernández-Marín et al., 2006, 2015), a complex glandular structure exclusive to ants (Yek and Mueller, 2011). Workers use characteristic movements of their forelegs in the metapleural gland opening, transferring gland secretions to contaminated areas (Fernández-Marín et al., 2006, 2013).

Prophylactic behavior during the selection and preparation of plant substrate is equally important to prevent (or decrease) infection risks (Quinlan and Cherrett, 1977; Mangone and Currie, 2007; Van Bael et al., 2012; Rocha et al., 2017). Insects tend to avoid foraging sites and food that is contaminated by parasites or pathogens (de Roode and Lefèvre, 2012), as reported for leaf-cutting ants (*Acromyrmex echinatior*, Tranter et al., 2015; *A. sexdens*, Rocha et al., 2017) and for fungus-growing termites (*Macrotermes natalensis*, Bodawatta et al., 2019). Thus, choosing and preparing plant substrates for the fungus garden may be fundamental to avoid the introduction of alien microbes. This is also true for endophytes, because leaf-cutting ants spend more time processing leaves with high endophyte loads than those with a low abundance. The presence of endophytes may also influence ants' foraging preferences, as they tend to collect leaf material containing a low abundance of endophytes (Cobletz and Bael, 2013). For instance, workers avoid plant substrates enriched with *Trichoderma* species (Rocha et al., 2014, 2017), a recurrent endophytic fungus and potential antagonist of the fungal cultivar (Ortiz and Orduz, 2000; Silva et al., 2006). Besides the surveillance of what is entering the colony, it is also important to control what is being thrown away. Waste management by leaf-cutting ants is an important task to prevent the access of already removed microbes and reinfection with contaminated material (Bot et al., 2001a). Old or infected pieces of fungus garden, dead brood, corpses, and even dried or unsuitable leaves are carried away to underground dumps (Autuori, 1947; Hart and Ratnieks, 2001) or

disposed above the soil far away from the colony in some leaf-cutting ant species (Weber, 1972). Waste workers do not access garden chambers, preventing the introduction of microbes of the refuse material in the fungus garden (Bot et al., 2001a).

Considering the central role of the fungal crop for fungus-growing insects, it is reasonable to consider that individual and group-level mechanisms may have evolved to avoid disease outbreaks in the fungus garden, comprising an important trait of their social immunity. Leaf-cutting ants combine diverse chemical and behavioral mechanisms to protect the fungus gardens from infective pathogens. Chemical defenses involve ants applying secretions of their metapleural and labial glands, known for exhibiting fungistatic, fungicidal, and bacteriostatic activity, to prevent the growth of entomopathogenic microbes (Graystock and Hughes, 2011). Gland secretions are also applied to the fungus garden surface, inhibiting the development of recurrent antagonistic microbes (Ortius-Lechner et al., 2000; Bot et al., 2002; Poulsen et al., 2002; Fernández-Marín et al., 2003, 2006, 2015). When facing contaminations on the fungus garden, leaf-cutting workers may use behaviors such as grooming the garden by "licking" possibly contaminated areas (Currie and Stuart, 2001). They can also transplant a healthy piece of fungus garden to an infected area (known as fungus-planting behavior; Fernández-Marín et al., 2013). Depending on the extent of the contaminated area, ants may employ weeding, a multiple-step behavior performed as an effort to restrain an established garden infection (Currie and Stuart, 2001; Barcoto et al., 2017; Nilssøn-Moller et al., 2018). During weeding, minima workers chew the edges of contaminated garden fragments, holding and pulling until the fragment is detached, ultimately being carried to the waste chamber (Currie and Stuart, 2001). It is worth to note that the majority of these behaviors are observed in experimental fungal infections, especially against fungal contaminants that normally are found in this environment, including *Escovopsis* (Currie and Stuart, 2001; Fernández-Marín et al., 2006; Barcoto et al., 2017; Nilssøn-Moller et al., 2018; Bonadies et al., 2019).

Fine-tuned mechanisms for detecting and recognizing microbial threats to the fungus garden may be an important part of social immunity, modulating defensive strategies that allow an early avoidance and reduce the cost of infection (Cremer et al., 2007; Meunier, 2015; Tranter et al., 2015). As discriminating mutualistic microbes from antagonistic ones might be a recurrent task in a fungicultural system, an efficient recognition process may be required for the ants to decide which mechanism of their social immunity is the most suitable for a specific situation (Cremer et al., 2007). In leaf-cutting ants, workers present specific responses toward harmful microbes, preferentially removing from the colony those that could cause damage (Currie and Stuart, 2001; Mighell and Van Bael, 2016). Although ant workers are reported to detect infections threatening the colony (Currie and Stuart, 2001; Abramowski et al., 2011; Gerstner et al., 2011; Mighell and Van Bael, 2016; Rocha et al., 2014, 2017; Tranter et al., 2015), the specific mechanism behind the recognition of distinct microbes remains unclear. In this context, we pose the following questions: (1) How are the processes of detection and recognition of microbial threats triggered and executed? (2) Does the fungus garden influence these processes? In the following sections, we discuss possible scenarios that could explain how ants recognize and discriminate microbes that are harmful to the fungus garden.

# DISCRIMINATING BETWEEN MICROBES

## Through the Chemical Profiles of the Fungus Garden and Alien Microbes

Each fungus-growing ant colony has a particular odor (Jaffé and Villegas, 1985; Hernández et al., 2006; Richard et al., 2007a; Nehring et al., 2011). As the chemical blends from the garden have a higher diversity of compounds than the chemical blends of workers and brood, the fungal crop possibly influences the colony odor (Bot et al., 2001b; Richard et al., 2007a,b). Ants probably recognize these chemical cues and discriminate between their resident fungal cultivar and that of sympatric colonies (Bot et al., 2001b; Viana et al., 2001; Poulsen and Boomsma, 2005). Fungal crops of closely related ant species (e.g., *Ac. octospinosus* and *Ac. echinatior*) produce a similar set of compounds but in different concentrations, suggesting that the ants' process of recognition may be fine-tuned to qualitative and quantitative differences in the fungal chemical profile (Bot et al., 2001b; Viana et al., 2001; Richard et al., 2007a; Valadares et al., 2015). Also, the discrimination of volatile organic compounds (VOCs) seems to be used by insects to recognize and select their mutualistic fungus strain (Bot et al., 2001b; Viana et al., 2001; Mueller et al., 2004; Richard et al., 2007a), as demonstrated for some Macrotermitidae species that collect fungal spores from the environment every new generation (Biedermann and Kaltenpoth, 2014). The termites' fine-tuned ability to localize and recognize their mutualistic fungus is probably guided by specific odors (Biedermann and Kaltenpoth, 2014). Nevertheless, fungus-growing termites can distinguish scent profiles from their mutualistic and that from invasive fungus, rejecting the weedy fungus after recognition (Katariya et al., 2017).

Considering the diverse VOCs produced by microbes (Schulz and Dickschat, 2007; Feofilova et al., 2012; Morath et al., 2012; Davis et al., 2013; Schulz-Bohm et al., 2017) that could act as signaling molecules for insects (Rohlfs et al., 2005; Davis et al., 2013), we inquire whether leaf-cutting ants may distinguish between alien microbes and their mutualistic fungus by recognizing VOCs or chemical cues. The ants could detect volatile compounds or surface chemicals of invasive microbes, discriminating a chemical signature that does not match that of their colony, then triggering hygienic responses (**Figure 1**). Therefore, what has been reported as "specific removal" or "specific hygienic responses" (Currie and Stuart, 2001; Tranter et al., 2015; Mighell and Van Bael, 2016) could be related not only to the threat level of an alien microbe in the fungus garden but also to their distinct chemical profile. Future assays offering only "scents" from different microorganisms to leaf-cutting ant colonies could unveil if detection only depends on VOCs, or whether the presence of physical structures (e.g., spores, mycelia, or bacteria cells) is also required. Hence, the quantification of avoidance or repellence for each bait could clarify the potential of recognition. Also, electroantennogram assays are plausible

to compare responses from workers' antennae (receptor and action potentials) regarding the presence of different microbe species, seeking for species-specific odor detection. In cases where the microbe has coevolved with the leaf-cutting ants' fungiculture, like the genus *Escovopsis* (Currie et al., 2003; Gerardo et al., 2006b), research on the detection based on chemical profiles must be taken with caution. The recognition process of such microorganisms could be a result of genetically determined neurophysiological mechanisms that trigger a cascade of physiological reactions in ant workers, resulting in immediate actions to remove it. Therefore, comparative studies toward different strains of *Escovopsis* species and microbes that did not coevolve within the system will help to understand patterns in overall gene expression (transcriptome) during the ants' responses.

## Through Fungus Garden Semiochemicals

Leaf-cutting ant workers are capable of recognizing changes in the physiological conditions of the fungal cultivar (Ridley et al., 1996; North et al., 1999; Herz et al., 2008). When incorporating a substrate unsuitable for the cultivar (e.g., toxic leaves and baits containing fungicide), workers avoid foraging for this substrate for several weeks, even if it is not harmful to the ants themselves (Ridley et al., 1996; North et al., 1997, 1999; Herz et al., 2008; Thiele et al., 2014). Because workers cease to forage for the harmful substrate after recognizing the damage in the fungus garden, the avoidance comprises a phenomenon known as *delayed rejection* (Herz et al., 2008; Saverschek et al., 2010; Saverschek and Roces, 2011; Arenas and Roces, 2016a,b, 2017). The delayed avoidance of particular plant substrates suggests that the response is influenced by the fungus garden (Ridley et al., 1996; Herz et al., 2008). Such modulation can be explained by chemical compounds produced during harmful interactions, which may be recognized by ant workers, thus acting as semiochemicals (chemicals that convey a message from one organism to another; Knapp et al., 1990; Ridley et al., 1996; North et al., 1999; Green and Kooij, 2018).

We speculate that a similar mechanism could be involved in the ants' recognition of harmful microbes, triggering a generalist response by the colony. Negative interactions between the fungal cultivar and antagonistic microbes could be communicated to ant workers via detectable modifications on the chemical profile of the fungus garden, acting as semiochemicals (Green and Kooij, 2018). During cultivar–pathogen interactions, defensive metabolites or incompatibility compounds produced by the cultivar (Poulsen and Boomsma, 2005; Gerardo et al., 2006a), derived products of hyphae breakdown (North et al., 1999), and even responses to metabolites released by the pathogen (Dhodary et al., 2018; Heine et al., 2018) could shift the fungus-garden chemical profile. These alterations would be processed in the antennal lobes by comparing the detected blend to the colony template memorized by the ant. By differing from the colony template, the chemical from fungus gardens' infected portions would trigger hygienic behaviors (**Figure 1**). As above, discrimination of microbes would happen when semiochemicals are released from negative interactions. Mechanisms by which the fungal crop signalizes harmful interactions, as well as the compounds involved in this process, remain unclear (Green and Kooij, 2018). Analyzing metabolites produced by both "infected" and "uninfected" cultivars may reveal context-dependent molecules, which can be tested for having a direct influence on the ant's behavior (e.g., a semiochemical role). A whole branch of research could be derived from investigating the evolution of ant–fungus communication and its influence on social immunity.

## Through Associative Learning

Leaf-cutting ant workers learn to differentiate between suitable and unsuitable leaf substrates mainly through the olfactory system, associating the fungal crop response to the chemical profile of the foraged substrate (Herz et al., 2008; Saverschek et al., 2010). Chemical information characterizing the unsuitable substrate is stored in the ants' brain as "olfactory memory," coding a long-term memory that will be retrieved once the same detrimental plant is collected (Herz et al., 2008; Saverschek et al., 2010; Saverschek and Roces, 2011; Falibene et al., 2015). Learning from olfactory experience and formation of associative memories involve structural remodeling of brain centers for sensory integration and association, such as the mushroom bodies (Galizia and Rössler, 2010; Falibene et al., 2015). When leaf-cutting ants learn how to differentiate between substrates according to the suitability to the fungal crop, the development of long-term associative memories is correlated to transient modifications in the density of synaptic complexes in the mushroom bodies (Falibene et al., 2015). Similarly, chemical signals from the infected garden could be detected by olfactory neuron sensors in the ants' antennal sensilla, present throughout the ants' antennae, and ultimately reaching the olfactory glomeruli in the antennal lobe, where the information is processed (Kleineidam et al., 2005; Galizia and Szyszka, 2008; Galizia and Rössler, 2010; Carey and Carlson, 2011). Developing long-term memories associating with the odor of an infection as a threat for the garden health could involve transferring olfactory information from the antennal lobe to the mushroom bodies, where it would promote a reorganization of associative networks (Galizia and Rössler, 2010; Falibene et al., 2015). Thus, we suggest that olfactory associative learning, which comprises the cognitive ability to connect different stimuli and predict relationships between them (Giurfa, 2007; Leadbeater and Chittka, 2007; Dickinson, 2012), could be related to the recognition of harmful microbes.

We postulate that ant workers would learn and memorize the chemical profile of harmful microorganisms, associating it with the response of the fungal crop (**Figure 2**, step A4). Groups of tending workers that associate chemical cues with detrimental interactions would compose a "colony temporary memory." Hence, in subsequent contacts with a known pathogen, this mechanism would provide a faster response in grooming contaminated plant debris and foraging workers to prevent pathogens from entering the colony (**Figure 2**, step B1). If the microbe reaches the fungus garden,

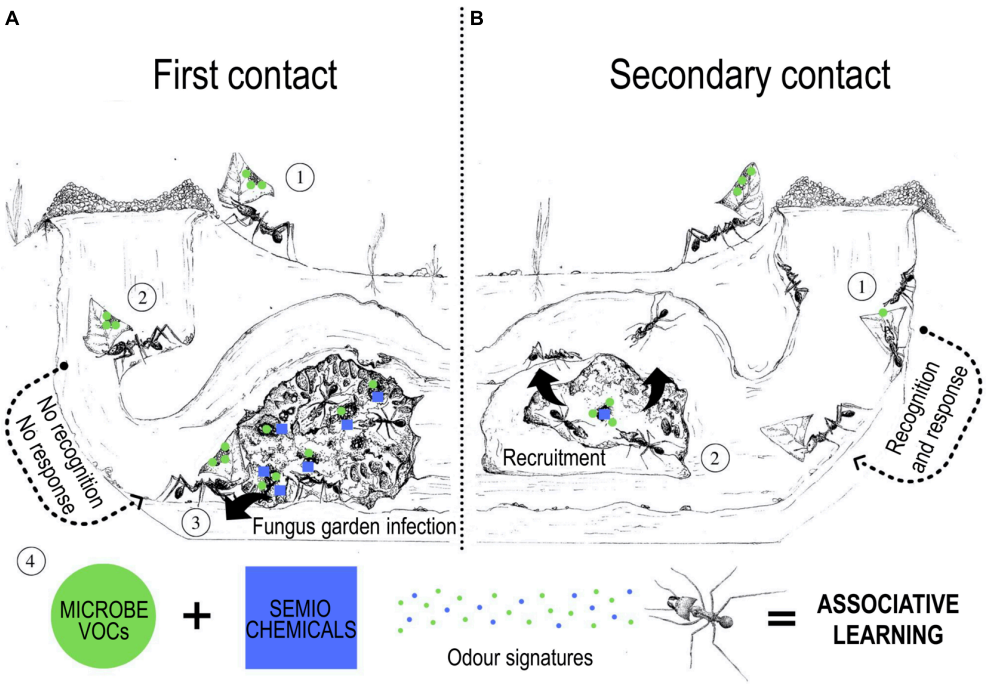


**FIGURE 2 |** Associative learning and the "colony temporary memory." **(A)** First contact: (1) a forager worker carries inside the colony a microbe on a leaf and/or on its integument. When workers have not experienced previous contact with this microbe, it may not be recognized by workers. Generalized hygienic behaviors may be employed for removing the microbe. If these mechanisms fail, (2) the microbe reaches the fungus garden and potentially harms the fungal cultivar (3). The "damaged area" of the fungus garden would signalize alterations to the tending workers. Recruited workers would interact and experience this feedback, linking it to specific chemical cues (volatile organic compounds - VOCs) of the harmful microbe through associative learning (4). By memorizing the pathogen's cues, these workers are capable to recognize this microbe in future encounters, associating its presence with damage to the fungus garden. **(B)** Secondary contacts: (1) in future contacts, workers would be able to recognize and remove the microbe from plant debris or even from themselves, preventing it from reaching the fungus garden. (2) In cases where the same harmful microbe reaches the fungal crop, workers who learned from previous contacts would recruit other nestmates (naive workers) for cleaning and removal, increasing the number of experienced ants to recognize this microbe as an antagonist. Therefore, over time and exposure to different antagonist species, the colony would acquire their own "immune memory." This colony-memory would be temporary, lasting during the lifespan of these workers, therefore putatively reprocessed during the lifetime of the colony. Illustration by Beatriz Garcia Gonçalves.

additional workers could be recruited to the infected area either by chemically interacting via antennation or by releasing alarm pheromones (Gerstner et al., 2011) from "memory workers" (**Figure 2**, step B2). Alternatively, in cases in which microbes never had caused negative outcomes before, the ants perhaps are only able to detect its chemical cues. Thus, strategies for preventing the infection would be more generalized (e.g., applying antimicrobials secreted from metapleural glands and microbe removal through fungus grooming).

For further investigation of this hypothesis, studies involving the structural alterations of the mushroom bodies (Falibene et al., 2015), in experimental setups where the fungus garden is threatened by pathogens, could answer questions on neurological activities and expressions during defensive responses in an individual-level perspective. Also, genomic and transcriptomic tools on active workers defending the fungus garden, or assays where the ants are exposed only to the pathogen, could fill a gap in our knowledge about physiological and genetic traits involved in their social immunity. For a colony-level perspective, responses could be verified through repeated inoculation of antagonistic microbes in the fungus garden, seeking evidence for learning processes, colony-memory to recognize the same pathogen or even "immunization" (Traniello et al., 2002; Ugelvig and Cremer, 2007; Walker and Hughes, 2009; Konrad et al., 2012).

## CONCLUSION

Ant–fungal communication and ants' ability to detect and recognize pathogens have played a key role in the success of the fungus-growing ants' symbiosis. Future research should address the ant–garden communication and defensive strategies across the attine ant lineages, investigating the evolutionary history of these mechanisms. Also, it remains unclear whether the defensive responses target specific pathogens genera or species and whether the hygienic behaviors and frequency of responses would vary accordingly. In an attempt to address such gaps, here we discussed the possible role of associative learning (to experience which microbes could be harmful to the fungus garden) and how chemicals could lead to microbe-specific recognition. The proposed mechanisms can be considered

frameworks to build experiments to understand how ants defend fungus gardens against harmful microbes. However, we cannot predict how costly or beneficial each of these mechanisms would be at both the individual and society levels. Nevertheless, addressing possibilities regarding learning due to recurrent infection to increase the survival and fitness of the colony will open new areas in social immunity knowledge. As pointed out in this review, we have only just begun to understand how social immunity evolved in leaf-cutting ants, and there is still a long way to go before we can form a full picture of the process from encountering a microbe to applying defenses.

## AUTHOR CONTRIBUTIONS

AG and MB conceptualized the hypothesis and reviewed the literature. AG, MB, PK, OB, and AR thoroughly discussed the hypothesis, wrote and reviewed the draft versions of the manuscript. MB conceived **Figure 1**. AG, MB, PK, and AR wrote the final manuscript. All authors approved the final version of the manuscript.

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# A Potential Collective Defense of *Drosophila* Larvae Against the Invasion of a Harmful Fungus

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The establishment of a collective defense is an important means of controlling the spread of harmful microbes in group-living animals. Collective defenses are associated with costs resulting from the investment in resources and the risk taking of infections or the exposure to microbial toxins for the performing individual and are often assumed to have evolved in (eu)social insects, like bees and ants, as a result of close contact and pathogen transmission between nestmates. We hypothesize that collective antimicrobial defense mechanisms are potentially also found in insects that exhibit simpler forms of sociality or even mere aggregation behavior. The larvae of the saprophagous fruit fly *Drosophila melanogaster* develop in high-density aggregations on rotting fruits, which are often colonized by insecticidal filamentous fungi. Here we show that fruit fly larvae suppress the invasion of a harmful fungus not only by the summative effect of individuals at high densities but also because larger groups of larvae at the same density can control fungal growth more efficiently. We achieved the necessary manipulation of the group size by increasing the number of larvae in proportion to an increase in habitat size, thereby excluding the effect of density changes on fungal growth as a confounding factor. We found evidence that part of the variation in the ability to suppress the fungus in this group size-dependent manner can be explained by genetic variation at the insects' *foraging* (*for*) locus. Group size therefore influences the extent to which the larval aggregates suppress the spread of a harmful fungus. This indicates a potential collective defense against habitat invasion by pathogenic fungi. The selection pressure on the efficiency of this potential defense strategy may contribute to the evolution of aggregation behavior in non-(eu)social insects.

**Keywords: aggregation, density dependence, *Drosophila*, foraging phenotype, group size, group living, pathogenic fungi, social immunity**

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## INTRODUCTION

Allogrooming, application of antimicrobial substances, "weeding," and removal of diseased broods are traits that enable group-living insects—dependent on their social organization—to defend themselves collectively against the invasion of harmful microbes (Cremer et al., 2007; Meunier, 2015; van Meyel et al., 2018). "Social" or "collective" imply that antimicrobial behavioral and physiological strategies are performed jointly and/or toward each other. Therefore,

collective defense constitutes an additional shield that complements the protection achieved by individual defenses.

The individual-level processes mediating the collective defense are thought to have evolved in response to increased pathogen transmission in eusocial insects—the eusocial framework (Cremer et al., 2007; Cotter and Kilner, 2010; Kappeler et al., 2015; Nuotclà et al., 2019). For this reason, collective antimicrobial defense is often seen in the context of social immunity (Cremer et al., 2007). However, as highlighted by Meunier (2015) and van Meyel et al. (2018), antimicrobial defense strategies, such as release of self-produced antibiotics, hygienic behavior, or allogrooming, are found not only in eusocial but also in non-(eu)social and even solitary insects. If these strategies are performed collectively and result in positive feedback on individual fitness, such a feedback may be a selective force favoring group living and the social complexity of such groups—the group living framework (Meunier, 2015; Biedermann and Rohlfs, 2017; van Meyel et al., 2018; Nuotclà et al., 2019). According to the group living framework, one would expect to observe collective defense strategies in insects that are non-eusocial or in those that form only semi-social aggregations (Nuotclà et al., 2019). In such aggregations, resistance against harmful microbes can be achieved passively due to density-dependent effects (**Figure 1**). However, this seemingly improved control of microbes is a mere numerical summation of the effects of otherwise competing individuals, i.e., an effect that would not be considered as collective defense or social immunity (Cotter and Kilner, 2010; Meunier, 2015; van Meyel et al., 2018). Thus, if an antimicrobial trait is important for a possible collective defense in a given system, one would expect that the efficiency of the expression of this trait increases with increasing group size, independent of the actual density of individuals (**Figure 1**). To test this, careful experimental manipulation is required to control for the confounding effect of density.

Within this group living framework, we hypothesize that an ancestral stage in the early evolution of collective antimicrobial defense is due to the advantages of simple semi-social aggregation behavior (Biedermann and Rohlfs, 2017). Semi-social aggregations are widespread in insects that breed in ephemeral resources, e.g., dung, carrion, fruits. On such resources, the developing insect larvae have to cope with numerous saprotrophic and often harmful microorganisms, insecticidal bacteria, and fungi (Janzen, 1977). Aggregative behaviors of non-social insects have frequently been observed in association with such microbes [numerous examples described in Wertheim et al. (2005)]. In the fruit-inhabiting *Drosophila* model system, the harmful effect of molds on larval development is based on a constitutive and inducible formation of insecticidal secondary metabolites (Caballero Ortiz et al., 2013), which trigger several disease symptoms or even kill the larvae (Wölfle et al., 2009; Trienens et al., 2010). However, fruit fly larvae in high-density aggregations can successfully suppress the spread of insecticidal mold fungi, e.g., *Aspergillus* sp. and *Penicillium* sp., in their feeding habitat and thus achieve higher per capita fitness (Rohlfs, 2005). The formation of fungus-controlling aggregations not only seem to matter in *Drosophila* (Hodge et al., 1999; Wertheim et al., 2002; Rohlfs and Hoffmeister, 2003) but also contribute to the management of detrimental fungi in facultative eusocial ambrosia beetles, for example (Biedermann and Taborsky, 2011; Nuotclà et al., 2019). The insects probably achieve the destruction of hyphal tissue by using their mouth parts, e.g., by chewing the mycelium. In addition, chemical components such as the release of antimicrobial peptides could also be involved in the inhibition of fungal growth.

As outlined above, in such aggregations, group size-specific effects could be masked by insect density-dependent suppression of harmful fungi or not exist at all, i.e., there may be no collective behavior resulting from interactions between group members that contributes to the suppression of harmful microbes. For this reason, we investigate here the hypothesis that larger groups of fruit fly larvae have a higher capacity to suppress the growth of a harmful fungus, which would indicate the involvement of an antimicrobial collective action. To test this, we manipulated the group size of *Drosophila melanogaster* larvae while not altering their density and quantified the suppression of the insecticidal fungus *Aspergillus nidulans* by these groups. This fungus species is representative of various taxa of widespread molds that negatively influence insect development in different decomposer systems by the formation of insecticidal secondary metabolites (Janzen, 1977; Hodge et al., 1999; Drott et al., 2017; Künzler, 2018). Additionally, in order to gain a first insight into how selection might favor different behaviors in the face of microbial invasion, we also investigated the extent to which genetic variation in movement behavior, determined by allelic variation in the *foraging* locus (Anreiter and Sokolowski, 2019), contributes to the suppression of fungal growth in the breeding substrate of *Drosophila*.

# MATERIALS AND METHODS

## Organisms

Experimental *D. melanogaster* larvae were obtained from an outbred lab population that was established from field-caught flies in 2006 (Wölfle et al., 2009).The population has been kept since with non-overlapping generations, where larvae were reared under moderate densities in several flasks containing breeding substrate. Subsequently, the enclosed flies have been joined as one population in a cage provided with food and water. A flask with breeding substrate was attached to the cage to allow egg laying. This strain was used in Experiment 1 and Experiment 2.

*Aspergillus nidulans* (strain RDIT2.3) colonies were grown on malt extract agar (30 g malt extract, 5 g peptone, and 20 g agar Kobe I, filled up to 1 L with purified water). Fungal conidiospores for inoculation of experimental setups were harvested from 7-day-old colonies by rinsing the colony with saline solution (8.6 g NaCl, 300 mg KCl, 350 mg $CaCl_2$ per liter of demineralized water). Conidiospore titer was estimated using a hemocytometer (Neubauer improved). Experiment preparations were conducted in a laminar flow cabinet. We used heat-sterilized tools and substrate. Eggs and plastic frames were treated with sodium hypochlorite to eliminate adherent microbes.

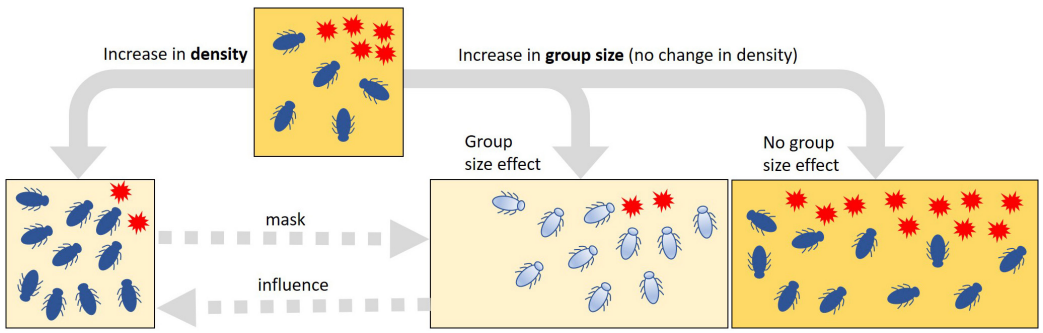


**FIGURE 1 |** Summative vs. collective effects of variation in the density or group size of insects on the spread of harmful microbes. Increased suppression of harmful microbes (red stars) can be the result of an increase in both density and group size in a habitat (squares). In a pure density effect, this change in suppression occurs only passively through the additive effects of the antimicrobial activity of the individuals; an increase in density (i.e., changes in the number of insects, but no change in habitat size as indicated by the square size) does not change the quality or the intensity of the antimicrobial properties. If there are elements of a collective defense in the control of harmful microbes that is based on an active change in the quality or the intensity of antimicrobial properties (depicted by the change in insect and habitat coloration), it is to be expected that the suppression of microbes is stronger in large than in small groups. If there is no such group size effect, the harmful microbes would spread in proportion to habitat size. To detect such group size effects and thus the potential collective actions, it is important to carefully control the effects of density changes experimentally. Density effects may mask potential group size effects, but group size effects can be part of and possibly amplify density effects (dashed arrows).

## General Experimental Setup

To manipulate insect group size rather than insect density, we designed experimental arenas of different sizes. For this, we created rectangular plastic frames that consisted of plastic strips (polyester strengthened with paper inlay, 15 mm height, 250 μm thickness, the length depends on the arena size), which were pleated at three folding edges and fastened at the fourth to obtain frames of respective sizes (**Figure 2A**). The plastic frames were filled with fruit agar (50/50 v/v% mashed banana, water, and 36 g agar/L) and placed in 50- and 90-mm Petri dishes, respectively. We created a clearly defined fungal growth zone by forcing the unidirectional growth of the fungi. Filter paper strips (3 mm × arena length) were soaked in a suspension of conidiospores (1 million conidia per microliter); this conidia titer was used to ensure the development of a homogeneous growth front of the fungus. The air-dried filter strips were then placed flush with the frame on the fruit agar (**Figure 2B**). All setups were pre-incubated at 25°C and constant darkness for 48 h. Then, the larvae were transferred to the arenas and further incubated at 25°C and constant darkness.

To quantify the expansion of the fungi, we photographed the arenas 12, 24, 36, and 48 h after larval transfer and measured the substrate area covered by fungal tissue ("a" in **Figure 2C**) growing out of the filter paper ("f" in **Figure 2C**; using ImageJ; //imagej.net for measurement). In the insect treatment, the peripheral growth zone of the fungi was severely damaged by the feeding activity of the larvae. There were still few hyphal fragments visible in this growth zone, but these were not quantifiable, so we did not consider these fragments to be part of an intact colony (**Figure 2C**). To reduce variation due to observational errors, we performed three independent measurements of each colony and used their means for statistical analysis. The expansion of fungi on the substrate was quantified after 12, 24, 36, and 48 h of confrontation with the larvae as the average expansion per millimeter of growth front (in mm).

## Experiment 1: Suppression of the Insecticidal Fungus *Aspergillus nidulans* by Differently Sized Groups of *Drosophila melanogaster* Larvae

To test the effect of larval group size difference on the expansion of a harmful fungus, in this first experiment, we used plastic frames of 25 × 25 mm and 50 × 25 mm, i.e., the latter was twice as large as the former. The plastic frames were filled with 4 and 8 ml fruit agar, respectively, inoculated, and incubated as described above. Before the transfer of larvae, the arenas were randomly assigned to the larval treatment. We transferred 10 first-instar larvae into the small arenas and 20 of them into the large arenas, resulting in a larval density of 0.4 larvae per millimeter of fungal growth front (larvae/mm) or 1.6 larvae per $cm^2$ arena in both arena types. In total, there were 10 replicates per treatment, with an additional 10 larval-free control colonies per arena type. Due to the aberrant growth of the fungus in one small arena, we were forced to reduce the number of the previously assigned replicates for the larval treatment to nine. The arenas were prepared and incubated, and fungal growth was quantified as described in "General Experimental Setup". The expansion of the fungi on the substrate was quantified after 12, 24, 36, and 48 h of confrontation with the larvae as average expansion per millimeter of growth front (in mm).

## Experiment 2: Suppression of the Insecticidal Fungus *Aspergillus nidulans* by Differently Sized Groups of *Drosophila melanogaster* Larvae at Two Levels of Larval Density

The aim of this second experiment was twofold: first, to verify the previous observation made in Experiment 1 by using a wider range of group sizes and, second, to test whether the effect of

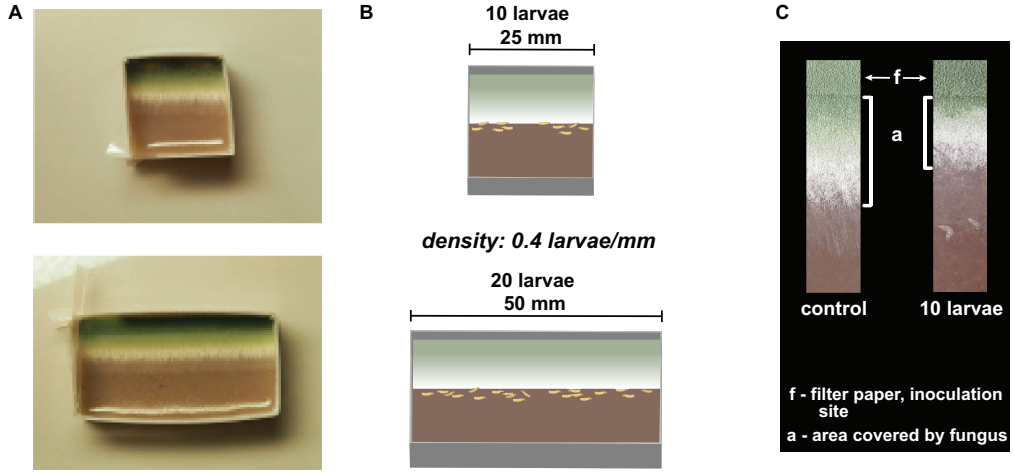


**FIGURE 2 |** Illustration of experimental setup. **(A)** Pictures of experimental arenas with unconfronted *Aspergillus nidulans* colonies. **(B)** Schematic drawing of the experimental arenas. **(C)** Sections of unidirectionally growing mold (from top to bottom) without (control) and with 10 larvae. The aging and the fruiting zones (top) are characterized by the development of increasingly greener conidiospores. The younger productive zone can be recognized by the formation of aerial whitish hyphae, while the hyphae of the outer zone lie flat on the fruit agar or are slightly embedded in it. The feeding activity of *Drosophila* larvae is largely restricted to this outer zone of the mycelium, which makes the mold colonies less invasive. "a" is the area of undamaged fungal tissue that we quantified; "f" is the area covered by the filter paper inoculated with conidia.

group size can be observed at different larval densities. For this purpose, arenas with a size of 25, 50, or 75 × 25 mm were filled with 4, 8, or 12 ml fruit agar, respectively. The filter paper strips inoculated with conidia were adapted to the size of the arena. By reducing and increasing the number of larvae, we changed the differences between the groups compared to Experiment 1. We transferred groups of 5, 10, and 15 larvae, resulting in a constant larval density of 0.2 larvae per millimeter growth front of the fungus, or 20, 40, and 60 larvae, resulting in a constant larval density of 0.8 larvae per millimeter growth front of the fungus (**Supplementary Figure S1**). We conducted 20 replicates per combination; however, in individual cases, the final replicate number was reduced due to contaminations of the substrate. The arenas were prepared and incubated, and fungal growth was quantified as described in "General Experimental Setup."

Moreover, to corroborate an overall density-dependent effect on fungal pathogen expansion in the insects breeding sites, we additionally transferred larvae in all group sizes to all arena sizes. This resulted, over all arena types, in a density range of 0.06 to 2.4 (that is, five larvae in 75-mm arenas to 60 larvae in 25-mm arenas; a full list of combinations is provided in **Supplementary Tables S1**, **S2** and **Supplementary Figure S1**). The results of this density range experiment can be found in the **Supplementary Material**.

## Experiment 3: Effect of Larval *Drosophila melanogaster* Foraging Phenotype on the Suppression of Insecticidal *Aspergillus nidulans*

Since the fly larvae actively seek out the fungus, the containment of the fungus is largely based on the behavior of the larvae. Based on this, we have analyzed whether genetic variation in the locomotor activity of *D. melanogaster* larvae contributes to differences in the suppression of fungal expansion. We used two *D. melanogaster* strains, "rover" and "sitter," which differ in allelic variation at the *foraging* (*for*) locus and express two different behavioral phenotypes: the larvae of the "rover" phenotype travel longer distances during foraging compared to the "sitter" larvae (Sokolowski, 1985; de Belle et al., 1989). We used arenas of 25 and 50 × 25 mm and transferred groups of 10 and 20 larvae, respectively, with 10 replicates each. Fungal growth was quantified at 12, 24, 36, and 48 h after larval transfer.

To test whether there is indeed a variation in larval behavior that can be correlated with the variation in fungal suppression, we used the images taken after 12, 24, 36, and 48 h to count the number of larvae that were in contact with the fungal colonies. The arenas were prepared and incubated, and fungal growth was quantified as described in "General Experimental Setup".

## Statistics

In Experiment 1, we first tested whether the arena size had an impact on the expansion of unconfronted fungal colonies and, further, the null hypothesis that differences in larval group size do not significantly affect fungal expansion by applying generalized estimating equations (GEE) and fitting marginal generalized linear models (GLM), which take the repeated measures into account. We specified the model with a Gaussian distribution and identity link function, an auto-regressive correlation structure (on the basis of equally spaced cluster), and a fully iterated *jackknife* estimator. After model selection, fitting was estimated with Q-Q plots.

In concordance, we tested in Experiment 2 whether larval group size and larval density contributed to the variance in the data using the GEE. Different to the above-stated model specification, here we fitted a Gaussian distribution with a log link function (all other settings were the same as above). The effect of

differences in the locomotion behavior of *Drosophila* larvae on fungal expansion (Experiment 3) was likewise analyzed using the GEE procedure with the same model settings as for Experiment 2.

The aggregation of larvae at the rim of the fungal colony was analyzed with a glm specifying a quasibinomial distribution of larvae counts in contact with fungal colony to account for overdispersion. In model specifications, larval group size was nested within foraging type, and time was specified as a random factor.

All statistic procedures were performed in R (R 3.6.1, R Core Team, 2019; "*geepack*" 1.2-1, Højsgaard et al., 2005; "*car*" 3.0-3).

## Bioethics Statement

All aspects of the present research have been conducted in compliance with national and international legislation and fundamental ethical principles. Experimental work with the invertebrate *D. melanogaster* does not require specific measures regarding the Animal Welfare Act.

# RESULTS

## Experiment 1: Suppression of the Insecticidal Fungus *Aspergillus nidulans* by Differently Sized Groups of *Drosophila melanogaster* Larvae

In our first experiment, the group size of *D. melanogaster* was 10 or 20 larvae with a constant density of 0.4 larvae per millimeter fungal growth front. Colony expansion was recorded 12 to 48 h post-larval transfer (**Figure 3**). This early phase of interaction of *Drosophila* larvae with filamentous mold fungi—about 25% of the insects' development time—determines whether the fly larvae are able to suppress the growth of insecticidal mold fungi sustainably and survive until pupation (Rohlfs et al., 2005). In unconfronted colonies, the arena size had no effect on fungal expansion (analysis of Wald statistic $\chi^2 = 0.031$, $p = 0.86$; **Figure 3**, **Table 1**, Q-Q plot **Supplementary Figure S2A**). Compared to these unconfronted controls, the colonies of fungi exposed to fruit fly larvae expanded more slowly. In addition, the spread of the fungus was more suppressed by the larger group, with the effect of group size increasing over time (analysis of Wald statistic, time × group size: $\chi^2 = 28.94$, $p < 0.001$; **Figure 3**; **Table 1**, Q-Q plot **Supplementary Figure S2B**).

## Experiment 2: Suppression of the Insecticidal Fungus *Aspergillus nidulans* by Differently Sized Groups of *Drosophila melanogaster* Larvae at Two Levels of Larval Density

To verify the group size effect observed in Experiment 1, we repeated and extended this experiment by increasing the number of arena types and thus the number of possible group sizes. Moreover, group size variation was tested at two different density levels, 0.2 and 0.8 larvae per millimeter growth front of the fungus. As in Experiment 1, the mycelial expansion was significantly reduced, with increasing group size as a function of time (analysis of Wald statistic, time × group size: $\chi^2 = 36.56$, $p < 0.001$; **Figure 4** and **Table 2**), thus verifying the

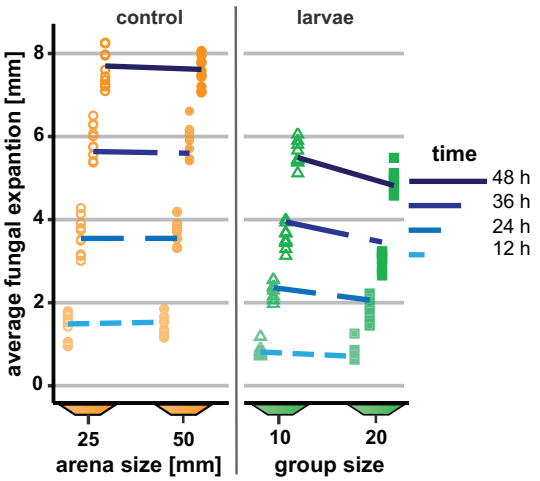


**FIGURE 3 |** Effect of *Drosophila melanogaster* larvae at two different group sizes on the expansion of the insecticidal filamentous fungus *Aspergillus nidulans*. Expansion of the fungus across the substrate in small (open symbols) and large (filled symbols) arenas without larvae ("control," left-hand side) and as affected by the presence of *Drosophila* larvae with 10 or 20 larvae ("larvae," right-hand side). Dashed lines depict the fitted model estimates for the four time points. For the insect treatment, the density of larvae was 0.4/mm fungal growth front and 1.6/cm$^2$ arena in both the small and the large arenas. The GEE was applied separately for arenas with and without larvae, while larva-free fungal colonies showed no significant difference between arena sizes; confronted colonies were more suppressed over time by larger larval groups (**Table 1**).

**TABLE 1 |** Factors influencing expansion of *Aspergillus nidulans* in Experiment 1. Results of the most parsimonious GEE model (after backward elimination of non-significant factors) for larvae-free and larvae-confrontedfungal colonies are shown.

| | **Larvae-free control colonies** | | |
|---|---|---|---|
| **Explanatory factor** | **Model estimate ± SE** | ***Wald*** | ***p*-value** |
| Intercept | −0.5180 ± 0.2250 | 5.29 | < 0.001 |
| Time | 1.7000 ± 0.0011 | 2463.87 | < 0.001 |
| Arena size | −0.0001 ± 0.0054 | 0.03 | 0.855 |
| Alpha | 0.840 ± 0.027 | | |
| $R^2_{marg}$ | 0.974 | | |
| | **Larvae-confronted colonies** | | |
| **Explanatory factor** | **Model estimate ± SE** | ***Wald*** | ***p*-value** |
| Intercept | −0.8097 ± 0.1396 | 33.63 | < 0.001 |
| Time | 0.1437 ± 0.0043 | 1134.10 | < 0.001 |
| Group size | 0.0049 ± 0.0088 | 0.31 | 0.580 |
| Time × Group size | −0.0016 ± 0.0003 | 33.68 | < 0.001 |
| Alpha | 0.152 ± 0.108 | | |
| $R^2_{marg}$ | 0.965 | | |

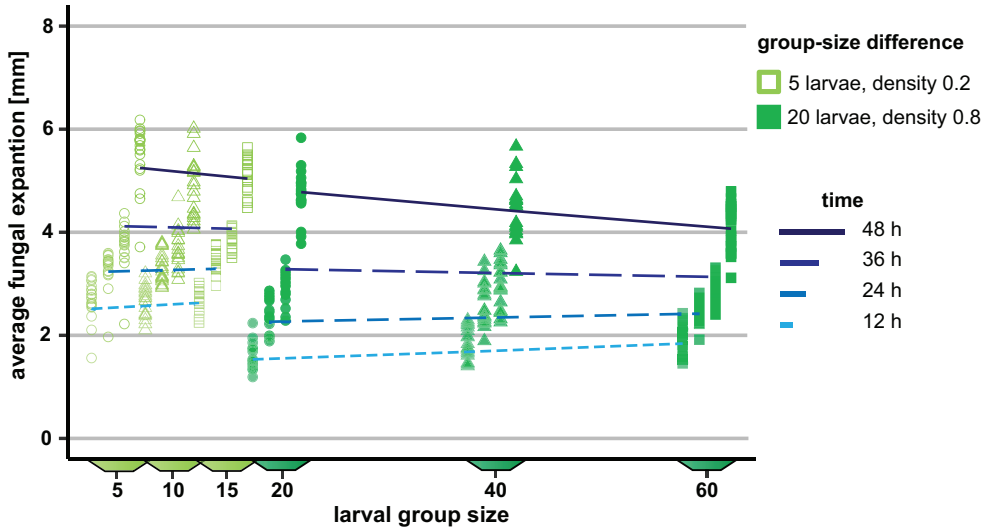


**FIGURE 4 |** Influence of variation in the group size of *Drosophila melanogaster* larvae on the spread of the insecticidal filamentous fungus *Aspergillus nidulans*. The influence of the three different group sizes on the expansion of the fungus was quantified at two different densities (0.2 and 0.8 larvae per millimeter of fungal growth front) at four different points in time. The differences in group size were 5 and 20 at the two density levels, respectively. For the sake of clarity, the data points within one group size per time are shown slightly shifted along the *x*-axis. Lines represent the adjusted model estimates for the four time points (generalized estimation equation).

**TABLE 2 |** Factors influencing the expansion of *Aspergillus nidulans* in Experiment 2. Results of the most parsimonious GEE model (after backward elimination of non-significant factors) are shown.

| Explanatory factor | Model estimate±SE | *Wald* | *p*-value |
|---|---|---|---|
| Intercept | 0.8890 ± 0.0278 | 1021.88 | < 0.001 |
| Time | 0.0167 ± 0.0007 | 571.92 | < 0.001 |
| Density | −1.2100 ± 0.0936 | 167.91 | < 0.001 |
| Group size | 0.0073 ± 0.0017 | 18.77 | < 0.001 |
| Time × Density | 0.0241 ± 0.0022 | 117.39 | < 0.001 |
| Time × Group size | −0.0002 ± 0.0001 | 31.38 | < 0.001 |
| Alpha | 0.534 ± 0.076 | | |
| $R^2_{marg}$ | 0.852 | | |

previously made observation. Moreover, density also contributed significantly to the reduction of fungal expansion in a time-dependent manner (analysis of Wald statistic, time × density: $\chi^2 = 67.40$, $p < 0.001$; **Figure 4**, **Table 2**, Q-Q plot **Supplementary Figure S2C**). This means that while the effect of group size is apparent at later points in time, the effect of larval density on fungal expansion is stronger at earlier points in time. However, the absence of a statistically significant three-way interaction between time × density × group size (analysis of Wald statistic $\chi^2 = 1.48$, $p = 0.22$, **Table 2**) suggests that the overall effect of larval group size on fungal control is not different at the two densities.

The density–range experiment revealed highly significant differences in fungal growth expansion. With increasing larval densities, fungal growth was increasingly strongly suppressed (analysis of Wald statistic, time × group size difference × density–range: $\chi^2 = 65.20$, $p < 0.001$; **Supplementary Figure S3**, **Supplementary Table S3**, Q-Q plot **Supplementary Figure S2D**).

## Experiment 3: Effect of Larval *Drosophila melanogaster* Foraging Phenotype on the Suppression of Insecticidal *Aspergillus nidulans*

To explore the possible contribution of genetic variation in larval locomotor activity to the suppression of *A. nidulans*, we quantified the effect of allelic variation at the *for* locus on the spread of the mold fungus. The *sitter* phenotype turned out to suppress the expansion of *A. nidulans* more than the *rover* phenotype (analysis of Wald statistic, rover vs sitter: $\chi^2 = 8.0$, $p = 0.004$; **Figure 5A** and **Table 3**, Q-Q plot **Supplementary Figure S2E**). Generally, larger groups suppressed fungal expansion more effectively than smaller groups in a time dependent manner (analysis of Wald statistic, time × group size: $\chi^2 = 6.0$, $p = 0.017$; **Figure 5A** and **Table 3**).

Variation in the *foraging* phenotype possibly leads to differences in larval behavior directed against the fungus, which might provide an explanation for why the genotypes differ in their quantitative effect on the fungus. To test this possibility, we calculated the proportion of larvae found to aggregate at the growth front of the fungus and are in touch with the mycelium. Based on the analysis of the images taken 12 to 48 h post-larvae transfer for fungal growth measurements, we found that a higher proportion of larvae of the *sitter* phenotype was in direct contact with the fungal colony than the larvae of the *rover* phenotype (analysis of deviance, type II: *rover* vs. *sitter* $F_{1,155} = 51.656$, $p < 0.001$; time: $F_{1,155} = 126.40$, $p < 0.001$; time × group

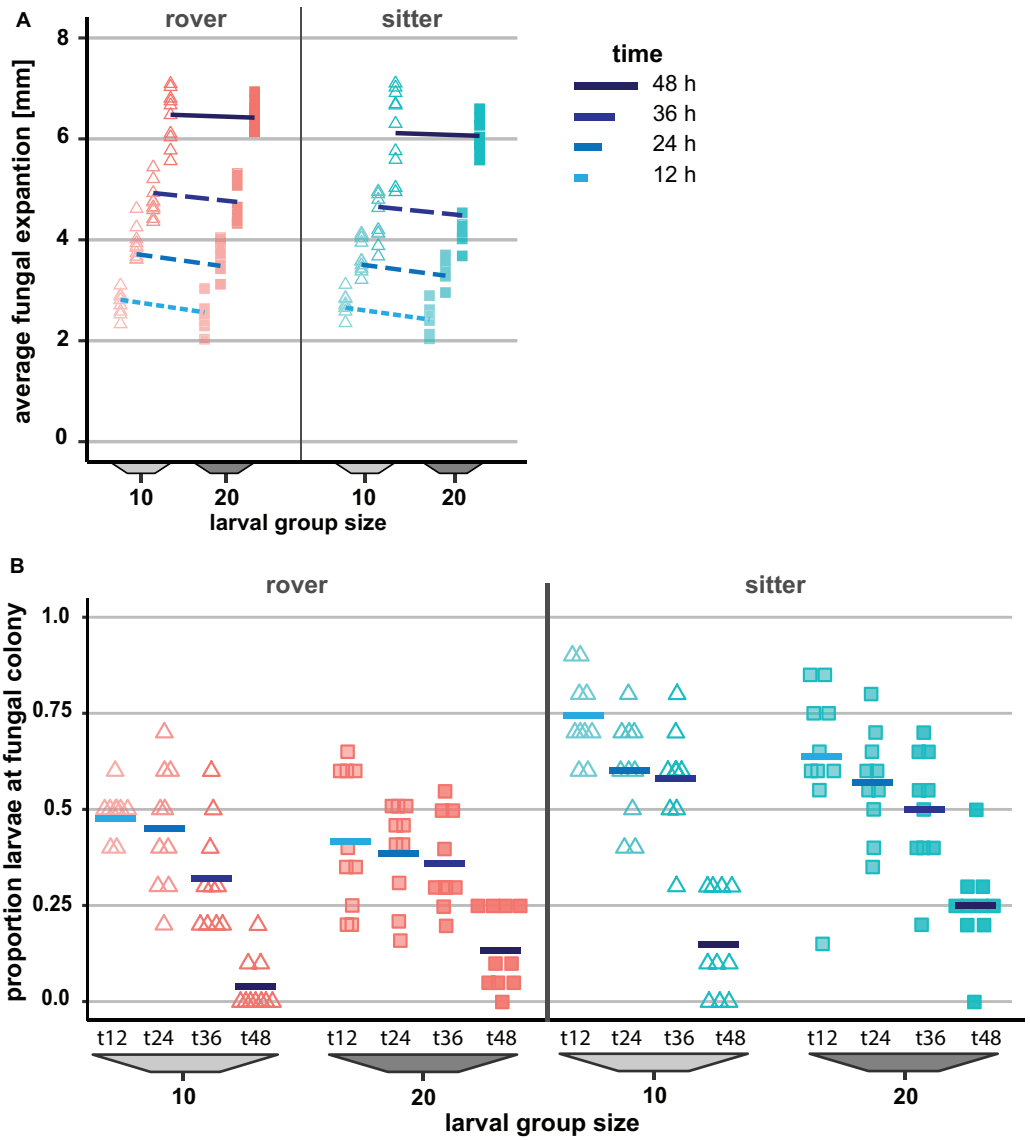


**FIGURE 5 |** Effect of genetically determined variation in the locomotion behavior of *Drosophila melanogaster* larvae on the spread of the insecticidal filamentous fungus *Aspergillus nidulans*. **(A)** Inhibition of the fungal expansion by *rover* and *sitter Drosophila* larvae in different group sizes (constant density = 0.4 larvae/mm). Fungal growth was determined for four time points after larval transfer as the average expansion of the fungus into the substrate surface per millimeter of growth front. Lines represent the fitted model estimates for the four time points (GEE; **Table 4**). **(B)** Proportion of larvae in direct contact with the fungal colonies at four different time points after larval transfer (**Table 4**).

size: $F_{1,155} = 6.449$, $p = 0.0121$; **Figure 5B**, **Table 4**, Q-Q plot **Supplementary Figure S2F**). Further, the decline of larvae in contact with the colony at time point 48 h coincides with the generic behavior of *D. melanogaster* larvae to leave the substrate surface around the beginning of the third larval stage, where they start digging deeper into the substrate.

## DISCUSSION

The existence and effect size of collective actions, such as defense, can be inferred from the extent to which the efficiency of performing a particular fitness-related "task" increases with increasing group size, while the density of individuals involved remains constant (Cotter and Kilner, 2010; Dornhaus et al., 2012). In a series of independent experiments, we tested whether larvae of the fruit fly *D. melanogaster* can collectively increase the efficiency of such a "task" that is directed against an insecticidal fungus invading the insects' habitat. While insect density clearly is, as expected, a significant factor in the suppression of the harmful fungus *A. nidulans*, we repeatedly found that larvae in larger groups, regardless of these density effects, suppressed the invasion of this fungus into their habitat more sustainably. This indicates that the emergence of a collective action in larger larval

**TABLE 3 |** Factors influencing expansion of *Aspergillus nidulans* in Experiment 3. Results of the most parsimonious GEE model (after backward elimination of non-significant factors) are shown.

| Explanatory factor | Model estimate±SE | *Wald* | *p*-value |
|---|---|---|---|
| Intercept | 0.8740 ± 0.0510 | 293.63 | < 0.001 |
| Time | 0.0210 ± 0.0014 | 214.97 | < 0.001 |
| Rover *vs*. Sitter | −0.0570 ± 0.0221 | 6.84 | 0.009 |
| Group size | −0.0120 ± 0.0035 | 11.61 | < 0.001 |
| Time × Group size | 0.0002 ± 0.0001 | 6.13 | 0.013 |
| Alpha | 0.476 ± 0.087 | | |
| $R^2_{marg}$ | 0.924 | | |

**TABLE 4 |** Factors influencing the proportion of *Drosophila melanogaster* larvae found in contact with *Aspergillus nidulans* colonies in Experiment 3. Results of the most parsimonious GLM (after backward elimination of non-significant factors) are shown.

| Explanatory factor | Model estimate±SE | *t* | *p*-value |
|---|---|---|---|
| Intercept | 1.8208 ± 0.5128 | 3.55 | < 0.001 |
| Time | −0.0860 ± 0.0165 | −5.23 | < 0.001 |
| Group size | −0.0732 ± 0.0292 | −2.51 | 0.013 |
| Rover *vs*. Sitter | 0.7985 ± 0.1125 | 7.10 | < 0.001 |
| Time *x* Group size | 0.0024 ± 0.0009 | 2.51 | 0.013 |

groups may constitute a central yet overlooked component of the suppression of harmful microbes in fruit fly larvae.

The aggregation of larvae on colonies of filamentous fungi probably results from a sensory response evolved in the context of the detection of food microbes, e.g., yeast fungi (Cooper, 1960), because both yeast and mold fungi emit volatile metabolites to which fruit fly larvae are attracted (Stötefeld et al., 2015). In previous observations, we found that a mold–fruit substratum can indeed serve as a benign dietary environment when *A. nidulans* is genetically (Trienens et al., 2010; Caballero Ortiz et al., 2013) or chemically (Caballero Ortiz et al., 2018) impaired in the production of toxic metabolites that otherwise deter, weaken, or even kill fly larvae. It therefore remains to be seen to what extent the aggregation of larvae on harmful fungi is due to an ancestral attraction to food microbes.

When *D. melanogaster* larvae reach the fungal growth front, they reduce their roving activity and display intensive mouth hook movements on the mycelium without crawling further to the center of the fungal colony (**Figure 2C**). This response leads to visible disruption of young exploitative hyphae, reduced formation of aerial hyphae, and impaired development of reproductive organs, i.e., a smaller fruiting zone (**Figure 2C**) (see also Trienens et al., 2010). To identify the exact mechanisms underlying the collectively caused suppression of the fungus, it will be important to test whether variation in group size induces changes in the expression of individual larval behavioral or physiological traits (e.g., release of antimicrobial peptides; Rolff and Schmid-Hempel, 2016). As it has been proposed for group size-dependent emergence of collective behaviors in ants (Dornhaus et al., 2012), we hypothesize that group size-dependent changes in the antimicrobial traits of fruit fly larvae would render larger larval interaction networks more effective in gaining control of their habitat.

Understanding how antimicrobial traits contribute to the formation of collective patterns from the interaction of individuals requires a combination of detailed observation of individual behavior and physiological approaches. Given that we found variation in larval locomotor activity—as determined by variation in the *foraging* locus—to differentially influence fungal growth suggests that behavior may indeed be a crucial factor in building up collective defense actions. This is corroborated by our observation that the larvae of the *sitter* phenotype appear to have more intensive contact with the fungus than that of the *rover* phenotype. That is, there seems to be genetic variation in how the insects respond to a harmful fungus at the level of behavior, which has consequences for the efficiency of microbial control. Whether this phenotypic variation has a function in the emergence of a collective defense in mixed genotype populations (Jolles et al., 2020) remains to be investigated for the *Drosophila* system.

Explaining the emergence of social complexity in insect communities is one of the chief problems in evolutionary ecology. By separating group size from density effects, our results suggest that non-(eu)social *D. melanogaster* fruit fly larvae harbor the potential to collectively suppress the invasion of a harmful filamentous fungus. It has been repeatedly stated that collective actions that keep harmful microbes in check are not limited to eusocial insects or those with sophisticated parental care in small family groups (Biedermann and Taborsky, 2011; Hwang and Lin, 2013; Meunier, 2015). On the contrary, it is assumed that collective actions have their evolutionary origin in comparatively simple semi-social aggregation behaviors, such as the *Drosophila* system, which lack comparatively complex behavioral structuring, nest building, and altruism (Meunier, 2015; Biedermann and Rohlfs, 2017).

Our finding thus supports the suggestions made by Meunier (2015) or van Meyel et al. (2018) that, in tracing the origin of complex collective defenses, such as social immunity, we need to better understand the extent to which non-(eu)social insects have evolved group-based strategies to manage the microbial environment and thereby stabilize habitat conditions to the benefit of their fitness (Biedermann and Rohlfs, 2017). Several recent studies have started revealing the mechanisms underlying social interactions, incl. collective actions, between *Drosophila* larvae, as related to food choice (Lihoreau et al., 2016), spatial foraging (Dombrovski et al., 2017), microbiome- and pheromone-mediated mutual attraction (Mast et al., 2014; Venu et al., 2014), and physical contact (Otto et al., 2016). *Drosophila* fruit flies and their interactions with microbes could thus be a suitable model system that would allow us to better understand how social complexity in insects can evolve from behavior in simple social precursor systems.

## DATA AVAILABILITY STATEMENT

Raw data are available as **Supplementary Material** in Table 1.XLSX and additional information in Data Sheet 1.docx.

## AUTHOR CONTRIBUTIONS

MR and MT designed the study, did the statistical analyses, and wrote the manuscript. MT performed the experiments and generated the data.

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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.2020.00079/full#supplementary-material

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# Cooperation and Conflict Within the Microbiota and Their Effects On Animal Hosts

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Symbioses between animals and microbes are ubiquitous, and often have drastic fitness effects on both parties. A rapidly growing body of research now shows that many of these effects are driven by social interactions among the symbionts. For instance, microbes frequently cooperate by producing shareable "public goods" that can mediate both virulence and host-beneficial functions. Conversely, hosts often exert control over symbionts by targeting their social interactions. Despite this pivotal role, we have only started to uncover the full diversity of microbial interactions, and many of the factors that shape variation in their effects on host function and evolution across different symbioses remain elusive. Here, we (i) review the known diversity of microbial interactions across different symbioses, and (ii) argue that variation in their nature and impact is often determined by differences in symbiont diversity. In particular, we first give a primer on the social lives of microbes, and then discuss how intraspecific and interspecific interactions among microbial symbionts affect – and are affected by – their host. Subsequently, we move to the evolution of symbiosis, and discuss the role of microbial interactions in symbioses that feature only few versus many different symbiont species. We show that symbiont-rich symbioses are shaped by strong interspecific competition, which selects against many host-beneficial forms of microbial cooperation, and thereby limits the scope for the evolution of strong host-symbiont dependencies. Conversely, symbioses involving only few symbiont species are often characterized by forms of microbial cooperation that mediate host-beneficial services, a situation that increases the scope for the evolution of host-symbiont dependencies. Overall, we infer that the explicit consideration of social dynamics within symbiont communities of varying complexity is crucial to advance our understanding of how microbes shape animal function and evolution.

**Keywords: symbiosis, microbiota, public goods, sociality, cooperation, competition**

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## SYMBIOSES: AN INTRODUCTION

Prolonged and intimate associations between animals and microbes are ubiquitous in nature and occur in a variety of different forms. They can involve both invertebrate and vertebrate hosts, and may comprise only few or many different species of microbe, including bacteria, protists, and fungi (Boucher et al., 1982; Douglas, 2018). Moreover, such symbioses can vary tremendously in terms

of their function for – and fitness effects on – the host and its symbionts. While some microbes benefit their host by supplying metabolic or defensive capabilities in exchange for nutrients and/or protection (Oliver and Martinez, 2014; Flórez et al., 2015; Mushegian and Ebert, 2016), others may drastically reduce host fitness by selfishly exploiting host resources without providing anything in return (Bull, 1994; Leggett et al., 2014). Finally, symbioses can also differ in the mode of symbiont transmission, the degree to which host and symbionts depend on each other, and the extent to which fitness effects on the host are largely determined by few versus many different symbionts (Fisher et al., 2017; Foster et al., 2017). Together, these differences give rise to a vast diversity of animal-microbe associations, ranging from the facultative parasitic "symbiosis" between humans and the opportunistic pathogen *Pseudomonas aeruginosa* (Andersen et al., 2015) to the obligate mutualism of certain cicadas with their organelle-like nutritional symbiont *Hodgkinia cicadicola* (McCutcheon et al., 2009).

Symbiotic associations often have substantial advantages for both the host and its microbe(s). From the host's viewpoint, associating with microbes can make it easier to cope with environmental challenges or spread to formerly uninhabitable environments. From the microbe's viewpoint, associating with animals can offer access to a "safe harbor" from which other hosts or environmental habitats can be colonized (Boucher et al., 1982; Douglas, 2018). The often substantial fitness effects of their interaction can cause the lives of host and microbes to become deeply intertwined (McFall-Ngai et al., 2013; Douglas, 2018). For instance, microbes can affect the development, communication, and behavior of their animal host (McFall-Ngai et al., 2013; Johnson and Foster, 2018), and might even drive host sociality if a repeated and reliable transmission of entire symbiont communities is necessary to ensure symbiont-mediated benefits to the host (Lombardo, 2008; Ezenwa et al., 2016). Conversely, animals can affect the density, distribution, and diversity of their microbial community (Hooper et al., 2012; Foster et al., 2017), and have been shown to interfere in social interactions among their symbionts (Ismail et al., 2016; Pietschke et al., 2017). Together, these observations suggest that studying the effects that animals and microbes may have on each other is crucial to understanding animal function and evolution.

The study of such reciprocal effects between symbiotic partners has traditionally focused on highly specialized associations featuring only a single, readily detectable type of microbe. However, recent years have seen a surge in research that deploys next-generation sequencing methods to investigate these effects in symbioses involving complex microbial communities (McFall-Ngai et al., 2013; Douglas, 2018). This new body of research has revealed that the composition and functioning of symbiont communities are crucial in determining the effects of the symbiotic associations on the host (Cryan and Dinan, 2012; Sharon et al., 2016; Johnson and Foster, 2018). Intriguingly, community composition and functioning are themselves shaped by competitive and cooperative interactions among the constituent microbes (West et al., 2006; Mitri and Foster, 2013; Nadell et al., 2016), and a number of recent studies highlights that such interactions frequently occur within animal hosts (Kommineni et al., 2015; Chatzidaki-Livanis et al., 2016; Rakoff-Nahoum et al., 2016; Wexler et al., 2016). Together with the observation that cooperation among symbionts often mediates host-beneficial services in "traditional" symbioses (Douglas, 1998; Schwartzman and Ruby, 2016), this suggests that social interactions among symbionts might be important factors shaping effects on the host across a wide range of symbioses (Costello et al., 2012; Coyte et al., 2015; Foster et al., 2017). It is hence crucial to unravel the occurrence and role of such interactions in symbioses with both simple and complex symbiont communities.

In this review, we showcase the diversity of microbial interactions across different symbioses and argue that the nature and impact of these interactions on host fitness are often determined by the diversity of the symbiont community. In particular, we (i) give a brief overview of social interactions among microbes, and then (ii) outline how social interactions among microbial symbionts affect – and are affected by – their host. Finally, we move to the evolution of symbiosis, and (iii) discuss the role of microbial interactions in two scenarios of symbiosis that represent opposing ends on a continuum of symbiont diversity, and hence differ in the relative scope for intraspecific versus interspecific interactions among the symbionts. Overall, our review highlights the diversity of symbiont social interactions, and shows that an explicit consideration of these interactions and their varying role in symbioses featuring few versus many symbiont species is crucial to advance our understanding of how microbes shape animal function and evolution. Note that although we mostly focus on interactions among bacteria, we expect our conclusions to be applicable to other microbes as well. A glossary with the definitions of important terms (in bold print below) is provided at the end of the manuscript.

## A PRIMER ON THE SOCIAL LIVES OF MICROBES

Contrary to the historically held view of microbes as solitary organisms, an impressive body of research now shows that microbial life histories are characterized by intricate webs of cooperative and competitive interactions. This new view of microbial life was initially popularized by the discovery of sophisticated cooperative behaviors in myxobacteria and eukaryotic slime molds, where single cells come together to form multicellular fruiting bodies that allow some cells to disperse as stress-resistant spores (Strassmann et al., 2000; Velicer et al., 2000). Over the last three decades, it has become clear that microbes typically live in dense and diverse communities in which **cooperation**, **competition**, and **predation** all occur frequently, and play a crucial role in shaping community composition and functioning (West et al., 2006; Little et al., 2008; Mitri and Foster, 2013; Nadell et al., 2016; Pérez et al., 2016).

Microbes can engage in a surprising diversity of cooperative behaviors. They regularly form multicellular structures such as biofilms, communicate with each other via chemical signals, and engage in group-coordinated motility, resource acquisition

and "chemical warfare" against predators or competitors (Crespi, 2001; Velicer, 2003; West et al., 2007; Foster, 2010; Granato et al., 2019). Most of these cooperative behaviors are mediated by the release of costly metabolites (**Figure 1A**). For instance, bacterial communication often involves the release and group-wide detection of small diffusible signal molecules that accumulate in the local environment, and thereby allow individual cells to collectively alter global patterns of gene expression once a concentration threshold is reached (quorum sensing; Williams, 2007; Whiteley et al., 2017). Similar secretion-dependent cooperative behaviors range from iron acquisition, where cells release siderophores to scavenge iron from environmental stocks (Griffin et al., 2004; Leventhal et al., 2019), to the formation of biofilms, where cells release structural polysaccharides to form an extracellular matrix (Greig and Travisano, 2004; Kearns, 2010; Nadell et al., 2016).

The secretion of costly metabolites often makes them accessible to other cells in the vicinity of the producer. Such "**public goods**" hence not only benefit the producer and its clonemates, but can also affect other community members (Kümmerli and Ross-Gillespie, 2014), and may then induce a variety of social interactions. In some cases, public goods production can spur mutually-beneficial division of labor involving the exchange of different types of public good between different phenotypes, strains, or species (Amin et al., 2009; van Gestel et al., 2015; Kim et al., 2016; Dragoš et al., 2018). For instance, the sliding motility of of *Bacillus subtilis* critically depends on an interaction between cells that produce matrix components to form migration loops, and cells that produce the organic "lubricant" surfactin to reduce the cell-surface friction (van Gestel et al., 2015). Other examples of such mutually beneficial interactions involve cooperative cross-feeding, a form of mutualism whereby each of the partners produces a costly metabolite that is consumed by the other (Shou et al., 2007; Pande et al., 2015; **Figure 1A**). Such cooperative cross-feeding is thought to evolve readily from by-product benefits arising where different partners feed on each other's waste products (Zelezniak et al., 2015; D'Souza et al., 2018).

Despite the frequent occurrence of cooperative interactions, the social lives of microbes are often far from peaceful. This is because cooperative behaviors can often be exploited by cheaters that reap the benefits of cooperation without cooperating to the same extent themselves (West et al., 2006; Ghoul et al., 2014; Özkaya et al., 2017; **Figure 1B**). The resulting tug-of-war between cooperators and cheaters can lead to a "tragedy of the commons," where cooperation collapses despite its group-level benefits (Rankin et al., 2007; MacLean, 2008). Although mitigating factors, such as increased spatial structure, often prevent the complete collapse of cooperation, cheating can have a profound influence on the evolutionary dynamics of microbial communities (Griffin et al., 2004; Ross-Gillespie et al., 2007; Kümmerli et al., 2009; Özkaya et al., 2017). For instance, cheating among members of one species can negate its competitive advantage over a second, usually inferior species, and might thereby foster species coexistence (Leinweber et al., 2017).

In addition to cheating, microbes may deploy a range of other antagonistic strategies to compete with non-clonemates for limited resources and space (Hibbing et al., 2010; Ghoul and Mitri, 2016; Bauer et al., 2018). Such strategies range from the release of surface-modifying polysaccharides that impede the attachment of competitors, over the secretion of antibiotics and other toxins, to various variations of contact-dependent killing (Valle et al., 2006; Granato et al., 2019; **Figure 1B**). Note that the investment into toxins and antibiotics is often cooperative from the producer's perspective. This is because these compounds are typically costly to produce and can – once secreted – benefit clonemates of the producer (and other resistant cells that stand in competition with the targeted adversary). Whether a costly secreted compound is an exploitable public good or an imminent threat is hence often a matter of perspective (Niehus et al., 2017).

Apart from competition and cooperation, predation (in which we include parasitism for the sake of brevity) is the third fundamental type of interaction shaping microbial communities (**Figure 1C**). Many bacterial predators deploy a solitary hunting strategy whereby they either attach to their prey and consume it from the outside (e.g., *Vampirococcus spp.*) or penetrate the periplasmic space to consume it from within (e.g., *Bdellovibrio bacteriovorus*). However, others can perform cooperative attacks involving the quorum-sensing regulated production of enzymes and other secondary metabolites that degrade the prey cells (e.g., *Myxococcus xanthus*; reviewed in Martin, 2002; Pérez et al., 2016). Together with cooperative and competitive interactions, such cases of predation give rise to an intricate network of social interactions that jointly determine the composition and functioning of microbial communities, and thereby shape all aspects of microbial life (West et al., 2006; Mitri and Foster, 2013; Nadell et al., 2016; Pérez et al., 2016).

## THE SOCIAL DIMENSIONS OF SYMBIOSIS

Microbes frequently interact with each other within or upon their animal host, and these interactions can mediate effects on – and serve as a target for – the host. The resulting complex web of effects can be broken down into three "principal" social dimensions of **symbiosis** delineating effects from (i) microbe to microbe, (ii) microbe to host, and (iii) host to microbe (Foster et al., 2017). Below, we separately introduce each of these dimensions and then discuss how they are shaped by intraspecific and interspecific interactions among microbes.

### Microbe to Microbe

Like microbes in natural habitats, microbial symbionts frequently engage in a variety of social interactions with other members of the **microbiota**. Interactions between conspecific symbionts are typically cooperative and often mediate interspecific interactions (see below) and direct effects on the host (see section "Microbe to Host"). By contrast, interactions between heterospecific symbionts may range from cooperation over competition to predation. Interspecific cooperation often occurs in the form of cross-feeding. For example, the human gut symbiont *Bacteroides ovatus* can break down the complex carbohydrate inulin to the benefit of its congener *B. vulgatus*. This behavior

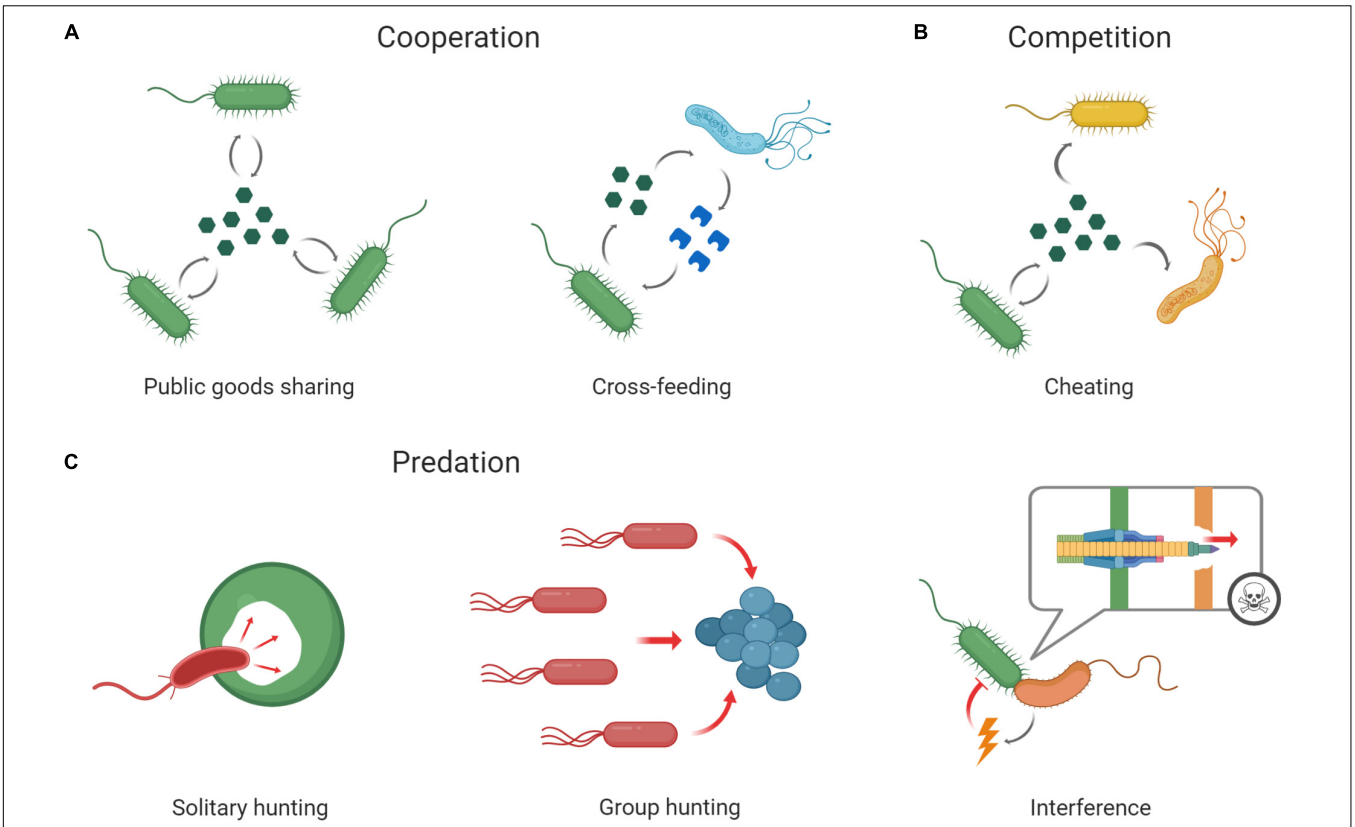


**FIGURE 1 |** Social interactions among microbes. Microbes frequently interact with clonemates or other microbes of the same or different species (represented by microbes of different colors and shapes, respectively). **(A)** Cooperation often involves *Public goods sharing*, where cells secrete a costly secondary metabolite that can be shared among cells. Alternatively, cooperation can involve *Cross-feeding*, where different genotypes or species exchange different metabolites. **(B)** Competition is often mediated by *Cheating*, where individuals capitalize on the public goods produced by others while not contributing (to the same extent) themselves. Other forms of competition involve the direct *Interference* with competitors, either via the secretion of diffusible toxins (lightning bolt), or via contact-dependent mechanisms (such as type VI secretion systems; speech bubble). **(C)** Predation frequently occurs in the form of *Solitary hunting*, and can, for instance, involve single predators that attack and digest their prey from within. Alternatively, it can involve *Group hunting*, where multiple cells coordinate their attack.

increases the fitness of *B. ovatus* despite the costs of inulin breakdown, since *B. ovatus* receives reciprocal benefits from *B. vulgatus* in return (Rakoff-Nahoum et al., 2016). Metabolic cross-feeding also occurs between symbionts of the glassy-winged sharpshooter *Homalodisca coagulata*, where the symbiont *Baumannia cicadellinicola* receives essential amino acids from *Sulcia muelleri* and provides vitamins and co-factors in return (Wu et al., 2006). The frequent occurrence of similar metabolic complementarities among microbes hosted by plant-sap feeding insects (McCutcheon and Von Dohlen, 2011; Douglas, 2016), marine oligochaete worms (Dubilier et al., 2001; Woyke et al., 2006), and vertebrates (Milani et al., 2015; Solden et al., 2018) indicates that cross-feeding among symbionts might be common and taxonomically widespread.

Another form of cooperation known to occur among microbial symbionts is coaggregation. This process involves individuals of different species attaching to each other via specific molecules, and thereby promotes the formation of mixed-species biofilms (Rickard et al., 2003; Kuramitsu et al., 2007). For instance, two early colonizers of the tooth surface, *Streptococcus oralis* and *Actinomyces naeslundii*, can only form stable biofilms on a tooth-like surface when coaggregated, suggesting that their coaggregation is mutualistic (Palmer et al., 2001). Intriguingly, interspecific biofilm formation and other forms of interspecific cooperation might often be regulated via interspecific quorum sensing (Rickard et al., 2006; Cuadra-Saenz et al., 2012). Specifically, different bacterial species might communicate using the auto-inducer AI-2, a signaling molecule that is produced and perceived by many different species (Pereira et al., 2013). In line with this idea, AI-2 expression has been shown to affect interactions among symbionts of the human gut (Thompson et al., 2015) and oral cavity (Cuadra-Saenz et al., 2012). Note, however, that it is often hard to determine whether the auto-inducer indeed serves as a signal in real communication (*sensu* Scott-Phillips, 2008), or merely as a cue allowing competing species to eavesdrop on one another.

Although microbes can cooperate with other microbes, competition for limited host resources and space might account for the greater part of microbe-microbe interactions (Coyte et al., 2015). The pervasive occurrence of interference competition is well documented among human gut symbionts. For instance, common bacteria such as *Enterococcus faecalis* and *Bacteroides*

*uniformis* can secrete a whole arsenal of toxins to combat other gut microbes (Kommineni et al., 2015; Roelofs et al., 2016). Conversely, *B. fragilis* uses specific Type VI secretion system to deliver toxins directly into competing species (Chatzidaki-Livanis et al., 2016; Wexler et al., 2016), an ability that it shares with many other gut Bacteroidales (Coyne et al., 2016; García-Bayona and Comstock, 2018). Similar cases of interference competition also occur in invertebrates. For example, sponge symbionts of the genus *Pseudovibrio* secrete toxins against sponge-derived *Bacillus* species (Esteves et al., 2017), and secretion systems for contact-dependent killing occur in *Snodgrassella alvi*, a gut symbiont of honey and bumble bees, and in *V. fischeri*, the defensive symbiont of the bobtail squid (Steele et al., 2017; Speare et al., 2018). Inter-species competition is hence a pervasive force shaping multi-species symbiont communities.

In contrast to cooperative and competitive interactions, the occurrence and role of predation among microbial symbionts has received little scrutiny. However, protist amoebas and bacterial predators have been detected in the microbiome of many animals, including corals, sponges, insects, and humans (Iebba et al., 2013; Welsh et al., 2016; Johnke et al., 2019). Intriguingly, the presence, abundance, and richness of predatory species is positively correlated with overall microbiome diversity in many cases (Johnke et al., 2019), suggesting that predation may shape the composition of symbiont communities. Overall, the above examples illustrate that the intricate web of interactions characteristic of microbial communities in natural habitats also occurs in animal hosts.

## Microbe to Host

Microbes can have both negative and positive effects on their host, and these effects are often mediated by social interactions among them. In general, negative effects predominate in parasitic symbioses and arise because microbes overexploit host resources (Murray and Murray, 1979; Dantzer et al., 2008). By contrast, positive effects predominate in mutualistic symbioses and typically arise because microbes complement the host's metabolic capabilities, contributing to (i) host metabolism, for example by digesting or synthesizing nutrients (Engel and Moran, 2013; Oliver and Martinez, 2014); (ii) host defenses, for example by conferring camouflage or producing defensive toxins (Oliver and Martinez, 2014; Flórez et al., 2015; Schwartzman and Ruby, 2016); (iii) host communication, for example by secreting metabolites that are used by hosts as sex or aggregation pheromones (Theis et al., 2013; Ezenwa and Williams, 2014; Wada-Katsumata et al., 2015); and (iv) host signaling networks, for example by serving as cues for the host to trigger the development of regulatory systems (Cryan and Dinan, 2012; Ezenwa et al., 2012; Hooper et al., 2012). Below, we review the role of intraspecific and interspecific interactions among microbes in mediating such effects and show that cooperation among microbes lies at the heart of key services that the microbes provide to their host.

### Intraspecific Interactions and Their Effects

Microbial effects on the host are often directly mediated by interactions among conspecific (clonal or closely related) microbes. This is best known from studies on host-pathogen interactions, where microbial cooperation is often crucial for virulence and disease progression (Buckling and Brockhurst, 2008; Leggett et al., 2014; Rezzoagli et al., 2020). Specifically, **pathogens** may deploy division of labor to thrive within the host (Ackermann et al., 2008; Diard et al., 2013), and often secrete "virulence factors" such as proteases, toxins, and siderophores that facilitate host colonization and exploitation (Rahme et al., 1995; Leggett et al., 2014; Rivera-Chávez and Mekalanos, 2019). Such studies are relevant for understanding symbioses, as the dynamics of pathogen infections are likely similar to those of infections with parasitic symbionts. Indeed, the malaria **parasite** *Plasmodium falciparum* secretes a whole arsenal of different compounds that not only remodel the host's red blood cells, but also cause them to stick to the blood vessel walls, thereby allowing the parasite to avoid splenic clearance (Tilley et al., 2011).

Similar interactions also occur in mutualistic symbioses, and often mediate nutritional or defensive services to the host. For instance, the obligate intracellular symbiont *Buchnera aphidicola* produces essential amino acids and makes them accessible to its aphid host via secretion (Douglas, 1998). From the perspective of an individual *Buchnera* cell, this behavior is not only cooperative toward the host, but also toward clonemates – i.e., it benefits them both and has at least partly been selected for because of these benefits. While the aphid benefits because it receives essential amino acids, clonemates of the focal *Buchnera* benefit because their fitness is closely linked to that of the host, such that the increase in host fitness due to the focal cell's secretion also increases their own fitness. The secretion of amino acids is partly selected for because of its benefits for the host; after all, it is the resulting increase in host fitness that directly increases the fitness of the secreting cell. However, the behavior is also partly selected for because of its benefits to clonemates, since such benefits to the fitness of relatives count toward the (indirect component of the) secreting cell's fitness (see West et al., 2006). Therefore, both direct and indirect fitness benefits jointly drive the evolution of symbiont cooperation. Notably, conceptualizing this behavior as cooperation with clonemates also highlights that it is in principle vulnerable to cheating (but see section "One Host – Few Microbes"). Specifically, it suggests that non-producing mutants that do not bear the costs of maintaining a dedicated enzymatic assembly for amino acid synthesis could potentially share into the benefits that the host provides to the symbionts in return for their service. The same logic presumably applies to many other symbioses of insects feeding on plant sap or other nutrient-poor resources (Baumann, 2005; Sabree et al., 2009; Salem et al., 2014).

In addition to their role in host nutrition, intraspecific cooperative behaviors also play a role in mediating host defenses. In the marine bacterium *Vibiro fischeri*, single cells use quorum sensing to regulate the bioluminescence that is thought to camouflage their host, the Hawaiian bobtail squid *Euprymna scolopes*, at night by distorting its dark silhouette within the water column (Verma and Miyashiro, 2013). Many other defensive symbioses are based on the symbionts producing dedicated antibiotics or toxins to the benefit of their host. For instance, symbionts produce antibiotics that specifically act against parasites of the host's cultivars in fungus-growing ants

(Currie et al., 1999; Haeder et al., 2009) and bark beetles (Scott et al., 2008; Oh et al., 2009), whereas the symbiont of the European beewolf *Philanthus triangulum* produces a cocktail of antibiotics that protect the beewolf's larvae from fungal infestation (Kaltenpoth et al., 2005; Kroiss et al., 2010; Engl et al., 2018). Like host provisioning, the secretion of compounds for host defense creates a (potentially cheatable) public good from the symbiont's perspective.

In contrast to effects on host nutrition and defenses, effects of symbionts on host signaling networks and communication are typically not directly mediated by social interactions. Instead, such effects primarily seem to reflect that animals evolved to integrate waste products of microbial origin and similar cues of microbial presence into their own development and functioning (Dillon and Charnley, 2002; Hooper et al., 2012; Theis et al., 2013; Wada-Katsumata et al., 2015; Douglas, 2018). For instance, the German cockroach *Blattella germanica* uses volatile carboxylic acids, common by-products of microbial metabolism, as an aggregation cue (Wada-Katsumata et al., 2015). Similarly, mice seem to use such by-products to induce the development of colonic regulatory T-cells (Smith et al., 2013). Nevertheless, the observation that some bacteria secrete host signaling molecules (Cryan and Dinan, 2012; Rastelli et al., 2019) suggests that microbes sometimes cooperate to manipulate host signaling networks. Indeed, the gut bacterium *Bacteroides fragilis* actively suppresses an inflammation response of its human host by releasing vesicles that contain the signaling molecule polysaccharide A (Shen et al., 2012). Conversely, the protist parasite *Toxoplasma gondii* increases dopamine titers in rodent hosts by releasing the rate-limiting enzyme for dopamine synthesis, thereby triggering changes to the rodent's behavior that are thought to increase the parasite's transmission to its definitive feline host (Vyas et al., 2007; Prandovszky et al., 2011). Note, however, that it is currently unclear how frequent such putative cases of manipulation occur, because they are as vulnerable to cheating as other cases of (public goods) cooperation (see section "One Host – Many Microbes" and Johnson and Foster, 2018).

### Interspecific Interactions and Their Effects

Many microbial effects are not directly mediated by intraspecific microbial interactions, but instead arise as (mostly indirect) aftereffect of interactions among different microbe species. Such multipartite effects have received most attention in studies on pathogenic microbes. This is because co-infections of one pathogen with other pathogens or members of the microbiota often display increased virulence and enhanced pathogen persistence in comparison to infections by single pathogens (Alizon et al., 2013; Murray et al., 2014; Tay et al., 2016). Such "polymicrobial synergy" can arise because interspecific competition promotes higher pathogen growth and virulence, or because pathogens can reap by-product benefits from co-infecting microbes (Frank, 1996b; Tay et al., 2016). For instance, *P. aeruginosa* can use peptidoglycans shed by Gram-positive bacteria as a cue to increase the production of compounds that not only harm potential competitors, but also exacerbate disease severity by inflicting damage on the host (Korgaonkar et al., 2013). Similarly, the virulence of the opportunistic pathogen *Aggregatibacter actinomycetemcomitans* is increased in co-infections with the resident symbiont *Streptococcus gordonii*, because the pathogen can metabolize L-lactate, a waste product of the symbiont's metabolism (Ramsey et al., 2011).

While interactions between pathogens and resident symbionts are detrimental in some situations, they can boost host defenses in others. First, hosts can benefit if their symbionts outcompete the pathogenic intruder (competitive exclusion; Koch and Schmid-Hempel, 2011; Buffie and Pamer, 2013; Fraune et al., 2015; Schwarz et al., 2016; Chiu et al., 2017; Oliveira et al., 2020). For example, the human gut symbiont *E. coli* can reduce intestinal colonization by *S. enterica* through siderophore-mediated iron competition (Deriu et al., 2013), while *Ruminococcus obeum* can hamper the colonization of *Vibrio cholerae* through the quorum-sensing-mediated repression of multiple virulence factors (Hsiao et al., 2014). Second, hosts can benefit if a symbiont induces a host immune response that is more deleterious to the pathogen than to itself (Douglas, 2018). Such "apparent competition" occurs in tsetse flies, where *Wigglesworthia glossinidia* triggers the development of the host's immune system, and thereby prevents the host from succumbing to *E. coli* infections (Weiss et al., 2012). Finally, hosts can also benefit if predatory symbionts target pathogens. Such "predatory exclusion" occurs in the coral *Montastraea cavernosa*, where *Halobacteriovorax* bacteria prey on the pathogenic *Vibrio coralliilyticus* (Welsh et al., 2017).

Multipartite effects can finally also occur in a non-pathogenic context. This is best exemplified by cross-feeding interactions – such as those among symbionts of the marine oligochaete *O. algarvensis* and plant-sap feeding insects like the sharpshooter *H. coagulata* (see section "Microbe to Microbe") – where the hosts critically rely on metabolites provided by all involved symbionts (Dubilier et al., 2001; Woyke et al., 2006; Wu et al., 2006). Similar effects might also underlie benefits the host derives in other contexts. For instance, cooperative (or by-product) cross-feeding among microbes could increase the availability of metabolites used in host communication. In general, such multipartite effects are likely pervasive in multi-partner symbioses (Zélé et al., 2018).

## Host to Microbe

Microbes can have a substantial impact on host fitness, and hosts therefore have a strong incentive to manage the abundance and composition of their microbiota (Douglas, 2018). In particular, hosts typically suppress the growth of detrimental microbes using antimicrobial peptides and other immune effectors (Login et al., 2011; Franzenburg et al., 2013; Peterson and Artis, 2014; Foster et al., 2017), but may also promote the growth of beneficial microbes by provisioning them with nutrients (Douglas, 1998; Graf and Ruby, 1998; Arike and Hansson, 2016). While many of the resulting effects on symbiont fitness likely arise independently of the symbiont's social behavior, hosts at least sometimes directly target symbiont social traits. Numerous studies on host-pathogen interactions lend credit to this notion. For instance, hosts regularly interfere with pathogen growth by sequestering the pathogen's siderophores and by producing their own (Flo et al., 2004; Fischbach et al., 2006). Moreover, hosts can reduce pathogen persistence and virulence by inhibiting

biofilm formation and by targeting additional virulence factors such as proteases (Singh et al., 2002; Overhage et al., 2008; Le et al., 2017). Finally, hosts can also reduce virulence by interfering with the pathogen's quorum sensing communication (quorum quenching; Chun et al., 2004; Grandclément et al., 2015; Weiland-Bräuer et al., 2019).

The occurrence of similar effects on symbiont behaviors has received little scrutiny in a non-pathogenic context. However, one example of quorum sensing manipulation has recently been reported in *Hydra vulgaris*: this freshwater polyp can modify the quorum sensing signal of its main colonizer *Curvibacter sp*. such that the modified signaling molecules promote host colonization by inducing a phenotypic change in the symbiont (Pietschke et al., 2017). A similar case of manipulation might occur in mammals, where epithelial cells produce a mimic of a common bacterial quorum sensing signal in response to secreted bacterial factors or epithelial breaches (Ismail et al., 2016). Although the benefits of manipulation in this latter case are thus far unclear, quorum sensing systems seem to be ideal targets for host control, because they serve as "master-switches" for the simultaneous regulation of many different microbial traits (Pietschke et al., 2017). Finally, note that hosts can also indirectly affect symbiont interactions. For instance, the mucus secreted by epithelial cells often promotes symbiont attachment in addition to serving as a food resource (Sicard et al., 2017). This arrangement may promote microbial cooperation in mucus digestion (Rakoff-Nahoum et al., 2014, 2016), while simultaneously providing the spatial structure that favors its maintenance due to an increased symbiont relatedness (West et al., 2006).

# THE EVOLUTION OF HOST-MICROBE INTERACTIONS IN THE INNER ECOSYSTEM

Animals diverged from their protist ancestor roughly 650 million years ago, and many animals have evolved in close association with microbes ever since. Although such associations often have substantial advantages for both parties, they are never entirely free of conflict because host and symbiont(s) are not perfectly related and may thus have diverging fitness interests (Leigh, 2010; Barker et al., 2017; McCutcheon et al., 2019). Adaptations of hosts and symbionts to each other's presence thus often evolve in a field of tension between cooperation and conflict. For instance, host adaptations include a variety of mechanism to manage the abundance and composition of the microbiota, and these mechanisms may either aim at promoting beneficial or at harming detrimental microbes. Conversely, symbiont adaptations center around the persistence in the **microbiome** (Webster, 2014; Foster et al., 2017), and thus often include mechanisms to compete or cooperate with the host or other members of the microbiota. However, microbiotas are strikingly diverse across animal groups in terms of the number of symbiont species (Engel and Moran, 2013; Colston and Jackson, 2016). For instance, vertebrates typically harbor more symbionts than invertebrates, presumably due to underlying differences in their morphology, physiology, and immunity (McFall-Ngai, 2007; Engel and Moran, 2013; Colston and Jackson, 2016; Woodhams et al., 2020). Conversely, animals feeding on complex diets typically harbor more symbionts than those feeding on simple diets, presumably because they have an increased diet-related uptake of environmental microbes or need to maintain a higher symbiont diversity to ensure the digestion of their diet (Ley et al., 2008a; Engel and Moran, 2013; Reese and Dunn, 2018). Irrespective of their origin, these differences in symbiont diversity affect the occurrence and nature of intraspecific and interspecific interactions in the microbiome (see the sections above) and might also come with different requirements on host control. Variation in microbial interactions and host control might, in turn, affect the scope for cooperation versus conflict between the symbiont(s) and the host. Overall, differences in symbiont diversity could hence profoundly affect many aspects of animal function and evolution via their impact on symbiont interactions.

Below, we explore this notion by discussing two types of symbioses on opposing ends of a continuum of symbiont diversity that feature, respectively, a "simple" inner ecosystem involving few microbe species and a "complex" inner ecosystem involving many different microbe species. We show that the inner ecosystems of these two types of symbioses differ profoundly in terms of symbiont interactions, which in turn have key effects on the evolution of host-beneficial services, host control, and host-symbiont dependencies.

## One Host – Few Microbes

Many symbioses involve few or only one species of (typically mutualistic and often intracellular) microbe and are characterized by heavily skewed symbiont-dependent effects on host fitness (**Figure 2A**). For instance, the microbiota of plant-sap-feeding insects such as aphids and whiteflies are dominated by only one or two obligate, mutualistic endosymbionts. These symbionts provide the host with nutrients and protection, and the effects of these services on host fitness dwarf any effects that occasionally detected gut microbes may have on their host (Engel and Moran, 2013; Jing et al., 2014). Symbiosis with such "simple" inner ecosystems occur in many other invertebrates, including certain squid, marine oligochaetes, and blood-feeding insects (Dubilier et al., 2001; Graf et al., 2006; Engel and Moran, 2013; Schwartzman and Ruby, 2016). In these symbioses, the scope for interspecific interactions among symbionts is limited, and symbionts are therefore primarily shaped by intraspecific interactions and the host environment (Foster et al., 2017). Interactions with conspecifics are usually cooperative, and often mediate host-beneficial nutritional or defensive functions (see section "Microbe to Host"). Such host-beneficial services are favored in "simple" inner ecosystems, because their low microbial diversity increases both the potential for microbes to affect host survival and reproduction, and the potential to benefit from cooperating to do so (Foster and Wenseleers, 2006; Johnson and Foster, 2018).

Microbial cooperation mediating host-beneficial services is potentially vulnerable to cheating even in "simple" inner ecosystems (Frank, 1997; Ghoul et al., 2014; Özkaya et al., 2017). Because partners can derive substantial benefits from these services, selection against cheating may often act on both the

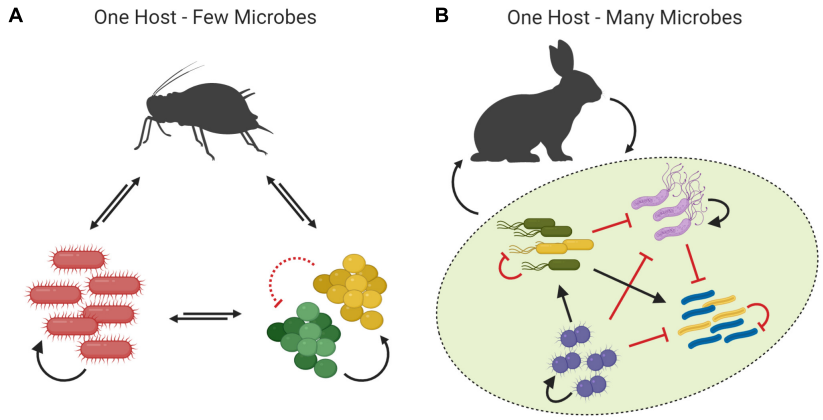


**FIGURE 2 |** The Inner Ecosystem. Different symbioses vary in the complexity of the inner ecosystem, the stage on which positive (black arrows) and negative (red arrows) interactions occur between microbes and their host and among microbes of the same or different species (represented by microbes of different colors and shapes, respectively). **(A)** Animals that harbor only few symbiont species have "simple" inner ecosystems. In such symbioses, interactions are usually mutually beneficial, and conflicts among microbes, or between microbes and the host, are limited due to strong, symbiont-specific host control and/or self-enforcement. **(B)** Animals that harbor many different symbiont species have "complex" inner ecosystems. In such symbioses, the host and individual microbe species are thought to benefit, on average, from their association, but symbiont-specific host control is limited such that the host can be considered to interact with the microbiota as a whole (green area). Because of the limited specificity of host control and the high diversity of the microbiota, interactions among different genotypes and symbiont species can be both cooperative or competitive.

symbiont(s) and the host. On the symbiont side, selection can favor "self-enforcement," i.e., the evolution of mechanisms that prevent cheating by pleiotropically linking selfish phenotypes to personal costs, or by limiting the phenotypic penetrance of mutations via redundancy (Ågren et al., 2019). A possible example occurs in *Buchnera*, where the synthesis of host-beneficial histidine is coupled to the synthesis of purins that *Buchnera* requires for growth. This coupling likely prevents freeloading, because a mutant that no longer produces histidine would also not acquire the purins it needs to sustain its own growth (Thomas et al., 2009). On the host side, selection can favor mechanisms that enforce symbiont cooperation, and allow the host to exert strong control over specific symbionts (Foster et al., 2017; Ågren et al., 2019). For instance, the aphid can control symbiont cooperation in the production of two essential amino acids, methionine and arginine, by adjusting the supply of the respective precursor, cystathionine and glutamine, to its symbiont (Price et al., 2014; Russell et al., 2014). Conversely, the bobtail squid likely controls symbiont cooperation in bioluminescence by monitoring symbiont oxygen consumption and killing cheaters that do not produce light and are thus unable to consume oxygen at a typical rate (Schwartzman and Ruby, 2016). The evolution of such symbiont-specific host control is possible in "simple" inner ecosystems, because the number of symbiont species that must be controlled simultaneously is relatively low (Foster et al., 2017).

Host control and symbiont self-control can promote the evolution of mutual dependencies, because they stabilize cooperative symbiont interactions, and may thereby ensure that the benefits of mutualistic symbioses to both partners persist over time (Douglas, 2018). Such dependencies, in turn, increase the alignment of host and symbiont fitness interests, and may thus reinforce selection for symbiont cooperation, host control, and symbiont self-enforcement. Accordingly, the origin of mutual dependencies can jumpstart a positive feedback-loop that promotes host-symbiont **coevolution**, and might ultimately render the symbiosis obligate for both partners by favoring an increasingly deep integration of host and symbiont into each other's development and function (Wein et al., 2019). Intriguingly, the evolution of such strong dependencies is often associated with a shift from horizontal (environmental) to vertical (parent-offspring) symbiont transmission (Frank, 1996a; Fisher et al., 2017). This shift can further reinforce the positive feedback between mutual dependencies and the mutual benefits of symbiosis, because vertical transmission increases the scope for host-beneficial cooperation among symbionts by ensuring high symbiont relatedness (Leeks et al., 2019). It is noteworthy that such high levels of symbiont relatedness can also be achieved among horizontally transmitted symbionts, for instance by imposing strong host control on immigration (as in the squid-*Vibrio* system; Nyholm and McFall-Ngai, 2004). In all these cases, host-beneficial services mediated by microbial cooperation are crucial drivers of a shift of selection to the aggregate level: hosts with "cooperative" symbionts are fitter than hosts harboring "selfish" (cheating) symbionts, favoring the propagation of "good" host-symbiont combinations.

Animals hosting few microbe species can gain substantial benefits of symbiosis, but they might also be especially vulnerable to pathogens and parasites. This is because the low diversity of their microbial community reduces the scope for the competitive exclusion of harmful microbes, and thus makes them vulnerable to manipulation (Foster et al., 2017; Johnson and Foster, 2018). In line with this idea, both pea aphids (*A. pisum*) and spider mites (*Tetranychus urticae*) do not increase their antibacterial defenses after an immune challenge, but instead seem to ramp up a terminal investment into reproduction (Altincicek et al.,

2008; Santos-Matos et al., 2017; Zélé et al., 2019). While the lack of antibacterial defenses in these (and other plant-sap feeding) arthropods has been ascribed to their limited exposure to food-born microbes, it might also reflect a shift in defensive strategies due to the limited chances for a successful defense against pathogens or parasites. This suggests that the benefits linked to harboring a "simple" inner ecosystem may come at the expense of defenses linked to hosting a diverse microbiota.

## One Host – Many Microbes

Many symbioses involve numerous species of (mutualistic, commensal, and/or parasitic) microbe, and are characterized by moderately skewed symbiont-dependent fitness effects (**Figure 2B**). For example, the human microbiota can comprise several hundred microbe species, of which many occur in considerable numbers and thus likely contribute significantly to the overall fitness effect of the microbiota as a whole (Qin et al., 2010). Symbiosis with such "complex" inner ecosystems are the norm among vertebrates (Colston and Jackson, 2016; Foster et al., 2017), and also occur in some invertebrates such as sponges, corals, and wood- or detritus-feeding beetles and termites (Engel and Moran, 2013; Thomas et al., 2016). In these symbioses, microbes are predominantly shaped by interspecific interactions (Foster et al., 2017). Although such interactions are sometimes mutualistic (Sachs and Hollowell, 2012; Zelezniak et al., 2015; Rakoff-Nahoum et al., 2016), they are thought to reflect competition for limited host resources in most cases (e.g., (Stein et al., 2013; Coyte et al., 2015; Roelofs et al., 2016; Wexler et al., 2016). Such interspecific competition has crucial consequences on the effects of symbionts on their host. This is because it puts symbionts investing in costly host-beneficial cooperation at a disadvantage relative to cheating conspecifics and other symbionts that refrain from investing in such behaviors (Johnson and Foster, 2018). In symbioses with "complex" inner ecosystems, positive effects of symbionts on host fitness are therefore usually mediated by products of microbial metabolism or general cues of microbial presence rather than by cooperative interactions (see section "Microbe to Host").

Animals harboring diverse microbial communities typically exert control over their symbionts by harming or promoting whole groups of microbes that occupy similar niches and fulfill similar ecological functions (Foster et al., 2017; Douglas, 2018). For instance, different *Hydra* species express different repertoires of antimicrobial peptides, and thereby support and maintain a species-specific microbiota (Franzenburg et al., 2013). Conversely, mammalian gut epithelial cells secrete complex glycans (Sicard et al., 2017) that can serve as food for gut *Bacteroides* species, and allow them to outcompete microbes lacking the enzymatic machinery for glycan breakdown (Xu et al., 2003; Pickard et al., 2014). While such broad-brush mechanisms allow hosts to keep their microbiota "on a leash" (Foster et al., 2017), they often cannot effectively target specific symbiont species. For instance, hosts seem to modify the structure of glycans in response to an immune challenge (Goto et al., 2014), which has been shown to increase the competitive ability of *B. thetaiotaomicron*, and thus indirectly benefits the host (Pickard et al., 2014). However, this provisioning does not allow for host control at the species level, as host glycans can be used by multiple competing *Bacteroides* species (Sonnenburg et al., 2010; Sicard et al., 2017). Notably, this limited precision of host control, which is presumably an unavoidable corollary of harboring a diverse microbiota, also prevents hosts from selectively reciprocating to host-beneficial cooperation by specific symbionts. In combination with strong interspecific competition, this further undermines the scope for host-beneficial cooperation among microbes.

Although symbioses with "complex" inner ecosystems are thought to benefit, on average, both the hosts and their microbes, they do not normally lead to strong (obligatory) dependencies on specific partners. For instance, symbionts are typically well adapted to general features of the animal habitat (Schell et al., 2002; Ley et al., 2008b), but can often colonize multiple host species (Ley et al., 2008a; Frese et al., 2011). Conversely, hosts are typically not dependent on the presence of a specific microbe, but instead seem to adapt to general cues of microbial presence and/or common products of microbial metabolism. This is likely because the (co)evolution of mutualistic host-symbiont interactions, which form the basis for the origin of strong mutual dependencies, are impeded by multiple hurdles. First, strong interspecific competition impedes the evolution of host-beneficial cooperation among the symbionts. Second, the limited precision of host control further exacerbates this impediment by preventing hosts from specifically reciprocating to beneficial microbes. Moreover, limited host control leaves room for stochastic effects, such that a specific symbiont species will not be present in all host individuals and/or at all times (Huttenhower et al., 2012). Finally, the evolution of mutualistic host-symbiont interactions is impeded by the predominantly horizontal transmission of symbionts. This is because horizontal transmission includes an environmental step that leads to mixing of symbionts (Browne et al., 2017; Björk et al., 2019), which further impedes the evolution of host-beneficial cooperation among the microbes by decreasing their average relatedness (Leeks et al., 2019). As a consequence of the overall limited scope for mutual dependencies, host and symbiont fitness in symbioses with "complex" inner ecosystems are often not well aligned – a notion that is underscored by the frequent occurrence of opportunistic pathogens in complex microbiota (Qin et al., 2012; Wang et al., 2012).

Animals with "complex" inner ecosystems may gain only limited benefits from individual symbionts, but the high diversity of their microbiota also offers ample scope for the competitive exclusion of pathogenic microbes, and thus reduces the host's risk of being manipulated (Foster et al., 2017; Johnson and Foster, 2018). This is the case because, like symbionts investing into costly host-beneficial behaviors, pathogenic microbes that cooperate to manipulate their host put themselves at a competitive disadvantage relative to other members of the microbiota, and thus risk being outcompeted. Manipulation is hence only expected to be favored if its benefits, such as an increased transmission or resource supply, predominantly fall back on the manipulator, a scenario that is most likely to occur if symbionts manipulate their local environment and face little competition from other symbionts (Johnson and

Foster, 2018). Notably, pathogens and parasites of hosts with species-rich microbiota often create a competition-free "simple" inner ecosystem for themselves, either by temporarily replacing competitors (e.g., *Salmonella enterica*; Ackermann et al., 2008; Diard et al., 2013), or by occupying a competitor-free niche (e.g., *Toxoplasma gondii*; Vyas et al., 2007; Prandovszky et al., 2011). Some aspects of pathogenesis might hence be similar in symbiosis comprising few versus many microbe species, with harmful microbes exploiting – and potentially manipulating – their host from competitor-free niches in both cases.

## CONCLUSION

Microbes frequently interact with each other within or upon their animal host, and a rapidly increasing number of studies now shows that these interactions can have substantial effects on host fitness. However, many of the factors that shape variation in microbial interactions and their effects on host function and evolution across different symbioses remain elusive. In this review, we have summarized the known diversity of microbial interactions, and argued that variation in their nature and impact on the animal host is often determined by differences in symbiont diversity.

The first part of our review shows that social interactions characteristic for microbial communities in natural habitats also occur in the microbiome (**Figure 1**), where they often mediate key effects on host functioning and fitness. While intraspecific microbial interactions often directly mediate key (nutritional or defensive) services, interspecific interactions typically affect the host indirectly through multipartite effects such as the competitive exclusion of pathogens by resident symbionts. In both cases, hosts may target symbiont interactions to exert control over their microbiota.

The second part of our review focuses on the evolution of animal-microbe associations and shows that the nature and impact of microbial interactions often differs between symbioses featuring only few versus many different symbiont species (**Figure 2**). In particular, it shows that the low symbiont diversity in symbioses with "simple" inner ecosystems allows for both strong host control over specific symbionts, and the evolution of cooperative behaviors among microbes that mediate host-beneficial services. These conditions increase the scope for coevolution, and thus ultimately favor the evolution of host-symbiont dependencies. In symbioses with "complex" inner ecosystems, on the other hand, the high number of symbiont species leads to strong interspecific competition and prevents hosts from exerting strong control over specific symbionts. These conditions render host-beneficial cooperation among the microbes unlikely, and thereby limit the scope for coevolution and the emergence of dependencies between the host and specific symbionts.

Overall, our review provides a perspective on the evolution of symbiosis that explicitly accounts for the occurrence and role of intraspecific and interspecific interactions within the microbiota across the whole taxonomical diversity of animal-microbe associations. Recent advances in the study of symbiosis have revealed the key role of microbial interactions for microbiota diversity and functioning (Kommineni et al., 2015; Chatzidaki-Livanis et al., 2016; Rakoff-Nahoum et al., 2016; Wexler et al., 2016); we hope that our perspective on the intricacies of the social lives of microbial symbionts complements this trend by raising awareness of the multifaceted nature of these interactions in different symbioses.

We believe that the further development of this perspective could follow three directions. First, it could involve studies investigating microbial interactions in species with moderately complex microbiota (such as those of honey bees; Bonilla-Rosso and Engel, 2018). This direction could reveal where on the continuum of microbiota diversity the dynamics shaping microbial interactions in "simple" inner ecosystems segue into those shaping highly "complex" inner ecosystems. Second, it could involve studies investigating microbial interactions across space, time, and varying conditions in wild animals (Amato, 2013; Coyte and Rakoff-Nahoum, 2019). This direction could reveal the stability of microbial interactions under natural conditions, and thus shed light on the reliability of their effects on the host. Finally, the further development of this perspective could involve studies investigating how interactions of animals with conspecifics affect – and are affected by – social interactions among the microbiota. This direction could most notably reveal the occurrence and nature of reciprocal effects between animal sociality and symbiont social interactions. In the light of these considerations, we believe that we only started to uncover the multifaceted role of social interactions within the microbiota for animal functioning and (social) evolution.

## GLOSSARY

- **cooperation:** a social behavior which provides a benefit to another individual and which has evolved and/or is currently maintained (at least partly) because of its beneficial effect on the recipient.
- **competition:** a situation that arises when two or more (con- or hetero-specific) individuals strive for the same limited resource, resulting in immediate costs for all individuals involved.
- **predation:** an interaction where one organism (the predator) kills and consumes another organism (the prey).
- **public goods:** costly resources that benefit not only the producer, but also other members of the population or local community.
- **symbiosis:** a prolonged and close association between organisms of two species.
- **microbiota:** a community of microbes associated with a particular (e.g., host) environment.
- **microbiome:** the community of microbes plus the particular (e.g., host) environment.
- **pathogen:** an organism that lives in or on another organism (the host), at a cost to the latter, often with severe consequences (disease) and for varying periods of time.

- **parasite:** a eukaryotic organism that lives in or on another organism (the host), at a cost to the latter, often for extended periods of time.
- **coevolution:** reciprocal evolutionary adaptations in different species, whereby adaptations in one party select for adaptations in the other party.

## AUTHOR CONTRIBUTIONS

Both authors reviewed the literature, developed the ideas, and wrote the manuscript.

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# From Symbionts to Societies: How Wood Resources Have Shaped Insect Sociality

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Sociality has independently arisen in several wood-dwelling insect lineages, yet little is understood about how the properties of decaying logs have favored the evolution of cooperative social groups. Here we evaluate the current literature on wood-dwelling social insects to identify the structural, nutritional, and microbial properties of decaying logs that have led to the repeated evolution of social behavior. Wood-tissue is structural resilient, and thus provided an enclosed, defensible nest site for early wood-feeding insect groups. This structural stability enabled the long-term persistence of family groups, and was likely a key feature in the transition toward more complex eusocial societies. The resilient structure and relatively poor nutritional quality of wood also likely provided a stable environment for the evolution of complex mutualisms with prokaryote and fungal symbionts to digest this resource. Parental care likely evolved as a means to protect the valuable nest site and ensure adequate nutrition for offspring in this environment by allowing parents to both provision and transfer microbial symbionts to offspring. Pathogenic microbes are also abundant in nests constructed in wood-tissue, and social adaptations such as allogrooming and nest maintenance may have evolved in response to microbial invaders. In general, the dynamic relationships between insects, microbes, and the wood-tissue that they inhabit was a critical component in the evolution of sociality in this habitat.

**Keywords: social evolution, social insects, log decomposition, saproxylic, microbial community, symbionts**

## INTRODUCTION

Of William D. Hamilton's many notable contributions to evolutionary biology, his most significant work was his formalized theory of kin selection (Hamilton, 1964). This theory provided an explanation for the evolution of altruism that was consistent with individual selection by demonstrating that helpers could produce copies of their own alleles by assisting non-descendent

kin (Hamilton, 1964). In addition to investigating the genetic processes of altruistic evolution, William D. Hamilton was also fascinated by the ecological conditions that facilitated the evolution of complex insect societies. In his lesser known 1978 paper, "Funeral Feasts: Evolution and Diversity Under Bark," Hamilton noted the peculiar diversity of social insects inhabiting decaying logs (Hamilton, 1978). He recognized that while close relatedness was essential to the evolution of worker altruism, these early social groups were more likely to arise in certain habitats. He suggested that decaying logs, due to their protective, long lasting properties, provided the ideal environment for the early evolution of highly altruistic eusocial societies, such as those of ants and termites.

Since Hamilton's observations nearly 40 years ago, much has been revealed about the intricacies of insect sociality under bark. New social wood-dwelling insects have been discovered (Kent and Simpson, 1992), fascinating details of different insect societies have been described (Biedermann and Taborsky, 2011; Smith et al., 2018), and the mechanisms driving the evolution of sociality in these environments are continuing to be explored (Thorne and Traniello, 2003; Inward et al., 2007; Korb et al., 2012; Nuotclà et al., 2019). We draw upon this growing literature to investigate the factors that have contributed to the diversity of insect societies inhabiting decaying logs within the context of William D. Hamilton's pioneering work.

## FAMILY AND GROUP FORMATION IN DECAYING LOGS

The evolution of complex sociality can be broken into three major phases: the onset of group formation (initially through extended parental care), maintenance of social groups, and the subsequent transition to complex, obligate social-living (Bourke, 2011; Korb and Heinze, 2016). Family groups consisting of parents and offspring are considered a critical prerequisite for the transition to more complex eusocial societies, and are generally more common in social log-dwelling lineages than aggregations of unrelated individuals (**Table 1**; Kent and Simpson, 1992; Inward et al., 2007; Nalepa et al., 2008; Biedermann and Taborsky, 2011; Suzuki, 2013; but see Zorapterans, Mashimo et al., 2014). Indeed, the two instances of eusociality that have arisen in wood-dwelling insect lineages evolved from ancestral family groups (ambrosia beetles, Smith et al., 2018; termites, Klass et al., 2008). Identifying the properties of the decaying log habitat that favored the evolution and maintenance of parent-offspring groups is thus key to understanding the prevalence of sociality in log-dwelling insects.

The selective pressures that favor the transition to cooperative societies from simple family groups in any environment can generally be classified as either benefits associated with staying in the natal nest or costs associated with dispersal (Koenig et al., 1992; Emlen, 1994). The benefits of remaining in family groups in log-dwelling lineages can generally be classified as structural, nutritional, or microbial benefits that the log resource provides to offspring that delay dispersal from the nest (**Table 2** and **Figure 1**). Logs provide a safe, sheltered, food-abundant nesting resource for retained offspring, allowing for prolonged parent-offspring interactions that set the stage for the subsequent evolution of more complex social behaviors. Dispersal costs can also be extremely high in wood-dwelling insects, drastically reducing the likelihood of independent establishment for those that disperse from the natal nest. Competition for log resources is fierce and some termite species experience a <1% success rate in establishing a new nest site following dispersal (Chouvenc, 2019). Below we discuss these specific attributes of wood environments that have shaped sociality in log-dwelling lineages in more detail.

## STRUCTURAL RESILIENCE OF NEST SITES CONSTRUCTED IN WOOD TISSUE

Wood tissue is a stable, long-lasting resource that provides both food and protection for nests constructed within. When used as a food source, decaying logs serve as plentiful bonanza resources, reducing competition among group members and favoring family group formation by limiting within-group conflict (Korb and Heinze, 2016). Wood-tissue, particularly large tree trunks, degrades slowly and is structurally resilient, allowing insect families to persist for several generations before the resource is depleted (Nalepa and Bell, 1997; Thorne, 1997; Korb et al., 2012). Both wood-dwelling passalid beetles and ambrosia beetles form families in which the social group and the nest persist well after offspring mature into adults (Schuster and Schuster, 1997; Biedermann and Taborsky, 2011). In both systems, young adults have been observed helping to care for younger siblings, highlighting the importance of prolonged family cohesion in the evolution of cooperation (Schuster and Schuster, 1985; Biedermann and Taborsky, 2011).

Structural stability also ensures that the nest site remains intact even after the death of a parent, providing offspring that remain in the nest the opportunity to inherit the breeding resource. For instance, competition between colonies for nesting resources in *Microcerotermes papuanas*, an extant termite species that forms colonies in a single log resource ("one-piece" nesting; Abe, 1987), often results in the death of one or more primary reproductive individuals in the colony (Thorne and Traniello, 2003). Young workers in these colonies can molt into reproductive adults and begin to reproduce in the nest via parthenogenesis to fill this breeding vacancy (Roisin, 1990; Fougeyrollas et al., 2017). Indeed, the likelihood of inheriting a breeding position within the natal colony is actually higher than that of successfully dispersing and establishing a nest in a new log for some lower termite species (Korb and Schneider, 2007; Korb and Heinze, 2016).

Nesting in log resources also provided early social lineages with valuable, defensible nest sites, facilitating the evolution of eusociality via a "fortress defender" route (Queller and Strassmann, 1998). In contrast to eusocial "life insurers" in which workers specialized into a foraging caste, fortress defenders, such as deadwood termites, favored soldier castes to defend the nest site against competitors (Queller and Strassmann, 1998). Low establishment success, high competition, and high value of the log resource resulting from its function as both food and shelter

**TABLE 1 |** Summary of the ecology and life history of the social and gregarious wood-dwelling insects.

| Group | Social system(s) | General ecology | References |
|---|---|---|---|
| Blattodea: Cryptocercidae and Blaberidae | Subsociality with biparental care. | Live and feed on wood in decaying logs and both parents provision dependent offspring, often for several years in some species. | Nalepa and Bell, 1997; Nalepa et al., 2008; Nalepa and Arellano, 2016 |
| Isoptera | Eusociality with biparental care in initial stages, then cooperative care. | Derived from social wood-feeding cockroach ancestors likely similar to *Cryptocercus*. All contemporary lineages are eusocial and many still primarily live in wood-tissue. Colonies are founded by a breeding pair and are assisted by both male and female workers, and have a highly diverse hindgut microbiome that aids in wood digestion. | Abe, 1987; Thorne, 1997; Klass et al., 2008; Korb et al., 2012 |
| Coleoptera: Passalidae | Subsociality with biparental care; Cooperative brood care. | Nest sites initiated in decaying logs by both parents who provision, defend, and construct pupal cases for offspring. Adult offspring and parents remain in contact for weeks or months, and may cooperate in brood care during this time. | Schuster and Schuster, 1985, 1997; Ento et al., 2008; Dillard, 2017 |
| Coleoptera: Curculionidae | Eusociality, Subsociality with either maternal or biparental care, Cooperative brood care. | Highly variable life history, but in general nests are constructed in living or recently dead tree trunks by a single female or a female joined by a male. Adult female offspring may remain in the colony to provide care for younger siblings in cooperatively breeding *Xyleborus* species and the eusocial *Austroplatupus incompertus*. | Kent and Simpson, 1992; Biedermann and Taborsky, 2011; Biedermann et al., 2011; Baruch et al., 2017; Smith et al., 2018 |
| Coleoptera: Tenebrionidae (*Phrenapates bennetti*) | Subsociality with biparental care | Subsocial behavior and ecology appears similar to that of passalids and involves prolonged parent-offspring interactions in heavily decayed log habitats. | Nguyen et al., 2006 |

**TABLE 2 |** Properties of wood tissue that may have facilitated the evolution of parental care and cooperative societies in wood-dwelling insects.

| Property | Relevance to the evolution of insect sociality |
|---|---|
| Structural resilience | Wood tissue is structurally stable, allowing for prolonged cohesion of family groups and overlapping generations. |
| Defensibility | Nests constructed in wood tissue are generally high value due to the processing investment, and may promote the evolution of social defense behaviors. |
| Dispersal costs | Leaving the safety of a log in search of new nesting resource is risky, and the probability of surviving dispersal and finding a suitable log to colonize are very low for many insects. |
| Poor nutritional quality | Wood tissue is nutritionally poor, providing selection for parental provisioning or resource processing for young. |
| Microbial symbionts | Microbial symbionts are required to digest nutrient poor wood, and the need to transmit these microbes to subsequent generations may favor prolonged family interactions. |
| Microbial defense | Nests in wood tissue are sensitive to growth of pathogenic microbes, providing selection for social microbial defenses such as allogrooming or egg grooming. |
| Resource modification | The physical and chemical properties of wood allow social insects that nest in this resource to drastically alter their chemical, microbial, and physical environment to meet the needs of the colony. |

makes this habitat particularly amenable to the evolution of fortress defense eusociality.

## SOCIALITY AND SYMBIONTS: ADAPTATIONS TO POOR NUTRITIVE QUALITY OF WOOD

Although wood is abundant and long-lasting in large tree trunks, most of the nutrients contained in wood tissue are not accessible to the digestive systems of animals (but see Tokuda et al., 2004), and feeding on this resource could select for a variety of social adaptations to overcome this limitation. Wood is generally nitrogen-poor and difficult to digest due to its high cellulose and lignin content (Tallamy and Wood, 1986). Generally, feeding on resources that are low quality can result in slower development of young, ultimately increasing the amount of time during which offspring are dependent on parental care (Beehler, 1985; Strahl, 1988; Nalepa and Bell, 1997; Nalepa and Arellano, 2016). In some wood-feeding taxa, juveniles that do not possess the enzymes or symbionts necessary to digest wood depend on parents to process the wood resource for them (King and Fashing, 2007; Mishima et al., 2016). Parental care in these systems may thus primarily function as a means to provide offspring with adequate nutrition. The evolution of both parents assisting in brood care (which has occurred in passalids, cockroaches, and termites) may have arisen to meet the high nutritional needs of the brood, with

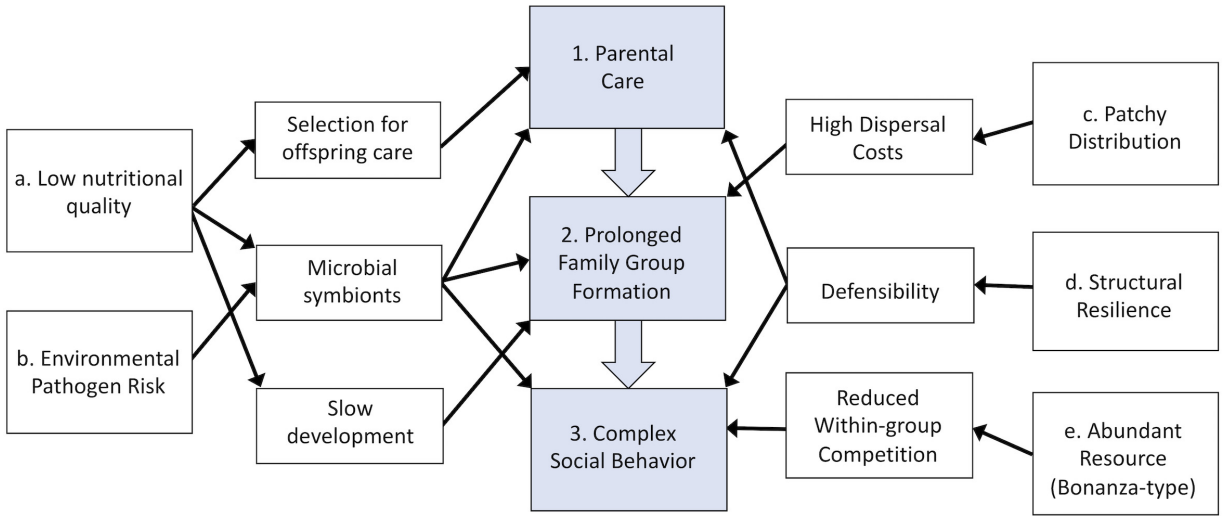


**FIGURE 1 |** Conceptual diagram of the factors associated with log-habitats that have shaped the evolution of parental care and the transition to more complex family and social groups. The physical properties of the log habitat (boxes a–e) favored the sequential evolution of complex family-based social groups from ancestors with simple parental care (boxes 1–3) by generally favoring prolonged family cohesion and reducing dispersal and independent establishment success.

increasing parental care setting the stage for the co-evolution of even more dependent (altricial) young (Nalepa et al., 2008). The nutritional deficiencies of wood may therefore have been a major determinant of the subsequent evolution of complex cooperation by initiating the evolution of extreme offspring dependency through parent-offspring feedback selection (Nalepa et al., 2001, 2008). Increased offspring altriciality likely then increased the benefits of offspring care, providing selection for the evolution of even more complex social traits, including cooperative breeding and eventually eusociality.

## SYMBIONT TRANSMISSION BETWEEN PARENTS AND OFFSPRING

To subsist on the generally nutrient-poor wood tissue, wood-feeding insects have evolved complex symbiotic relationships with bacteria and fungi that allow them to extract nutrients from these largely indigestible resources (Nalepa et al., 2001; Suh et al., 2003; Biedermann et al., 2009; Mishima et al., 2016). Although these microbial symbionts allow their insect partners to thrive in the nutrient-poor wood tissue environment, maintaining the integrity of the microbial communities and transmitting them to subsequent generations can be challenging.

Intraspecific coprophagy (feces ingestion) and anal trophallaxis were key adaptations allowing for the transfer of beneficial microbial communities from parents to offspring. In the *Cryptocercus* cockroaches and termites, the need to share symbionts between parents and offspring was likely a major driver of prolonged parental care (Nalepa et al., 2001). Juveniles are fed microbe-rich secretions produced by their parents via anal trophallaxis to initiate and maintain their own hindgut community of wood-digesting microbial symbionts (Thorne, 1997; Klass et al., 2008). This mode of direct transfer rendered these microbes dependent on their hosts, and contributed to the strong mutualism observed between host and symbiont observed in both cockroaches and termites (Nalepa et al., 2001; Nalepa, 2017). Passalids, in contrast, share symbionts with offspring via ingestion of feces (coprophagy) and processed wood in the nest (Suh et al., 2003; Mishima et al., 2016). *Phrenapates bennetti*, a tenebrionid that strongly resembles passalids in morphology, life history, and subsocial behavior, also uses similar xylose fermenting yeasts (Nguyen et al., 2006). Although little is known about the social behavior of *Phrenapates*, given their convergent evolution with the passalids it is possible that they transfer symbiotic yeasts from parents to young via coprophagy as well, with subsociality potentially arising as a consequence.

Other wood-dwelling lineages, including the Xyleborine and Platypodine ambrosia beetles, feed on fungus that they cultivate in tree trunks rather than on the wood tissue itself (Kent and Simpson, 1992; Biedermann and Taborsky, 2011). Xyleborine ambrosia beetle females disperse as young adults to initiate their own colonies, and solve the transmission problem by carrying "starter" cultures from their natal nest that they store in either the gut or fungal storage organs called mycangia prior to dispersal (Batra, 1963; Biedermann et al., 2009; Seibold et al., 2019). As with the other microbial-dependent, wood-feeding insects, this form of symbiont transmission requires prolonged interaction between parents and offspring in the natal nest.

Interestingly, not all insects that breed in wood that rely on symbionts to aid in digestion live socially. Females of many stag beetle species, for instance, do not remain with their offspring after oviposition, although they appear to inoculate the oviposition site with xylose-fermenting yeasts from a fungal storage structure (mycangium) before departing to transmit the symbionts to their young (Tanahashi et al., 2009, 2010). Stag beetles only consume decaying wood as

larvae, however, and this difference in feeding modes between adult and neonates may partly explain how they have adapted to transmit endosymbionts to young without prolonged parent-offspring associations (Tanahashi et al., 2009, 2010). In contrast, all of the social wood-dwelling insects that feed on wood or microbes cultivated in wood do so as both adults and larvae (Nalepa and Bell, 1997; Schuster and Schuster, 1997; Thorne, 1997; Biedermann et al., 2011). Remaining in the log nest to feed as adults may have been an important pre-requisite in the evolution of parental care as well as more complex social behaviors. Further research comparing the ecological, physiological, and microbial differences between wood-feeding insects that transmit symbionts to offspring socially to those that can do so without prolonged parent-offspring contact may reveal valuable insight into the predisposing factors that have promoted sociality in certain wood-feeding insect groups.

## MICROBIAL DEFENSE AGAINST PATHOGENS IN SOCIAL GROUPS

Although wood tissue is amenable to the growth and maintenance of beneficial microbes, these environments also provide suitable habitat for growth of harmful or pathogenic microbes. These challenges might be exacerbated for lineages that facilitate the growth of beneficial microbes in the nest site (Nuotclà et al., 2019). Many wood-feeding insects have evolved social defenses against these deleterious microbes such as corpse management (López-Riquelme and Fanjul-Moles, 2013; Sun and Zhou, 2013; Sun et al., 2018), allogrooming (Rosengaus et al., 1998; Wilson-Rich et al., 2009; Meunier, 2015), and chemical defenses (Biedermann and Rohlfs, 2017). While many of these social adaptations most likely arose after the onset of group-living, they suggest ways that living in resources like wood could provide feedback selection on incipient social groups, reinforcing and elaborating social traits. Female ambrosia beetles of the species *Xyleborinus saxesenii*, for instance, delay dispersal for longer periods of time when their nests have been infected with *Aspergillus* spores (Nuotclà et al., 2019). Infection of ambrosia beetle nests with *Aspergillus* also leads to greater expression of allogrooming and corpse maintenance behavior, providing evidence that social behavior can be enhanced when microbial defense is needed. The gut symbionts of dampwood termites (*Zootermopsis nevadensis*) also have been shown to have anti-pathogen properties. In addition to their digestive function, the intestinal microorganisms of these dampwood termites increase intestinal acetate which has inhibitory effects on the opportunistic pathogen *Serratia marcescens* (Inagaki and Matsuura, 2018).

## DYNAMIC STRUCTURE OF DECOMPOSING LOG RESOURCES

Feeding within decaying logs either on the wood tissue itself or on the microbes cultivated in the nest site results in complex, dynamic resource properties. First, by boring into the wood, insects increase both the surface area and physical heterogeneity of the resource, allowing wood-degrading microbes (i.e., bacteria, protists, and fungi) to more readily colonize the resource (Ulyshen, 2016). Many insects alter the wood via physical processing, chemical additives, and inoculation of beneficial microbes to either enhance the quality of the wood (external rumination; Suh et al., 2003) or to cultivate food resources (fungal farming; Batra, 1963; Biedermann et al., 2009). Insect tunneling also leads to better aeration and fragmentation of the wood, improving habitat quality for both the insects and microbes (Ulyshen, 2016). This fragmentation, however, may reduce the structural integrity of the resource over time, causing the wood to collapse or the bark to slough off. Log degradation from colonization, which can be rapid when colonized by large or efficient social insects such as the passalids or termites, respectively, may thus reduce the total lifespan of the colony resource. Each log likely has a carrying capacity that varies not only with the abiotic conditions of the surrounding habitat, but also the use and transformation by the inhabiting insects through activities such as tunneling, enhancing, and cultivating the resource. This eventual degradation of the log resource by wood-feeding insects is perhaps one reason why the most complex termite societies evolved following the transition from log-nesting to inhabiting more permanent, self-constructed nest sites (Korb et al., 2012).

## SOCIAL INSECT AND MICROBIAL COMMUNITY ECOLOGY IN DECAYING LOGS

Microbial communities of logs and other large and recalcitrant wood debris are complex and not well understood (see review by Johnston et al., 2016). However, they are known to be mediated by organisms that have evolved to either consume the organic matter directly or use it as a relatively stable habitat (e.g., compared to carrion or leaf litter) for breeding or nest establishment (Ulyshen, 2016). Habitat stability has been proposed to provide the circumstances for positive evolutionary feedback between insect sociality and microbial community management (Biedermann and Rohlfs, 2017). However, as wood becomes more fragmented either through abiotic damage (e.g., wind, damage by falling, and rain) and insect tunneling activities (e.g., ambrosia beetles), the area available for microbial colonization increases (Ulyshen, 2016), suggesting that the importance of microbes to wood inhabiting insects becomes increasingly relevant during later decomposition. For many wood feeding insects, such as some Passalidae, those microbes likely make up a considerable portion of their nutritional needs (Castillo and Reyes-Castillo, 2009; Filipiak, 2018). Thus, the microbial communities and how they change during the long course of wood debris decomposition are thought to play at least a nutritional role in the ecology of wood feeding insects. There is also evidence of additional relationships between microbes and wood feeding insects, especially within the

context of co-evolution of endosymbionts (Suh and Blackwell, 2005; Nardi et al., 2006; Warnecke et al., 2007; Ulyshen, 2016) and fungal ectosymbiosis (Krivosheina, 1991; Mueller et al., 2005; Biedermann and Vega, 2020).

## CONCLUSION AND FUTURE DIRECTIONS

Some of the most complex, cooperative insect societies have arisen from ancestors that lived and fed in the wood tissue of large trees (Kent and Simpson, 1992; Klass et al., 2008). William D. Hamilton largely attributed this occurrence to the structural longevity of wood tissue, and the defensibility of nest sites constructed within (Hamilton, 1978). Newer discoveries reveal a critical role of both symbiotic and pathogenic microbes in the evolution and maintenance of parental care and sociality in wood-dwelling lineages (Biedermann and Rohlfs, 2017; Nuotclà et al., 2019). Parental care and other forms of sociality afforded these insects the means to provide adequate nutrition of young through provisioning and symbiont transfer and to defend their nest sites from biological threats (microbial and otherwise). The structural longevity of large tree trunks likely allowed these small family groups to persist for multiple generations, facilitating the transition from subsociality to eusociality in some lineages.

Despite this developing framework, much still remains to be understood about the nuanced interactions between social insects and their microbial and physical environment. Not all wood-dwelling insects have evolved social behavior to better exploit wood-tissue, and identifying the fundamental similarities and differences between the non-social and social wood-dwelling species may provide insight into the factors critical to the evolution of sociality in wood (Tanahashi et al., 2009, 2010). For instance, the microbial communities employed by different species to aid in wood digestion may differ drastically between social and non-social species, and the properties of these microbial symbionts may have been a critical factor determining whether or not a lineage became social. Fundamental differences in life history may also distinguish social from solitary wood-feeding insects, including adult feeding behavior. Further investigations into the coevolution between microbes and the behavior of their insect hosts will likely yield tremendous insight into the ways that microbes have contributed to the evolution of insect societies.

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REVIEW
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# Superorganism Immunity: A Major Transition in Immune System Evolution

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Social insect colonies can express adaptive, organism-like design. In some cases, colonies so resemble a unique, cohesive and integrated "individual" that they are termed superorganisms. The major evolutionary transitions framework explains, via inclusive fitness theory, how new levels of biological individuality, including genes into genomes within cells, cells into multicellular organisms and organisms into superorganisms can emerge. Importantly, it highlights how at each major transition similar challenges arose and why seemingly convergent solutions evolved. One challenge faced at each transition is exploitation, caused internally by social cheaters and externally by parasites and pathogens. To overcome the problem of exploitation transitions in biological individuality required novel immune systems to maintain the integrity of newly emerged individuals. Multicellular organisms evolved an immune system while social insect colonies evolved a social immune system. In this review, we take a major transitions perspective of immunity to highlight the interdependency between the evolution of immune systems and the emergence of biological individuality. We build on the notion that superorganisms have evolved an immune system to promote the fitness of the colony. We draw parallels between the evolution of the metazoan immune system and the social immune system, and their expression as cognitive networks. Moreover, we discuss how research on other group-living species, such as family based cooperative breeders, can inform our understanding of how social immune systems evolve. We conclude that *superorganism immunity* is an adaptive suite of organismal traits that evolves to maximize the fitness of advanced social insect colonies, fulfilling the same function as the immune system of Metazoa.

**Keywords: superorganism, disease, social evolution, major evolutionary transition, social immunity**

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## INTRODUCTION

*"Fixing attention on the honeybee. . .nothing like the immune system for detecting and combatting microbial enemies is known to exist."*

–Hamilton, 1987

*"The most general organismal character of the ant-colony is its individuality. Like the cell or the person, it behaves as a unitary whole, maintaining its identity in space, resisting dissolution and, as a general rule, any fusion with other colonies of the same or alien species."*

–Wheeler, 1911

Major evolutionary transitions are a series of defining moments in the history of life on earth where new, more complex forms of life emerged (Smith and Szathmary, 1997; West et al., 2015). At each transition, groups of previously free-living and self-replicating individuals cooperated to such a degree that they lost their independence, coalescing into a single distinct entity that functions and replicates as one. Conflict between lower-level constituent parts is negligible, such that the new higher-level life form becomes the fitness-maximizing "biological individual" (Queller and Strassmann, 2009; West et al., 2015). The underlying process governing each of these transitions is social evolution (Bourke, 2011; West et al., 2015) and examples include: genes into genomes within cells giving rise to single-celled life; cells into organisms resulting in the complex multicellular plants and animals; and organisms into superorganisms, the evolution of social insect colonies with obligate reproductive and worker roles.

One factor that has the potential to both hinder and encourage evolutionary transitions, particularly at the organismal and superorganismal level, are disease-causing pathogens and parasites. Hamilton recognized that, due to low levels of genetic diversity within these groups, disease should be a major constraint on the evolution of multicellularity and insect sociality (Hamilton, 1987). However, as the quote at the beginning of this review reveals, Hamilton did not believe anything comparable to the metazoan immune system to protect against disease had evolved in social insects. Hamilton instead proposed outbreeding to increase genetic diversity as the main mechanism that prevents pathogens driving social insects to extinction (Hamilton, 1987). Although increased genetic diversity via polygyny and polyandry as a means to protect against disease is well supported both theoretically and empirically (Baer and Schmid-Hempel, 1999; Schmid-Hempel and Crozier, 1999; Seeley and Tarpy, 2007), it nevertheless remains true that the majority of social Hymenoptera retain a single-queen genetic bottleneck at some point in their lifecycle, and most queens still mate with fewer than two males on average (Boomsma and Ratnieks, 1996; Queller, 2000; Hughes et al., 2008a). Measures to increase genetic diversity such as polyandry and polygyny seem to be especially rare in the termites as colonies are usually founded by a single monogamous pair (Shellman-Reeve, 1997). Interestingly, although termites from outbred colonies exhibit reduced fungal susceptibility, unrelated monogamous pairs are more likely to perish during colony foundation than related pairs (Calleri et al., 2005, 2006). Genetic diversity has, therefore, clearly not evolved as a universal mechanism to reduce disease susceptibility in insect colonies.

Since Hamilton's predictions, our understanding of how social insects avoid, tolerate and resist pathogens and parasites has expanded greatly. We now know that social insects have evolved a variety of mechanisms to prevent and/or mitigate the impact of disease. In 2007, Cremer et al. (2007) coined the term *social immunity* to describe the collective immune defenses present in insect colonies. Later, Cremer and Sixt (2009) took the immunity analogy further, pointing out the many ways in which social immunity in superorganisms plays a functionally equivalent role to metazoan immunity (see also Cremer et al., 2018; Cremer, 2019). In this review, our aim is to build on the original hypothesis asserted by Cremer et al. (2007) that, in superorganisms, social disease defense has evolved beyond simple cooperation to function instead as a systemic and indispensable "social immune system." As we will see, the evolution of biological individuality has repeatedly resulted in the parallel emergence of a bespoke defense system that operates at the level of the new individual (Bourke, 2011; Pradeu, 2013). This defense system maintains the integrity of the individual, be it a cell, organism or superorganism, by providing protective functions that extend beyond disease defense. It may, therefore, be possible to talk of a true immune system that provides *superorganism immunity* in advanced social insect colonies (Aanen, 2018), which could be used as a criterion for defining what a biological individual is in social insects. We hope that our review will be able to contribute positively to the debate on the defining features of major evolutionary transitions (Godfrey-Smith and Goodnight, 2013; Pradeu, 2013; Boomsma and Gawne, 2018), whilst providing clear directions for future research on superorganism evolution, the most recent and arguably least understood of the major evolutionary transitions.

## SUPERORGANISMS AS BIOLOGICAL INDIVIDUALS

Before we can explore the role of immunity in superorganism evolution, we first need to discuss what we mean when we talk of *superorganisms*, and, more broadly, *organisms* and *individuality* (**Figure 1**). Debate and discussion surrounding these terms abound, and there is seemingly no one answer that satisfies both philosophers and biologists (Godfrey-Smith and Goodnight, 2013). Most people have an intuitive understanding of the term organism, which is exemplified by the paradigmatic metazoan animal, yet universal classification remains difficult. An often accepted hallmark of multicellular organismality is the permanent sequestration of the germline early in development, but this criterion excludes many plants, fungi and even some animals, including sponges (Funayama, 2010), cnidarians (Nishimiya-Fujisawa and Kobayashi, 2012), Acoela (De Mulder et al., 2009), some helminths (Rink, 2013; Fields and Levin, 2018), and possibly echinoderms and annelid worms (Solana, 2013; Dannenberg and Seaver, 2018). These all appear to harbor totipotent somatic cells but are clearly organisms (Clarke and Okasha, 2013 and references therein). Such examples support the view that the germline has not been unambiguously sequestrated in either the metazoan or the urbilaterian ancestor of animals (Extavour, 2007; Clarke and Okasha, 2013; Solana, 2013; Fierro-Constaín et al., 2017). Similarly, Wheeler defined superorganisms as advanced social insects societies where worker and queen castes are permanently differentiated into "soma" and "germline" components, such that the worker can never mate nor give rise to a new superorganism (Wheeler, 1911; Boomsma and Gawne, 2018). However, cases that are difficult to classify exist: many termite species – for example, most multi-piece foraging/subterranean lower termites in the families Mastotermitidae and Rhinotermitidae (Roisin, 2000) – form complex, thousands-strong nests that are maintained, protected and supplied with resources by a "true worker" caste. True

termite workers split early on in development from the winged dispersing caste that do not work (Shellman-Reeve, 1997; Korb and Thorne, 2017). Nonetheless, true workers, including those in some higher termite species (Roisin, 1990; Myles, 1999; da Silva et al., 2019), can yield reproductively competent ergatoid neotenics under certain conditions, such as the death of the primary reproductive, which seems analogous to emergency queen rearing in honeybees (Shellman-Reeve, 1997; Myles, 1999). Despite this totipotency, true workers typically behave as altruistically as the permanently differentiated workers in other social insect lineages: they perform brood care, leave the colony to forage, and engage in altruistic hygiene (Chouvenc and Su, 2012; Korb et al., 2012; Davis et al., 2018). True workers also have an extremely limited chance of independent reproduction; less than 1% are estimated to become reproductive (Shellman-Reeve, 1997; Thorne, 1997; Korb et al., 2012; Korb and Thorne, 2017). In contrast, less advanced single-piece nesting/wood-dwelling lower termites have helper offspring ("false workers") that are fully totipotent: helpers and even pre-soldiers instars can reproduce through nest inheritance, colony fusion events, or dispersal (Shellman-Reeve, 1997; Thorne, 1997; Myles, 1999; Korb and Schmidinger, 2004; Korb, 2006, 2007; Hoffmann and Korb, 2011; Korb and Roux, 2012). Hence, termites with true workers appear as superorganismal as species with permanently differentiated castes, in that the reproductive-worker role is obligate and specialized. To avoid excluding these potential superorganisms and to aid comparisons across the major transitions, we will adopt the *biological individual* terminology used extensively in evolutionary biology, which defines the unit of selection as a meaningful measure of individuality. A biological individual can thus be a gene, cell, organism, or superorganism (Bourke, 2011; West et al., 2015). The important point is that natural selection acts on and between these biological individuals, so that it is the biological individual as a consolidated whole that responds most to selection, accrues adaptions to maximize its fitness, and so evolves over time (Queller, 2000; Bourke, 2011). One such adaptation that is thought to be essential for biological individuals to emerge is immunity (Pradeu, 2013). In the following section, we will see that immunity is an evolutionary widespread adaptation, which can also help to define when a major evolutionary transition has occurred.

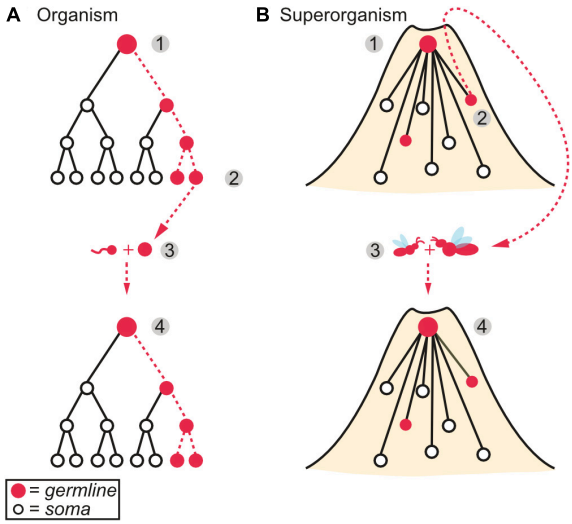


**FIGURE 1 |** Analogous features between **(A)** multicellular organisms and **(B)** social insect superorganisms. (1) An organismal/colony existence is obligate with distinct germline (red dots) and soma (white dots) roles. The germline gives rise to both the sterile soma and additional germline entities. (2) The germline reproduces the (super)organism. (3) Lifetime commitment, through the fusion of gametes as a zygote in organisms and strict lifetime monogamy between mated pairs in superorganisms, creates the conditions necessary for the evolution of germline-soma differentiation, which in many, but not all, metazoan and superorganismal lineages has also become fully sequestered (see main text). (4) Superorganism fitness, akin to the fitness of an organism, is measured not by the number of new insects it creates, but by the number of new, independent superorganisms it produces.

## IMMUNOLOGY AND INDIVIDUALITY

The concept of biological individuality is tightly linked to immunology (Pradeu, 2012). For biological individuals to emerge, evolve and adapt, they need to: (i) suppress the independent evolution of their constituent parts, (ii) develop a clear delineation of the "self" that makes them biologically unique, and (iii) prevent exploitation by infectious diseases (Bourke, 2011; Pradeu, 2013). In the evolution of multicellularity it is the immune system that acts as "policer," eliminating non-cooperating cells to prevent conflicts of fitness; "delineator, setting the limits and boundaries of the organism (i.e., acting as gatekeeper); and "eradicator," preventing infections from spreading within, and causing the dissolution of, clonal aggregations (Pradeu, 2013). It is hypothesized that multicellular immunity may have first evolved to prevent selfish mutations arising that replicate at cost to the whole organism (though modeling indicates this probably only occurs under specific circumstances or as multicellular organisms grew larger; Queller, 2000) and to prevent fusion with, or invasion by, nonkin cells, before secondarily evolving the ability to fight pathogens (Pradeu, 2013).

We, like Pradeu (2013), argue that since immune systems are so important for maintaining individual integrity – and are universally present among all multicellular organisms including plants, fungi and animals – that they may even precede other more patchily distributed criteria, such as a sequestered germline (Clarke and Okasha, 2013). Immune systems should hence be considered key mechanisms that enable and maintain transitions in individuality. For example, some form of crude immunity seems to even be present in facultatively multicellular organisms, such as *Dictyostelium*, suggesting that immunity evolves concomitantly with the emergence of multicellularity (Chen et al., 2007). Moreover, Pradeu (2013) reasons that immunity has likely played a similarly pivotal role in other major evolutionary transitions, in particular, the emergence of unicellularity and superorganismality: in prokaryotes, Pradeu hypothesizes that their form of immunity (the CRISPR-Cas system) may have evolved to protect the biological individuality of unicellular life (Horvath and Barrangou, 2010); whilst in

superorganisms, "the immune system of the colony will make it strongly cohesive in such a way that the colony will qualify as an organism."

Wheeler, as quoted at the beginning of this review, recognized that social insect colonies possess the fundamental characteristics of a biological individual, exhibiting a tight unity and functional integration of its constituents (**Figure 1**). Additionally, he noted that they remain the same whilst changing through time and rarely fuse with other colonies. In Wheeler's era, less was known about the cooperative disease defenses of social insects. Now, we are a better position to examine whether superorganismal social insect colonies have evolved an immune system that perpetuates the individuality of colonies, and, whether this "social immune system" was as instrumental in the evolution of superorganismality as immunity is thought to have been for multicellularity (Pradeu, 2013).

## SUPERORGANISM IMMUNITY

If superorganisms possess an immune system, we expect it to exhibit certain properties. Chief among these is an ability to police its constituent parts, mechanisms to maintain the superorganism's uniqueness and an ability to detect and eradicate harmful entities; namely, parasites and pathogens (Bourke, 2011; Pradeu, 2013). Based on the best studied immune systems, those of the vertebrates, we might also predict other qualities and phenomena, such as decentralized control and immunological memory (Hofmeyr and Forrest, 1999). Moreover, we would expect information sharing and the emergence of similar network-based rules (Moses et al., 2019; Piñero and Solé, 2019). In **Table 1** we summarize some of the convergent properties of multicellular and superorganismal immunity (based partly on: Hofmeyr and Forrest, 1999). Below we discuss some of these aspects in more detail. Although originally considered in early examinations of colony-level immune systems (Cremer et al., 2007; Cremer and Sixt, 2009), the role of immune policing and the immunological delineation of the individual have been largely neglected in recent discussions of social immune systems (Cremer et al., 2018), which is likely due to a research focus on microbial pathogens. Thus, in the following section, we highlight the broader protective role of superorganism immunity for superorganism integrity, expanding on earlier assessments by Cremer et al. (2007), Cremer and Sixt (2009), and Bourke (2011).

### Immune Policing of the Superorganism

Biological individuals cannot emerge and evolve if there is significant selection and evolution of their lower-level constituent parts (Gardner, 2013; Pradeu, 2013; West et al., 2015). Preventing social cheaters with differential fitness is thus a reoccurring challenge across the major evolutionary transitions (Queller, 2000; Bourke, 2011; Ågren et al., 2019). Genetic bottlenecking in modern-day multicellular organisms severely limits selective opportunities for selfish mutants (e.g., cancers) beyond one generation (Queller, 2000; Michod, 2007). However, there was likely to be more opportunity for selfishness and conflict in the early stages of multicellularity, so that policing of constituent parts was important to prevent selfish elements overwhelming the germline (Michod and Roze, 2001; Michod, 2007). It is also unclear how often social parasites of somatic origin (next section) would evolve in the absence of modern-day immune policing mechanisms that prevent cancers from evolving (Ågren et al., 2019). In superorganisms, their modular structure, a degree of individual control over caste fate, and the ability of workers to lay haploid, unfertilised eggs (in hymenopteran societies) – coupled with polyandry and/or polygyny in some species – creates more potential for conflict and hence worker selfishness (Beekman and Oldroyd, 2019 and references therein). Worker reproduction is usually inhibited by the presence of a reproductive queen that emits a pheromone signaling her fertility. These conserved signals suppress the activation of worker ovaries and so act as a form of "policing" (Van Oystaeyen et al., 2014). Reproductive workers are rare when fertile queens are present; for example, less than 1% of honeybee workers lay eggs (Bourke, 2019 and references therein). Reproducing workers have been compared to cancerous, somatic cell lineages in multicellular organisms, which also selfishly replicate at catastrophic cost to the organism (Tsuji and Dobata, 2011; Teseo et al., 2013). In multicellular organisms, constant immunosurveillance by the immune system identifies and eliminates mutant cells before they develop into cancers via tumor-specific antigens present on malignant cells (Dunn et al., 2002; Pradeu, 2013; Corthay, 2014; Feng et al., 2018). In superorganisms, the main form of policing is workers seeking out and eating the eggs of other workers, which they distinguish from queen-laid eggs by specific chemical odors (Ratnieks and Visscher, 1989). In clonal raider ants, where individuals are genetically identical, all ants reproduce during specific phases of the colony's lifecycle. However, some ants continue to reproduce uncontrollably outside of these phase, as they fail to respond to regulating signals that control reproductive synchrony in the colony (Teseo et al., 2013). These aberrantly replicating ants are detected through divergent cuticular hydrocarbons (CHCs) and killed by nestmates, similar to the organismal immune system detecting malignant cells via cancer-specific antigens (Teseo et al., 2013; Feng et al., 2018). Mutations that cause ants to behave selfishly have also emerged in this species: by producing more "germline-like" reproductive individuals, mutant lines can monopolize reproduction in chimeric colonies and create an opportunity for the evolution of social parasites (Teseo et al., 2014); a similar result has been found in multicellular cooperation, suggesting that mechanisms to supress the evolution of cheaters is paramount to social stability (Teseo et al., 2013; Bastiaans et al., 2016; Ågren et al., 2019). The removal of dead, non-infectious insects has parallels with the removal of dead, damaged, or dangerous cells in a body, which can also be considered a form of policing (Pradeu, 2013). Apoptotic cells not removed by phagocytes release noxious chemicals that cause tissue inflammation (Nagata and Tanaka, 2017), whilst dead insects left in the colony reduce worker and larvae survival (Diez et al., 2014). Although historically studied from a conflict resolution perspective, policing is clearly a general mechanism to prevent constituent evolution and conflict of fitness within superorganisms, which has direct analogs to the immune policing that prevents intercellular conflicts in the

**TABLE 1 |** Convergent properties of organismal and superorganismal immune systems.

| Immune system property | Role in the (super)organism | Mechanisms in organisms | Mechanisms in superorganisms | Examples of evidence in superorganisms | Possible research questions |
|---|---|---|---|---|---|
| Immunological policing | Maintains cooperation of constituent parts and prevents them from evolving/having differential fitness | Immunosurveillance of the body for malignant cells that could lead to cancer | Surveillance by nestmates for worker reproduction | Honeybee worker policing (Ratnieks and Visscher, 1989) | Do specific workers survey their colony in search of intruders/cheaters like immune cells? |
| | | | | Clonal raider ant policing (Teseo et al., 2013) | |
| Immunological identity | Establishes the boundary of the biological individual and its unique identity, despite undergoing change over time | Specific recognition of self and gate-keeper function, determining what is accepted into the organism and what is rejected/tolerated | Unique colony odor allows for nestmate recognition. Prevents colony fusion and both intra and interspecific parasitism | Colony odor prevents colony fusion (Lenoir et al., 1999) | Why do some species exhibit stronger kin discrimination than others? Is this related to levels of parasitism/risk of colony fusion? |
| | | | | Cape honeybee soma parasites circumvent recognition system and lead to colony collapse (Martin et al., 2002) | Do parasitized colonies develop stronger or specific recognition of parasites? |
| | | | | Specific recognition of ant social parasites (Brandt et al., 2005b) | |
| Diverse immunological protection | Protects against any harmful elements (parasites & pathogens) that affect fitness | Innate and adaptive protection against a vast variety of parasites and pathogens | Many defenses are broad-spectrum and effective against a diversity of microorganisms | Metapleural gland secretions in leaf cutting ants (Bot et al., 2002) | Are commonly studied defenses such as grooming effective against other pathogens than fungi? |
| | | | | Incorporation of broadly antimicrobial substances into nests (Chapuisat et al., 2007; Chouvenc et al., 2013) | How do colonies overcome the problem of antibiotic resistance to self-produced and environmental antimicrobials? |
| | | | | | What are the costs of deploying a specific immune response? Are there trade-offs between immune defenses? |
| Distributed, systemic immunological protection | Multiple immune components interact locally to provide systemic, global protection, meaning there is no central control, and hence no single point of failure | The immune system is an example of a cognitive living network, which operates without central control | Social insects coordinate all their activity through local interactions without global oversight – superorganism immunity is expected to be no different, but colony-level studies of immunity are rare | Immediate spatial effects and global interaction network change upon pathogen exposure (Ugelvig and Cremer, 2007; Stroeymeyt et al., 2018) | How do superorganisms coordinate global responses to infection? |
| | | | | | How to superorganisms communicate about infections? |
| | | | | | How do the many lines of defense interact to produce colony-level immunity? |
| Error tolerant | A few mistakes in classification and response should not be catastrophic. Moreover, collateral damage due to an immune reaction should be tolerable | Generally, the immune system does not harm the organism. However, immunopathology does occur when immune responses are inappropriate | Non-infected brood may be removed alongside infected brood, seemingly without drastic consequences for the colony. Likewise, kin may be rejected/accepted into the colony, apparently without large impacts on fitness | Ants accepting and rejecting sick and healthy brood (Tragust et al., 2013b) | Do colony-level immune responses cause collateral damage? |
| | | | | Guard bee recognition errors (Couvillon et al., 2013) | How do colonies balance their use of toxic compounds to reduce self-harm? |

*(Continued)*

**TABLE 1 |** Continued

| Immune system property | Role in the (super)organism | Mechanisms in organisms | Mechanisms in superorganisms | Examples of evidence in superorganisms | Possible research questions |
|---|---|---|---|---|---|
| | | | | Possible collateral damage due to nest sanitation in ants (Pull et al., 2018a) | Can autoimmunity emerge in superorganisms? |
| Self-protecting | Same mechanism that protects the (super)organism also protects the immune system | By protecting the organism, the immune system is also preserved. If the immune system is compromised this severely limits organismal protection | By protecting the superorganism, the per capita risk of disease decreases; since it is the workers that provide this protection, superorganism immunity is thus also maintained. Superorganism immunity can also become compromised | Beyond a certain point in colony infection, superorganism immunity seems to collapse (Chouvenc and Su, 2012; Loreto and Hughes, 2016) | Can the social immune system become compromised by parasites/stress? |
| | | | | Parasitism reduces ability of colony to discriminate kin from nonkin (Beros et al., 2015) | How does systemic protection emerge? |
| Immunological adaptability and memory | Identification of previously unencountered pathogens and retained memory of those pathogens facilitates future responses | Universal and conserved recognition of pathogen-associated molecular pattern molecules (PAMPs) and adaptive immunity | Possible recognition of diversity of microbes and limited evidence of immunological memory | Ants groom contaminated nestmates more if they have previously encountered the same pathogen (Walker and Hughes, 2009; Konrad et al., 2018) | Do superorganisms exhibit immunological memory? |
| | | | | Micro-infections cause changes in how ants respond to contaminated nestmates in future (Konrad et al., 2018) | How do workers identify pathogens that they have never encountered? |
| | | | | | What receptors govern pathogen recognition in superorganisms? |
| Immune privilege | Certain subsets of the (super)organism receive additional immune protection and/or are protected from potential immune damage by physical barriers | Immune privilege of especially important components of the body, including germline, brain and eyes | The queen, along with the susceptible brood and possibly harder-to-replace young workers (nurses) may be subject to immune privilege | Queens and young nurses occupy a central position in ant colony social network, whilst foragers occupy periphery positions (Mersch et al., 2013; Baracchi and Cini, 2014; Quevillon et al., 2015) | How are oral food-sharing networks protected from disease to prevent queen/nurse infection? |
| | | | | Queens receive reduced pathogen load during a colony epidemic due to network reorganization (Stroeymeyt et al., 2018) | Is the queen's diet processed by workers to reduce the risk of infection? |
| | | | | Honeybee queens upregulate immune response when workers are sick (Hernández López et al., 2017) | Are certain worker task groups better protected than others? |
| | | | | | Are physical structures such as the "royal chamber" of termite nests "immune barriers" that control contact rates/restrict movement around the queen/king? |
| Apoptosis | Mechanisms for compromised constituent parts to self-remove from the (super)organism | "Cellular suicide" in organisms prevents damaged or infected cells from releasing toxins or pathogens into the body. Reduces the need to | Moribund insects "leave" the nest when close to death, including when infected. Reduces the need for nestmates to remove dying/dead individuals, | Moribund ants become isolated from nestmates (Heinze and Walter, 2010; Bos et al., 2012) | How early on does "social apoptosis" evolve in the transition to superorganismality? |

*(Continued)*

**TABLE 1** | Continued

| Immune system property | Role in the (super)organism | Mechanisms in organisms | Mechanisms in superorganisms | Examples of evidence in superorganisms | Possible research questions |
|---|---|---|---|---|---|
| | | mount an immune response Expected to evolve early on in the evolution of multicellularity | thus reducing their risk of infection | | |
| | | | | Moribund honeybees "leave" the hive and infected honeybee progress to out-of-hive tasks faster (Rueppell et al., 2010; Natsopoulou et al., 2016) | Is "social apoptosis" mediated by simple loss of attraction to nestmate/colony odors in all species? |
| | | | | Theoretical model predicts the evolution of social apoptosis in superorgansims (Rueppell et al., 2010) | Is the loss of attraction cues in ant due to being moribund or caused by infection? |
| | | | | In ants, "self-removal" appears to occur through a simple loss of attraction to nestmate and colony odors, reduced locomotion and possibly phototropism (Leclerc and Detrain, 2017) | |
| Functional redundancy | Layers of protection with multiple fail safes | Skin, cilia, mucus, local inflammation, cellular responses, humoral responses and adaptive immunity, combined, ensure that there are multiple hurdles for pathogens to overcome, reducing the likelihood of successful infection | Nest architecture, nest antimicrobials, avoidance, constitutive and induced worker behavior, and network plasticity are all examples of "layers" of defense that pathogens must cross in order to successfully infect a superorganism | Colonies exhibit pathogen avoidance, grooming to preventing infection, social network plasticity to mitigate spread and destruction of infected individuals, depending on the stage of pathogen infection (Tragust et al., 2013a; Stroeymeyt et al., 2014; Tranter et al., 2015; Davis et al., 2018; Pull et al., 2018b) | What is the role of nest architecture in the social immune response? |
| | | | | Nest architecture predicted to mitigate disease spread (Pie et al., 2004) | How do workers determine when to care for nestmates and when to destroy them as infections progress? |

*Here we have highlighted what we believe to be the key properties that define what an immune system is and does. We give the ultimate function of these properties and their proposed proximate mechanisms in organisms and superorganisms, but limit example references to superorganisms. Finally, we highlight some of the gaps in our knowledge on superorganism immunity. For a more detailed appraisal of the functional similarities between organismal and colony-level immunity see Cremer and Sixt (2009).*

evolution of multicellular organisms (Bourke, 2011; Pradeu, 2013; Ågren et al., 2019). In both cases, policing by the immune system ensures the cooperation of constituent parts and hence maximal (super)organism fitness (Bourke, 2011). Accordingly, we consider actively performed derived traits as part of superorganism immunity (e.g., worker policing), whilst traits that reduce conflict by default are not (e.g., a single-queen bottleneck). This is consistent with the suppression of tumors in multicellular organisms being part of organismal immunity, whilst unitary inheritance (single zygote-bottleneck) is not (Bourke, 2011 and references therein).

## Immunological Uniqueness of the Superorganism

It is essential that biological individuals establish a "boundary" within which cooperation between constituents occurs so that the benefits of cooperation circulate between kin (Queller, 2000; West et al., 2015). Immune systems delimit these boundaries in multicellular organisms by deciding what is accepted and rejected as part of the organism (Pradeu, 2013) and allorecognition systems seem to pre-date the evolution of obligate multicellularity (West et al., 2007 and references therein). It is through this process that the immune system maintains the unique identity of the organism across its life, despite it continually changing through growth, soma replacement and aging (Pradeu, 2012). Superorganisms establish their identity through chemical signatures that permit similar "self" recognition (Lenoir et al., 1999). Each insect produces colony-specific, long-chain CHCs on the surface of its body, which are mixed between individuals to create a uniform colony odor. This odor changes over time depending on the nest environment and the food consumed so that it is truly unique to each colony (Lenoir et al., 1999). Like the multicellular immune system that learns to recognize

itself early during embryonic development, individual insects are thought to learn their colony odor early in life, by developing an internal representation of the odor, known as a template (Lenoir et al., 1999; Bos and D'Ettorre, 2012). This template can be acquired during the larval stage but seems to become fixed during a time-sensitive window, shortly after adult emergence. The template can still be updated though as colony odor gradually changes over time. Workers discriminate self from nonself by comparing the odor of individuals they meet with their stored template; this results in cooperation when they match and aggression when they differ (Lenoir et al., 1999; Bos and D'Ettorre, 2012). Although it is possible to artificially fuse colonies and replace queens (e.g., during beekeeping), this requires a period of forced habituation, so that odors can presumably become mixed or the template is updated. Under natural conditions, colony fusion is prevented by guard insects that decide who enters the colony and the identification of intruders by all workers within the nest (Lenoir et al., 1999; Bourke, 2011). The "boundaries" of the superorganism are hence established through this odor-based recognition or colony surveillance system (Bourke, 2011).

Although colony fusion events are relatively rare in superorganisms owing to their effective self-nonself recognition system (Lenoir et al., 1999; Kronauer et al., 2010), colony identity and stability is at risk from interspecific social parasitism (Teseo et al., 2014), caused by "soma" and "germline" parasites. In the cape honeybee, for example, a strain of parasitic workers has evolved that transmits horizontally between colonies of a closely related subspecies to lay female eggs via thelytokous parthenogenesis. They give rise to more parasitic workers (Martin et al., 2002) and too many can cause colony collapse. This is remarkably similar to transmissible cancers in organisms, such as the facial tumors of Tasmanian devils (Tsuji and Dobata, 2011; Teseo et al., 2013). In both cases, these parasites are an asexual lineage of somatic origin that has broken free from their natal (super)organism to infect other (super)organisms (Bourke, 2011). Although our understanding of how somatic parasites evade the immune system is still developing, the high virulence of such diseases places a strong selection pressure on hosts to rapidly adapt (Epstein et al., 2016). We predict that workers evolve stronger discrimination abilities in populations where parasites are present, as well as rapid behavioral responses to kill parasites before they infect the colony. Germline parasites are queens that invade colonies of closely related species. Germline parasites either take up residence alongside the true queen or kill her to monopolize reproduction. They then either replace the colony soma with their own offspring or rely on the hosts' leftover workforce to raise their sexual brood (Brandt et al., 2005a). Germline parasites that hijack host reproduction are not, as far we are aware, known in metazoan organisms, but exist in colonial organisms (Cremer and Sixt, 2009 and references therein). Increased self-nonself discrimination, enhanced defensive behaviors, and the evacuation of the host queen are all mechanisms that colonies utilize to prevent germline infections. It is believed most germline parasites are successfully killed when trying to invade a colony (Brandt et al., 2005a).

## Immune Elimination of Pathogens and Parasites

Protection against microbial pathogens has been reviewed extensively elsewhere (Cremer et al., 2007, 2018; Cremer and Sixt, 2009; Wilson-Rich et al., 2009; Evans and Spivak, 2010; Rosengaus et al., 2011; Cremer, 2019), but to summarize the key findings, we see the evolution of colony-level resistance and possibly tolerance mechanisms against microbial diseases in social insects. Resistance encompasses all traits that limit the probability of infection, as well those that reduce pathogen load and lead to pathogen clearance. For example, this includes infection avoidance (Tranter et al., 2015; Pereira and Detrain, 2020), grooming (Hughes et al., 2002; Reber et al., 2011; Tragust et al., 2013a,b), the use of antimicrobials (Stow et al., 2007; Tragust et al., 2013a; Pull et al., 2018b), and reorganization of social networks (Stroeymeyt et al., 2018). Resistance mechanisms affect pathogen fitness and so can select for higher pathogen virulence over time (Cremer et al., 2018). Tolerance is the ability of organisms to cope with the damage caused by a pathogen, rather than targeting the pathogen itself. Consequently, tolerance mechanisms do not reduce pathogen load, hence relaxing selection on pathogen virulence (Råberg et al., 2009; Kutzer and Armitage, 2016). Although rarely studied, there is some evidence that colonies can tolerate the impact of infected workers on colony fitness (Scharf et al., 2012). Indeed, recent work has shown that "lazy" workers specializing on inactivity act as a reserve for when the rest of the worker force is compromised (Charbonneau et al., 2017). This could be a faster mechanism of "soma" replacement than raising new workers from eggs (Cremer et al., 2018). Whether a colony opts for resistance or tolerance will depend on pathogen–host ecology and life history. Highly virulent pathogens should generally always elicit resistance, though annual societies may opt for tolerance over costly resistance, where possible, to maximize reproduction over their comparatively short colony lifespans. Schmid-Hempel (1998) and Boomsma et al. (2005) give extensive summaries on how host life history affects social insect pathogen assemblages and disease protection mechanisms.

Social insect colonies are infested with a startling diversity of other organisms, which range from benign, non-specific associations, to extremely host-specific, co-evolved parasites (Schmid-Hempel, 1998). Although some parasites cause considerable damage to colony health when they act as vectors of other diseases or prevent queen reproduction, they generally seem to have low impacts. Like larger intercellular parasites of animals, which often exist "outside" of the body (e.g., worms in the digestive tract, lice in feathers), these larger colony parasites may be harder to remove because they are not susceptible to many of the superorganism's immunity defenses. Indeed, many social insect parasites have morphological adaptations to protect them from attack and/or develop chemical profiles that closely match their host, making them undetectable (Schmid-Hempel, 1998). Consequently, many parasites, especially those that have low levels of virulence, are likely to be tolerated. However, encapsulation of parasites (Neumann et al., 2001) and, in heavy infestations, nest abandonment are more drastic options (Cremer

et al., 2007). In general, although some systems are well studied (e.g., the *Varroa* mite; Rosenkranz et al., 2010), less is known about how social insects cope with macro parasites, and this remains an area for exciting future research.

## Immunological Cognitive Networks

Organismal cellular immunity is an example of a living cognitive network (Piñero and Solé, 2019). Living cognitive networks are defined by the ability to process information (carry out computations) and draw on past events (possess a form of memory) in order to optimize group-level decision-making. Although the brain is a classic example of a cognitive network that is neuronaly based, this is not a requirement for cognitive network formation in general. We propose that, like the organismal immune system, superorganism immunity functions as an aneural "liquid brain," where, unlike neuronaly based "solid" brains, the nodes of the cognitive network (immune cells or workers) have no fixed physical location. As in traditional solid brains, interactions based on simple rules between individuals can lead to complex emergent cognitive outputs at the superorganism-level, which are not inherently known to the individuals that make up the network (Couzin and Franks, 2003; Piñero and Solé, 2019), i.e., computation by distributed processing (Gordon, 2016). Collective cognition in both social insect and immune networks share additional key traits aside from their liquid-like nature. The first is somatic division of labor, which is epitomized by the different task-performing groups of social insects and the diverse effector cells found in the immune system. The second trait relates to the similarities in the algorithms that describe the search dynamics of social insects and immune cells. Recent work has highlighted how both adopt a variety of directional as well as random search strategies dependent on the environment and availability of stimuli (Moses et al., 2019). Both rely on frequent contacts between nodes (individuals/cells) as well as on physical structures, such as nest tunnels or vasculature, for guiding movements. A key unifying property is that "there is no one best search strategy that can be used for all search problems [. . .]; instead searchers change how they move and interact with each other and the physical environment in response to specific search problems in specific environments." (Moses et al., 2019). A search problem common to both types of network is the existence of a trade-off between search speed and accuracy, with optimal algorithms depending in both cases on the spatial layout and temporal stability of targets in the environment. With respect to random searches, studies combining modeling and empirical approaches have revealed important similarities in the random search strategies adopted by ants and T cells (Fricke et al., 2016), though it is unknown if workers utilize such search strategies to conduct colony-level immunosurveillance (**Table 1**). These findings reveal that fundamental shared tasks between individual and social immune systems (such as the differentiation between self/non-self and the treatment of invading microorganisms) may be regulated by convergent mechanisms of collective action.

In summary, there is compelling evidence that superorganisms have evolved an immune system that contributes significantly to their unique individuality. Although more comparative data are needed, it may even be possible to determine when insect societies have reached key milestones in superorganism evolution by examining the status of their colony-level immune system development. Hence, as discussed in more detail in sections to come, we suggest that, as with the evolution of multicellularity, immune system evolution could be an important criterion for determining when key steps along the road to superorganismality have been taken.

# THE ROLE OF IMMUNITY IN THE MAJOR TRANSITION TO SUPERORGANISMALITY

In this section, we examine how disease and selection for immunity may have shaped the transition to superorganismality (**Figure 2**). Major evolutionary transitions can be broken down into three steps, as defined by Bourke (2011): (1) group formation concerns the genetic and ecological factors that initially favor social life, (2) group maintenance prevents exploitation of the social group (e.g., from selfish and parasitic elements), and (3) group transformation is the development of the group into a complex, integrated biological individual. Although group formation and maintenance possibly overlap (West et al., 2015), we consider them separately in order to partition traits that promote group-living from post-group formation adaptations that evolved specifically to protect the new individual. Following Bourke, we use the broad term "group" to describe these processes, though only groups formed

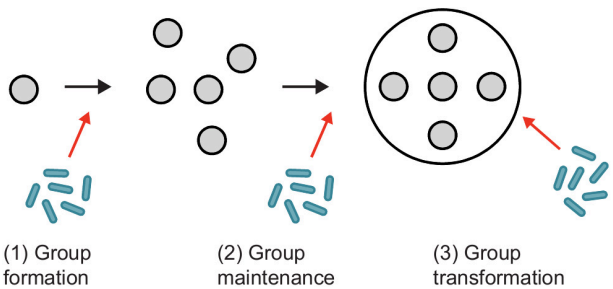


**FIGURE 2 |** Disease and the emergence of a major transition. (1) Disease causing agents have the potential to both hinder and promote the formation of a social group. For example, an increase in the risk of disease due to the close proximity of relatives may select against group formation, whilst additive effects of individual immunity and cooperative disease defense may positively select for sociality. (2) Once a group has formed, the aforementioned increase in the cost of disease may outweigh the benefits afforded by sociality. Consequently, pre-existing individual-level disease defenses should be co-opted and elaborated to maintain group integrity instead. Additional disease defenses that function specifically to protect the group are therefore predicted to evolve, such as self/non-self recognition and an ability to deal with infected group members. (3) Group transformation into a cohesive, complex biological individual. Increases in complexity (e.g., number of cell or worker types) and size both encourage and require a corresponding increase in immune defenses, as larger, more complex biological individuals are expected to encounter a greater diversity and number of disease-causing agents. (Figure inspired by West et al., 2015).

through direct co-ancestry (e.g., aggregations of clonal cells or families with retained offspring) can potentially undergo a major transition in individuality (Boomsma and Gawne, 2018 and references therein).

## Group Formation

Formation of a cooperating group is the first step towards a major transition (Bourke, 2011; West et al., 2015). Two conditions favor the evolution of cooperation: ecological and efficiency benefits that encourage cooperation and a mechanism to direct those benefits back to the actor or its relatives (West et al., 2015). The latter is achieved through kin selection where helping relatives ensures shared genes are passed on to the next generation. Benefits promoting cooperation are numerous and well-studied, but the role of disease has largely been neglected (but examples include, Rosengaus et al., 2013; Cole and Rosengaus, 2019; Nuotclà et al., 2019). Although disease has long been considered an antagonist that hinders group formation (Freeland, 1976; Hamilton, 1987), the view that group-living increases infection risk is over-simplistic; studies searching for a correlation between group living and disease risk have produced mixed results (Wilson et al., 2003; Nunn et al., 2015; Ezenwa et al., 2016). On the one hand, this may be due to the evolution of derived traits in social animals that compensate for an increased infection risk (Ezenwa et al., 2016), but on the other, there are likely innate benefits to group-living that reduce the impact of infectious disease. For colonies of cells and insects, the additive effects of individual defenses, i.e., autonomous immunity in cells and individual immunity in insects, may reduce the overall risk of infection in the group and so foster cooperation (e.g., percolation within a heterogenous network; Sander et al., 2002). For example, the individual use of antimicrobial substances by cells and insects in the same space could create a barrier against disease transmission in their nests. Indeed, many solitary and subsocial insects have been shown to use antimicrobial substances, which, in family groups, have important impacts on offspring growth and development (Cotter et al., 2013; Diehl et al., 2015; Meunier, 2015). When close kin live together with little competition, there is no disadvantage to providing direct aid to others; for example, grooming can easily be directed to others instead of oneself. Group-living may also provide indirect benefits that aid recovery from infections, since resource acquisition and other tasks that trade-off with immunity are shared, allowing sick individuals to invest resources into clearing and recovering from infection (Hart, 1990; Ezenwa et al., 2016). Additionally, this may confer improved tolerance to infections where the impact it has on the host is ameliorated, e.g., through increased food consumption (Almberg et al., 2015), leading eventually to reduced pathogen virulence. Pathogen evolution and diversity could be altered by simple social behaviors, such as grooming, leading to disrupted within-host pathogen dynamics during coinfections that result in altered pathogen communities and virulence (Milutinović et al., 2020). This could select for group-living if it positively affects host fitness. Much like during the evolution of the individual immune system, many of the building blocks of superorganism immunity (e.g., hygienic behaviors, external immune activity, etc.) are widespread in non-social insect lineages (Bulmer et al., 2012; Otti et al., 2014; Meunier, 2015). Therefore, initially simple, cooperative behaviors such as grooming, combined with means to deploy disinfectants into the external environment, could both protect the group and encourage further cooperation (Esparza-Mora et al., 2020).

## Group Maintenance

Once a group has formed, mechanisms to maintain cooperation should evolve that prevent social cheaters, pathogens and parasites from driving newly emerged groups to extinction (Godfrey-Smith and Goodnight, 2013). Social cheaters harm the group by using its resources but not contributing back. True parasites and pathogens are more likely to spread in groups of relatives, since there is no to little genetic diversity in a colony of clonal cells or insect relatives (Hamilton, 1987). Moreover, in groups of mutually dependent individuals, be they cells or insects, a single infected individual is a hazard to all others: cells and insects that have lost their totipotency cannot simply disperse and reproduce elsewhere when infection breaks out (they are "all in the same boat"; Ågren et al., 2019), even within the more modular insect colonies (Chouvenc and Su, 2012; Loreto and Hughes, 2016). Hence, at this stage, selection should produce defenses that evolve specifically to promote the health of the group, via inclusive fitness benefits, as well as ways to deal with lethal infections. This requires an ability to detect social cheaters and diseases, as discussed above. Recall that cancerous cells possess specific antigens that guide their immune elimination (Urban and Schreiber, 1992; Feng et al., 2018), worker laid eggs an odor that differs to queen laid eggs (Ratnieks and Visscher, 1989), and aberrantly reproducing workers in clonal raider ants have divergent CHC profiles (Teseo et al., 2013). Diseased insects also smell differently to their nestmates and so can be actively eliminated from the colony (Richard et al., 2008; Swanson et al., 2009; Baracchi et al., 2012; Pull et al., 2018b). There is also evidence for the mechanical "pushing out" of cancerous cells clumps from proliferative tissue (Hogan et al., 2009; Ågren et al., 2019). At this stage chemical communication is most likely to be based on simple cues triggered by illness/damage, but, because groups are composed of kin, the use of signals that are actively broadcast may also evolve in more complex groups (Cremer et al., 2018). Signaling of intracellular infections is also paramount in the elimination of compromised cells in a body (Grimsley and Ravichandran, 2003; Ravichandran, 2010; Feng et al., 2018). Moreover, at this step, immune defenses help to delineate the group, i.e., determine what is self and what is non-self, a feature that also emerged very early during the evolutionary origins of animal multicellularity (Müller et al., 1999; Pradeu, 2012). Over time, the accumulation of immunity adaptations that police social cheaters, maintain group identity and prevent infection, led to the formation of stable groups beginning to show organism-like design.

## Group Transformation Into a Biological Individual

In rare instances, some stable, cooperating groups developed permanent separation of helper and reproductive roles and

underwent a major transition, forming a new kind of biological individual. In many multicellular organisms (such as higher metazoans), the soma component loses its totipotency completely during this transition, and soma and germline roles become irreversibly independent. However, a fully sequestered germline is not generally required for major transitions in fraternal organism evolution (Queller, 2000), as evidenced by its absence in plants and early branching metazoan phyla (Extavour, 2007; Radzvilavicius et al., 2016; Fierro-Constaín et al., 2017). In contrast, group-adapted immune systems are conserved and taxonomically widespread, so may be a more useful criterion for defining major transitions in biological individuality (Pradeu, 2013). This is not to denigrate the importance of germ-soma segregation during the evolution of (metazoan) complexity, where it is clearly correlated with greater individual size and complexity, but we hypothesize that this emerges in parallel to or even after the foundations of a group-level immune system have been established. In metazoan immunity, evidence of a core ancestral immune system is well described (see next section), yet effector cell and immune pathway diversity varies greatly between animal phyla, with mechanisms of adaptive immunity appearing to be restricted to arguably more complex animal lineages (Müller et al., 2018). A comprehensive quantitative comparison of immune system and organismal complexity (as defined by cell-type, tissue diversity and degree of germ-soma segregation) has to our knowledge not been conducted in Metazoa, though, generally, larger organisms have more cell types (Bonner, 2004; Strassmann and Queller, 2007) and a fully sequestered germline. Hence, we predict that gradients of animal complexity, size and immune specialization should be positively correlated with each other. A gradient of complexity is also apparent among social insects (based on colony size and the number of different worker types; Bonner, 1993; Strassmann and Queller, 2007) including species whose colonies have ostensibly passed an evolutionary point of no return in individuality (Ferguson-Gow et al., 2014; Bourke, 2019). With an increase in size and complexity the probability of infection is likely to rise, and available data on parasite richness suggest this relationship holds true (Schmid-Hempel, 1998). A higher density of hosts with more intricate interactions facilitates greater disease transmission in larger colonies; additionally, they are a larger target with more niches for parasites to exploit. For example, different parasite taxa preferentially select small or larger worker castes within a colony as hosts (Schmid-Hempel, 1998). In the fungus-growing ants (higher attines), larger workers that are morphologically specialized for leaf-cutting have small metapleural glands relative to their size and more porous infrabuccal filters than smaller workers, rendering them more susceptible to parasites (Hughes et al., 2002, 2008b). Furthermore, higher levels of foraging and nest expansion in large colonies should increase pathogen exposure and transmission. Consequently, escalating colony size, complexity, and integration will require a corresponding scaling in immunity. Comparative studies between species demonstrate an increase in the strength of antimicrobials used by bees that correlates which correlates with colony size and complexity (Stow et al., 2007). Within species, denser and/or larger colonies produces higher immunocompetence in workers (Ruiz-Gonzalez et al., 2009; Armitage and Boomsma, 2010) and larger colonies have increased survival when exposed to pathogens compared to small ones (Leclerc and Detrain, 2018). Since larger colonies have more workers, exhibit advanced self-organization and increased task specialization, immunity itself should also become more integrated and complex with colony size. With more workers some colony members may be able to specialize on immunity roles (e.g., waste management; Eyer et al., 2013) and novel disease-related tasks can evolve, such as colony-level medication with substances collected from the environment (Chapuisat et al., 2007; Simone-Finstrom and Spivak, 2012; Bos et al., 2015); such behavior is less likely to evolve in smaller societies as the limited number of workers should prioritize essential functions such as food collection. As well as the active elimination of sick individuals by nestmates, self-removal mechanisms should also occur: cellular apoptosis (Michod and Roze, 2001) and social apoptosis ("self-removal"; Rueppell, 2004; Heinze and Walter, 2010; Rueppell et al., 2010; Bos et al., 2012; Page et al., 2016; Leclerc and Detrain, 2017) allow individuals to isolate themselves, thereby protecting their kin (Ugelvig and Cremer, 2007; Stroeymeyt et al., 2018). The causal relationship between the emergence of these advanced traits and superorganism complexity is unclear, and we are open to the view that selection on social immune traits due to ecological pressure could have also facilitated subsequent increases in complexity, rather than vice versa. Although an entirely open area of research, we expect large, more cohesive colonies to have efficient, rapid responses to disease, greater worker specialization for dealing with disease, and an increased protection of their highly specialized but more vulnerable queens, compared to smaller, less complex species.

## EVOLUTION OF SUPERORGANISM IMMUNITY

Several interesting and open questions arise from an immunological perspective of the major evolutionary transitions: do immune systems evolve before a full transition to individuality? Is it possible to undergo a major evolutionary transition without some form of immunity? In the transition to multicellularity some form of immunity probably evolved before biological individuality became fixed and pre-adaptions existed that facilitated immune system evolution. Given their strong, evolutionary convergences (Cremer and Sixt, 2009), organismal and superorganismal immune system evolution likely followed similar patterns.

In organismal immunity, many of the building blocks that make up antimicrobial defense mechanisms can be traced back to progenitor elements found in simpler ancestors. A classic example of this is the RNAi pathway, a conserved intracellular defense system of eukaryotes, which consists of an evolutionarily agglomeration of components derived from diverse prokaryotic ancestors (Shabalina and Koonin, 2008). The co-option and reuse of the same, probably ancient, protein domains appear widespread in the evolution of eukaryotic innate immunity (Richter and Levin, 2019). Importantly, the emergence of novel forms of immunity occurred concomitantly with the major

transitions in eukaryotic complexity, and many of these immune functions have been retained ever since. For example, in animals, the Toll-like receptor (TLR) signaling pathway is a key host immune receptor cascade involved in vertebrate innate immunity (Akira, 2003). But fully functioning Toll/TLR signaling pathways are also found in distantly related animal phyla such as *Drosophila* (Lemaitre et al., 1996) and non-bilaterian animals such as Porifera and Cnidaria (Wiens et al., 2006; Hemmrich et al., 2007; Franzenburg et al., 2012). This demonstrates that an immune role for TLR-signaling evolved early in animal evolution and has been conserved across Metazoa ever since. Effector immune cells are also present from sponges through to mammals and are essential for innate immunity (Buchmann, 2014). Highly conserved cysteine-rich scavenger receptors, as well as G-protein coupled receptor genes, are found across the metazoans, which facilitate effector cell adhesion, self/non self-recognition, phagocytosis and melanization (Müller et al., 1999; Dzik, 2010; Pita et al., 2018). The importance of effector cells in the evolution of multicellular life is highlighted by the slime mould *Dictyostelium*, a facultatively multicellular organism possessing sentinel cells that engulf bacteria and sequester toxins (Chen et al., 2007). Some sentinel cell and phagocytic functions in *Dictyostelium* (Chen et al., 2007) involve a TIR-domain containing protein, as well as other signal transducers and activators of transcription that have gene homologs in animals (Dunn et al., 2018), again hinting that these may have evolved early on in multicellular evolution.

Three conclusions emerge from research on organismal immune system evolution that might provide useful insights for studying the evolution of immunity during the major transition to superorganismality: (i) New forms of immunity are constructed from diverse and often unrelated building blocks found in simpler ancestors; (ii) Progenitor building blocks are repeatedly co-opted into immune roles in independent evolutionary lineages; and (iii) New forms of immunity are highly conserved following a major transition, although modifications and additions to core immune processes are widespread. Although the study of disease defense in group-living insects is an important and fascinating area of research in its own right (Cotter and Kilner, 2010; Van Meyel et al., 2018), studies on subsocial and family based cooperative breeders – the most likely ancestors of superorganisms (Linksvayer and Wade, 2005; Nalepa, 2015; Boomsma and Gawne, 2018; Cole and Rosengaus, 2019) – could prove especially useful comparative models for exploring the evolution of social immune systems (e.g., Nuotclà et al., 2019). Indeed, many solitary, familial and aggregative insects possess behavioral and physiological adaptations, such as pathogen avoidance, grooming and the production of antimicrobials (de Roode and Lefèvre, 2012; Meunier, 2015). How these traits become co-opted and modified with the transition to superorganismality, so that they provide colony-level immunity instead of individual protection, is unknown. However, many of the adaptations that we believe are necessary for a social immune system (**Table 1**) seem to be missing in most non-superorganisms (Meunier, 2015), appearing only in advanced taxa "close" to the superorganismal threshold. Whether this is a true reflection or sampling bias remains to be seen but suggests that novel evolutionary innovations are also important. Although we lack sufficient data to make firm conclusions about the evolution of immunity in social insects, we can speculate, broadly, how superorganism immunity likely emerged concomitantly with the evolution of superorganisms, from their family based ancestors (**Figure 3**).

## Families With Parental Care

Families are temporary associations between parent and offspring, where either one or both parents provide care to young that disperse once independent (Clutton-Brock, 1991). All individuals are totipotent and the group dissolves once parental care is no longer required. In insects, families with parental care are often termed subsocial to highlight their incipient role in social evolution (Wilson, 1971). Family life with parental care is the first step towards the evolution of complex sociality across the animal kingdom (Hughes et al., 2008a; Chak et al., 2017; Downing et al., 2020). Lifetime monogamy between parents generates maximal sibling relatedness (Boomsma, 2009; Downing et al., 2016, 2020), whilst parental care creates an avenue for helpers to evolve, by putting off dispersal and providing sibling care instead (Holman, 2014).

Parental care seems to have also played an incipient role in the evolution of social immune systems. Based on extant examples, we expect the subsocial ancestors of superorganisms to have already possessed personal immunity (physiological and behavioral defenses) and exhibited at least some form of parental behavior (either direct brood rearing or nest defense), including extended disease protection of offspring (Linksvayer and Wade, 2005; Trumbo, 2012; Nalepa, 2015; Cole and Rosengaus, 2019). One hypothesis, backed by transcriptomic data, is that an earlier expression of parental care genes in retained offspring provided the substrate upon which helper behavior in societies evolved (Linksvayer and Wade, 2005; Rehan et al., 2014). Both ant queens and termite pairs undertake a variety of behavioral disease defenses during colony foundation that are later performed by workers (Chouvenc et al., 2011; Pull and Cremer, 2017; Cole and Rosengaus, 2019), but whether this is evidence that social disease defenses emerge from parental care requires elucidation.

In many Hemiptera, the only parental care provided by mothers is the guarding of eggs from parasitoids, with mothers immediately abandoning their young when they hatch (Wilson, 1971). More widely, preventing microbial or parasitic infection of eggs and young is observed in many taxa (Trumbo, 2012; Cotter et al., 2013). Constructing nests in which to lay eggs and raise young is common and often takes place in microbialy rich soil or wood (Trumbo, 2012). Such behavior imposes a need on parents to evolve antimicrobial defenses that keep the nest environment sanitary; consequently, the use of exocrine gland compounds, antimicrobial faeces and the segregation of potentially harmful waste are commonly observed (Meunier, 2015). Moreover, when food is provided to developing brood it also requires processing so that it does not become a source of contagion, and to prevent microorganisms outcompeting the insect's young. This has been well studied in burying beetles and beewolfs, where parents use chemicals and/or form permanent symbiosis with antibiotic-producing microbes, to manage microbial communities on food

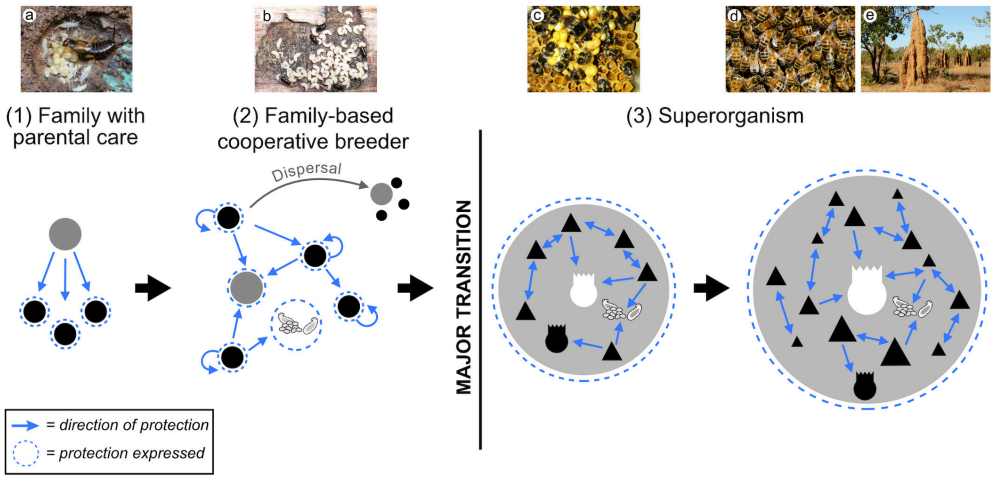


**FIGURE 3 |** The evolution of superorganism immunity. Superorganisms evolve from subsocial and family based cooperatively breeding ancestors with full-sibling helpers, termed the "subsocial route" to sociality. (1) In insect families [e.g., earwig mother and offspring (a)], extended parental care is primarily administered from parent to offspring to increase the chances of offspring survival and thus parent reproductive success. Both solitary and subsocial insects express many behaviors that appear to have acted as the building blocks upon which social immune systems later evolved. (2) In family based cooperative breeders, some totipotent offspring are retained as helpers at the nest to raise additional siblings. There is considerable variation in insect cooperatively breeding systems, with helpers in some species gaining most of their fitness through independent reproduction, whereas others derive most, if not all, fitness indirectly. Consequently, the evolution of incipient social immune systems in more advanced societies may be observed (i.e., the single piece nesting lower termites). However, in less complex societies where most/all individuals readily disperse [e.g., *Xyleborinus* ambrosia beetles (b)], social disease defenses are predicted to be less advanced. Hence, we expect social disease protection of individuals, but not a true social immune system. (3) In contrast, obligate worker altruism and the subsequent "transfer of fitness" to the colony-level selects for the evolution of a true immune system in superorganisms. This superorganism immunity is a higher-level adaptation that maximizes superorganism fitness. We predict significant variation in immune system protection that reflects the degree of "individuation" found among superorganisms, i.e., from less advanced [e.g., bumblebees (c)] to more advanced linages [e.g., honeybees (d) and fungus-growing termites (e)]. Photos by: (a) Tom Oates, 2010, available at Wikimedia Commons (CC BY-SA 3.0: https://creativecommons.org/licenses/by-sa/3.0/deed.en), (b) Peter Biedermann, (c) Victoria Blanchard, (d,e) Pixabay.com.

and prevent spoilage (Kaltenpoth et al., 2005; Rozen et al., 2008; Herzner et al., 2011; Rosengaus et al., 2013; Shukla et al., 2018).

As a consequence of parental care, many pre-adaptations, such as grooming, antimicrobial secretions, the formation of stable microbial symbioses, and rudimentary nest structures existed that could have facilitated transitions in insect social complexity, by limiting the impact of disease in prototypical colonies (**Figure 4**). Moreover, when independent reproduction is risky (Kennedy et al., 2018), the enhanced protection provided by extended parental care may have acted as an additional incentive for offspring to stay with their parents instead, where they can gain both indirect fitness through raising siblings and potentially direct fitness through nest inheritance (Downing et al., 2018; Cole and Rosengaus, 2019). When this switch occurs, family life evolves beyond simple parent-offspring associations, to cooperatively breeding families.

## Family Based Co-operative Breeders

Cooperative breeders are social groups that exhibit alloparental care. Some individuals help raise offspring that are not their own but, importantly, retain the ability to reproduce in the future (Crespi and Yanega, 1995; Clutton-Brock, 2002). Only family based groups that originate through offspring retention express complex sociality with reproductive division of labor; this is apparent in cooperatively breeding insects (Hughes et al., 2008a; Boomsma, 2009; Boomsma and Gawne, 2018), crustaceans (Chak et al., 2017), birds (Downing et al., 2020), and mammals (Jarvis, 1981; Lukas and Clutton-Brock, 2013). Family based cooperative breeders are the most likely pre-cursors of superorganisms (Boomsma and Gawne, 2018), and have hence also been termed facultatively eusocial (Crespi and Yanega, 1995; Boomsma and Gawne, 2018). Insect examples include polistine wasps, the gall-forming thrips and aphids and ambrosia beetles (Choe and Crespi, 1997; Boomsma and Gawne, 2018).

In these family based cooperative breeders, hygienic measures that are otherwise performed as part of parental care can now be used instead as part of sib-care. Due to pre-existing adaptations from parental care (previous section), each individual is already equipped with behaviors and morphological structures that can provide cooperative disease care within their natal nest, which may result in enhanced disease protection of the family group (Nuotclà et al., 2019). For example, helpers grooming the brood and one another, social microbial management, proactive nest hygiene and the production of antimicrobial substances are frequently found (Benton and Foster, 1992; Turnbull et al., 2012; Biedermann and Rohlfs, 2017; Nuotclà et al., 2019). Within the cooperative breeders, we find a gradient of social complexity, with the social aphids and thrips and their physically differentiated soldier castes, sitting close to the superorganismal border (Boomsma and Gawne, 2018). Closer still are the many one-piece/wood-dwelling lower termites, such as *Zootermopsis*. In such species, colony life is obligate and perennial, queens are moderately specialized for reproduction and pre-soldier instars can become reproductive

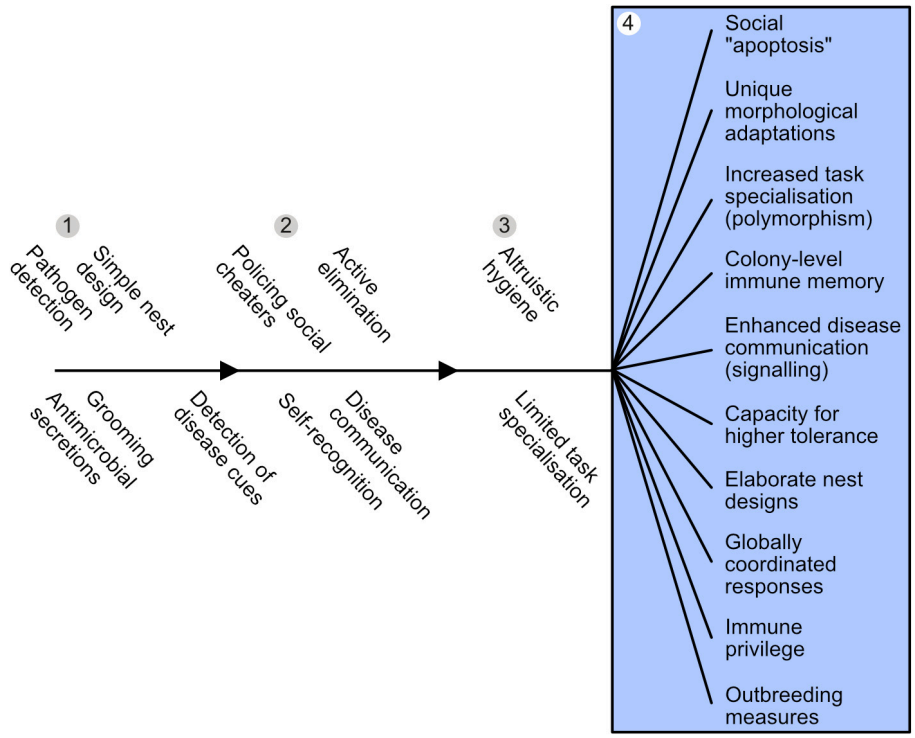


**FIGURE 4 |** Emergence and diversification of superorganism immunity. Adaptations that may have evolved prior to – and thus enabled – the transition to superorganismality are listed in the order we hypothesize they might appear, as an insect family approaches superorganismality. Colony-level immunity undergoes a systemic diversification and specialization to become a social immune system in superorganisms (blue box) that is functionally equivalent to an individual immune system. (1) Numerous pre-adaptions present in subsocial ancestors, such as pathogen detection, grooming and antimicrobial use, may have facilitated initial insect cooperation. (2) As insect family groups become larger and more interdependent, adaptations are predicted to evolve to counter the increased risk of disease inherent to kin-structured groups living in close social proximity, i.e., detection and/or elimination of sick family members (e.g., either avoidance or cannibalism/exclusion from nest). Additionally, mechanisms to police social cheaters and allorecognition should emerge to maintain social stability. (3) The evolution of altruistic hygiene, where helpers risk survival to augment the fitness of siblings is predicted in species where worker individuals accrue a large but not total proportion of fitness indirectly. Specializations for disease-related tasks are predicted and an incipient social immune system emerges. (4) Where reproductive-worker roles become effectively obligate, social immune systems with corresponding levels of diversification, specialization and intricacy are predicted to emerge (blue box).

(Myles, 1986), although this capacity is lost following the final soldier moult (Thorne, 1997). The helpers ("false-workers") behave altruistically but are totipotent and, aside from individuals destined to become soldiers, can independently reproduce under the right colony conditions (either by dispersing or through nest inheritance; Shellman-Reeve, 1997; Myles, 1999; Korb et al., 2012; Boomsma and Gawne, 2018). Their colonies are also small, have simple nest architecture, and helpers lack any clear task specialization (Rosengaus and Traniello, 1993). Although social disease defenses are clearly documented in other cooperative breeders (Benton and Foster, 1992; Turnbull et al., 2012; Nuotclà et al., 2019), decades of work by Rosengaus (Rosengaus et al., 2011 and references therein) shows that collective immune defenses are very well-developed in *Zootermopsis*: for example, grooming is effective against highly virulent pathogens (Rosengaus et al., 1998), they inform nestmates of the presence of pathogens (Rosengaus et al., 1999) and they produce effective antimicrobials (Rosengaus et al., 2004).

It is probably no coincidence that *Zootermopsis* and one-piece nesting termite species (Calleri et al., 2010), which seem to have progressed some way towards superorganismality due to the presence of a permanent and irreversible physical soldier caste, have considerable cooperative disease defenses. This suggests that collective immune defenses evolve before – and probably thus enable – transitions to superorganismality. We believe there are several reasons for this: when offspring are retained at the nest as helpers, the per capita risk of disease increases due to the density of potential hosts and the frequency of social interactions between them (Schmid-Hempel, 1998); nests begin to accumulate larger amounts of dangerous waste; and, chiefly, since family based groups are by definition closely related, they are more susceptible to the same pathogens, which is especially true in clonal and inbred species (Hamilton, 1987; Chapman et al., 2000; Abbot et al., 2001). When the presence of helpers reduces the cost of disease for parents, even initially small effects on parental fitness could select for further social immune elaborations in retained offspring (Holman, 2014). This positive feedback on fitness might then strengthen selection for reproductive division of labor, as disease-related tasks are inherently risky (Cooper and West, 2018); indeed, specific adaptations against disease seem to be more apparent in physical castes that are sterile (Benton and Foster, 1992; Turnbull et al., 2012). Division of

labor may also free reproducers of some of the costs of immune investment – possibly improving fecundity – whilst increasing their dependency on the helpers for immune protection. The more advanced a cooperatively breeding society becomes (i.e., larger with more division of labor; Cooper and West, 2018), the greater the interdependency between reproductive and helpers will be, and the likelihood that a social immune system thus evolves as part of the process of individuation leading to superorganismality. Immunity-driven changes in reproducer-helper dependency may even accelerate this transition (based on positive feedback; Holman, 2014).

## Superorganisms

Superorganismal adaptations can only evolve when within-colony selection of constituents is negligible (Gardner and Grafen, 2009). In superorganisms, within-colony selection is negated by strict lifetime monogamy of colony founders, resulting in maximal sibling relatedness equal to that of parent and offspring, and genetic bottlenecking during colony foundation (Gardner and Grafen, 2009; Boomsma and Gawne, 2018). However, recall that policing mechanisms are still necessary to mitigate lower-level constituent evolution (Ågren et al., 2019). During the process of individualization, there is a "transfer of fitness" from the lower-level constituents to the new, higher-level entity, so that it becomes the fitness-maximizing biological individual. When this process is complete, such that queen and worker roles become totally interdependent, workers become as irrevocably committed to their natal colony as a somatic cell is to a human body (Boomsma and Gawne, 2018). This eliminates conflict over the performance of somatic tasks by workers, since it is in the interest of all colony members to grow the colony, find food, maintain nest homeostasis and prevent disease (Boomsma and Gawne, 2018). Moreover, it is likely that queens in mature colonies become heavily dependent on the workers to keep them healthy due to extreme adaptations for reproduction (e.g., physogastry in termite queens prevents self-grooming).

Unlike a totipotent helper, superorganism workers are free to evolve specializations that solely enhance the fitness of the superorganism, as they are unrestricted by potential costs to independent reproduction (Gardner, 2013). For example, physical worker castes that are morphologically specialized for certain tasks only evolve in Hymenoptera once superorganismality is achieved (Beekman and Oldroyd, 2019). Superorganismality subsequently selects for "better" workers (and queens) with specific adaptations for superorganism immunity, such as the potential evolution of "social apoptosis" (support for which is seen in: Heinze and Walter, 2010; Bos et al., 2012; Page et al., 2016; Leclerc and Detrain, 2017; Pull et al., 2018b), disease-reducing morphological adaptations (Stow et al., 2007; Morgan, 2008), physical task specialization (Hughes et al., 2010), sophisticated disease communication (Richard et al., 2008; Baracchi et al., 2012; Hernández López et al., 2017; Pull et al., 2018b), and globally coordinated responses to infection (Hart and Brown, 2002; Stroeymeyt et al., 2018), among multiple possible traits (**Figure 4**). In other words, disease defense in superorganisms can undergo a complete developmental overhaul, to become as specialized, diversified and integrated as necessary, according to a superorganism's life history and pathogen pressure (Boomsma et al., 2005; Boomsma and Gawne, 2018; Cremer et al., 2018). This advanced, derived form of immune protection – or *superorganism immunity* – hence constitutes a colony-level immune system that exists to provide systemic colony protection, resulting in maximal superorganism fitness.

Once obligate queen-worker roles evolve, measures such as polyandry can also develop that increase within-colony genetic heterozygosity. Not only does polyandry decrease a colony's disease susceptibility (Tarpy, 2003; Hughes et al., 2004; Seeley and Tarpy, 2007) and make it harder for pathogens to adapt to their hosts (Hughes et al., 2004), it is also likely that increased colony heterozygosity improves the efficacy of other traits. For example, it has been shown that tramp ant colonies with higher heterozygosity have improved pathogen removal abilities than colonies with lower heterozygosity (due to experimental inbreeding; Ugelvig et al., 2010; but see also: Reber et al., 2008) and that polyandrous leaf cutting ants have larger metapleural glands than monogamous species (Hughes et al., 2008b). Polyandry also enhances task specialization: in desert ants, task specialization including waste management is partly controlled by patriline (Eyer et al., 2013) and recent work on altruistic rescue behavior reveals that such specialization is both heritable and genetically determined, with some patrilines being more likely to act as "first responders" (Andras et al., 2020). Consequently, polyandry – which can only evolve in superorganismal colonies (Hughes et al., 2008a; Boomsma, 2009; Beekman and Oldroyd, 2019) – may further increase the effectiveness and complexity of superorganism immunity.

# FUTURE DIRECTIONS

The idea we have endeavored to advance in this review, that superorganisms have evolved an immune system and that immunity enables major transitions, are hypotheses that require rigorous testing. Of particular importance is determining when a social immune system has evolved and how this relates to the transition to superorganismality; that is, does immunity evolve before, with or after this transition? Whilst it is likely that many ant, bee and termite species do indeed have a social immune system, a major difficulty is that, like the metazoan immune system, it also represents a distributed "organ": it is an incredibly diverse and diffuse self-organized set of adaptations that emerges from local interactions between individuals with their nestmates, the brood, reproductives, nest architecture and the parasites/pathogens themselves, in addition to the actions of contaminated and sick nestmates. This makes it difficult to pin down exactly when an immune system has emerged, since it needs to be studied holistically and should be backed up with comparative analyses across species. Despite these challenges, we are optimistic that a set of criteria can be established for determining when colony-level immunity has evolved (**Table 1**). We hope that by highlighting the general importance of immunity in the major transitions (Pradeu, 2013), it can be

used alongside other important criteria such as queen-worker differentiation (Wheeler, 1911; Boomsma and Gawne, 2018), to determine which species are superorganisms.

We currently lack good data showing that superorganism immune systems affect colony fitness and are heritable (Cremer et al., 2018). Specific behaviors have been shown to have an inherited genetic component (Rothenbuhler, 1964; Eyer et al., 2013), but studies are still needed that show social immune systems are shaped by natural selection. We can theorize that colonies with enhanced superorganism immunity should exhibit lower worker and brood mortality rates (Diez et al., 2014); higher levels of worker productivity, because activity and lifespans of healthy workers will not be affected by immunity trade-offs or pathogen-induced cognitive impairment (Moret and Schmid-Hempel, 2000; Gomez-moracho et al., 2017); fair better in intra- and interspecific competition for resources; and have well provisioned healthy queens that lay more eggs. A relatively unexplored aspect of colony immunity is the cost it incurs. For example, increased levels of grooming and the production of expensive glandular secretions will likely entail productivity and metabolic costs that trade-off with other colony-level tasks. Nest disinfection with caustic chemicals that harm nestmates (Poulsen et al., 2002; Theis et al., 2015; Pull et al., 2018a) and excessive use of destructive hygienic behaviors, such as cannibalism (Davis et al., 2018), could represent forms of superorganism autoimmunity. At a macroevolutionary scale, the presence of a sting for example in ants is costly, and precludes the evolution of other traits, such as larger colony size and defensive spines (Blanchard and Moreau, 2017). Consequently, just as immune systems trade-off with other fitness determining traits in organisms, we expect colony-level immunity to cause life history trade-offs at the level of the superorganism.

A major transitions perspective has helped in our understanding of the ultimate explanations for the evolution of superorganism immunity (Bourke, 2011; Cremer et al., 2018). But the recent suggestion that the evolution of biological individuality begins at the switch to superorganismality (Bourke, 2019) opens up a new line of research that is so far relatively unexplored; namely, that increases in colony complexity (measured as colony size and number of worker types; Bonner, 1993, 2004; Strassmann and Queller, 2007; Ferguson-Gow et al., 2014) will have strong influences on the evolution of colony-level immunity, or vice-versa (Burchill and Moreau, 2016). We would even suggest, based on work in single-piece nesting lower termites (Rosengaus et al., 2011), that the process of individuation begins before a major transition occurs. This is because an incipient immune system of some form is likely necessary to enable a major transition and so probably evolves during the group maintenance stage as it transitions into a biological individual, along with other adaptations that accrue to benefit the survival of the group. Still, we expect that once a major transition has occurred, superorganism immunity can undergo expansive evolutionary innovation and diversification, as workers become as specialized as necessary for the task of immunity (**Figure 4**; Gardner, 2013; Boomsma and Gawne, 2018). This may in part explain informal observations that some superorganismal taxa, such as the small, relatively simple societies of bumblebees, appear still to rely more on individual immunity than their colony-level immunity compared to the more cohesive, larger colonies of honeybees, i.e., honeybees have undergone greater individuation (Bourke, 2019). Rigorous testing and comparative analyses across species are, therefore, needed to examine whether an immunity–complexity correlation exists, and such research will undoubtedly uncover important relationships about the direction of the causes and consequences of immunological complexity, which may also help to explain patterns of complexity observed in the multicellular domain.

Another important aspect is understanding superorganism immunity evolution. One approach to this question is to examine organisms that most likely resemble the family based ancestors of modern day social insects (Meunier, 2015). Burying beetles with extensive parental care are now well-established models of cooperation and conflict in families (Scott, 1998), and, more recently, earwig family life has become another promising model system to study parental care in insects (Diehl et al., 2015). Both taxa use antimicrobial chemicals to control and shape the microbial community that surrounds developing offspring (Hoback et al., 2004; Gasch et al., 2013), with interesting trade-offs between individual immunity and how much care they can invest into to their young (Cotter et al., 2013; Diehl et al., 2015). Another promising but understudied system are the wood-eating cockroaches of the genus *Cryptocercus*. Phylogenetic approaches group these insects together in a clade with termites, indicating the latter likely evolved from subsocial wood eating cockroach ancestors (Lo et al., 2000). Indeed, *Cryptocercus* exhibit extended parental care and have faeces with antifungal properties; it has been suggested that these and similar pre-adaptations were factors encouraging the evolution of sociality in termites (Rosengaus et al., 2013; Nalepa, 2015). Studying family based cooperative breeders, such as ambrosia beetles (Nuotclà et al., 2019), will undoubtedly shed light on the role of relatedness, cooperation and conflict in the evolution of social disease defenses. In most of these models, the whole lifecycle can be observed in the laboratory, offspring relatedness may be manipulated through cross fostering and in- or outbreeding of parents, and selection experiments with pathogens are also possible. Moreover, studying social disease defense will help identify the adaptations that served as pre-cursors to superorganism immunity and facilitated superorganism evolution. Such an approach could also be used to determine the criteria used to define major transitions in biological individuality, which has represented a source of controversy in recent years (Godfrey-Smith and Goodnight, 2013; Boomsma and Gawne, 2018). For example, is a strict segregation between germ and soma really necessary for explaining a major transition, or could immune system evolution be better placed to define this boundary (Pradeu, 2013)? Patterns of immune system and germ-soma evolution in Metazoa suggest that the former holds greater explanatory power. This question could be addressed comparatively in the termites, where species have either helpers that almost all eventually try to breed, species where $\leq$1% of true workers become reproductive, and species with total worker sterility (Shellman-Reeve, 1997). Additionally, do the social disease defenses of cooperative breeders such as

ambrosia beetles, thrips and aphids constitute crude collective immunity, comparable to the crude immunity of facultatively multicellular organisms (Chen et al., 2007)? By taking such a broad, comparative approach, we might be able to answer general questions about immune system evolution that are difficult or impossible to address using multicellular models alone.

## CONCLUSION

In the years since Hamilton's original work identifying disease as a major constraint on the evolution of insect sociality (Hamilton, 1987), our view of how social insects overcome the problem of disease has shifted dramatically, from a focus on genetic resistance to a well-developed, comprehensive understanding of the role of disease defense in social insect evolution. By viewing immunity through the lens of the major transitions, and unifying it with the recent resurrection of Wheeler's original superorganism concept (Wheeler, 1911; Boomsma and Gawne, 2018), there is growing evidence that social insects have evolved an immune system that is convergent with the individual immune system of multicellular organisms. Since immune systems are crucial to the evolution of individuality and provide a level of protection that goes beyond mere infectious disease defense (Pradeu, 2012, 2013) – the major focus of social immunity research – we suggest the use of the term *superorganism immunity* to describe the protection arising from a fully functioning (social) immune system (Aanen, 2018). We hope that future research on social disease defenses in family based groups can begin to reveal how disease selection pressures initially promote group living, and how, in cooperatively breeding species, these behaviors maintain sociality once a stable group has formed; both of which are necessary steps for a major transition in superorganismality to occur. Importantly, such research would help provide us with fundamental insights into how selection acting on groups of cooperating relatives produces complex, higher-level adaptations, such as immunity in (super)organisms. Researchers have only just begun to scratch the surface of the evolution and ecology of superorganism immunity in recent years, and we hope that this review helps to stimulate further work in this burgeoning field.

## AUTHOR CONTRIBUTIONS

Both authors contributed to the original idea, graphs, writing, researching, and editing of the manuscript.

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# Cooperative Breeding in the Ambrosia Beetle *Xyleborus affinis* and Management of Its Fungal Symbionts

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Fungus-farming is known from attine ants, macrotermites, and ambrosia beetles (Scolytinae, Platypodinae). Farming ant and termite societies are superorganismal and grow fungal cultivars in monocultures. Social organization of ambrosia beetle groups and their farming systems are poorly studied, because of their enigmatic life within tunnel systems inside of wood. Ambrosia beetle-fungus symbioses evolved many times independently in both the beetles and their fungal cultivars. Observations suggest that there is evolutionary convergence between these lineages, but also a high variation in the degree of sociality and the modes of fungiculture. Using a laboratory observation technique, I here tried to give insights into the social system and fungus symbiosis of the sugar-cane borer, *Xyleborus affinis* Eichhoff (Scolytinae: Curculionidae), a currently poorly studied ambrosia beetle. The study revealed a cooperatively breeding system characterized by delayed dispersal of adult daughters, alloparental brood care by larvae and adults, and about half of the totipotent adult daughters laying eggs within the natal nest. Most interesting, there was a tendency of egg-laying females to engage more commonly in mutually beneficial behaviors than non-egg-layers. Fungus gardens covering gallery walls composed of five different filamentous fungi. A *Raffaelea* isolate was predominant and together with an unidentified fungus likely served as the main food for adults and larvae. Three isolates, a *Mucor*, a *Fusarium* and a *Phaeoacremonium* isolate were most abundant in the oldest gallery part close to the entrance; *Mucor*, *Fusarium* and the *Raffaelea* isolate in diseased individuals. Additionally, there was correlative evidence for some fungal isoaltes influencing beetle feeding and hygienic behaviors. Overall, *X. affinis* is now the second ambrosia beetle that can be classified as a cooperative breeder with division of labor among and between adults and larvae.

**Keywords: cooperative breeding, bark beetle, insect agriculture, symbiosis, fungus community, social behavior, fungus-farming, mutualism**

## INTRODUCTION

Advanced fungus agriculture by insects has evolved once in ants, once in termites and more than a dozen times independently in wood-boring weevils (Mueller et al., 2005; Hulcr and Stelinski, 2017; Vanderpool et al., 2018; Biedermann and Vega, 2020). The latter are jointly termed ambrosia beetles, even though they are a polyphyletic group and their mutualisms with fungi evolved multiple times independently (in both beetles and fungi; Hulcr and Stelinski, 2017) and every beetle-fungus combination may have a different farming system (Harrington, 2005; Six, 2012; Hulcr and Stelinski, 2017; Biedermann and Vega, 2020). In the best studied ambrosia beetle lineage, the Xyleborini (Curculionidae: Scolytinae), fungus-farming evolved along with social behavior and in some cases division of labor between and within developmental stages and adults (Mueller et al., 2005; De Fine Licht and Biedermann, 2012; Biedermann and Rohlfs, 2017; Nuotclá et al., 2019; Biedermann and Vega, 2020). At least one Xyleborini species classifies as cooperatively breeding (*Xyleborinus saxesenii*; Biedermann and Taborsky, 2011; Biedermann et al., 2012), which is defined in ambrosia beetles by adult daughters delaying dispersal from the natal nest and engaging in in hygienic brood care and fungus-farming tasks, while the mother is still present and breeding (Peer and Taborsky, 2007; Biedermann and Taborsky, 2011; Nuotclá et al., 2019). Some of the daughters may co-breed with the mother (Biedermann et al., 2012). They may disperse anytime to found their own nests (Biedermann et al., 2009). The remainder of described social systems in Xyleborini comprise maternal care and some kind of communal breeding (all daughters that stay co-breed with their mother in *Ambrosiophilus* spp.; Kirkendall et al., 2015; Kasson et al., 2016).

The morphology of ambrosia beetle galleries (i.e., tunnel systems within wood, where they nest) is highly variable (Kirkendall et al., 2015). Most common types in Xyleborini are brood-chambers as in *Xyleborinus* or *Xylosandrus* species and branching tunnel systems as in *Xyleborus* and *Euwallacea* species (Roeper, 1995; Biedermann, 2012). As adults, brood and fungus gardens are spatially more separated in branching tunnels than in communal chambers, gallery morphology has probably a major influence on social interactions and modes of farming. Cooperative breeding has been reported from *X. saxesenii* with a brood-chamber type of breeding system (Biedermann and Taborsky, 2011; Biedermann et al., 2012), but the social and farming behaviors of species that construct branching tunnel systems remain enigmatic. The sugar-cane borer *Xyleborus affinis* (Xyleborini, Scolytinae, Curcilionidae) is one of those species making branching tunnel systems.

Healthy fungus gardens of ants, termites, and ambrosia beetles harbor a rich community of fungi and bacteria, even though typically one or rarely two main cultivar fungi dominate (Francke-Grosmann, 1967; Haanstad and Norris, 1985; Kajimura and Hijii, 1992; Mueller et al., 2005; Rodrigues et al., 2008; Biedermann et al., 2013; Saucedo-Carabez et al., 2018; Skelton et al., 2018; Biedermann and Vega, 2020). In ambrosia beetles, these food fungi are transmitted by adult females from the natal to a newly established nest either within the gut (Francke-Grosmann, 1975) or more commonly within a specialized cuticular pouch, the mycetangium (Francke-Grosmann, 1956, 1967; Hulcr and Stelinski, 2017). The whole mycobiome and how monocultures of cultivar fungi are established by the beetles remains poorly studied (Hulcr and Stelinski, 2017; Biedermann and Vega, 2020). In the cooperatively breeding *X. saxesenii* hygienic behaviors are induced in response to fungal pathogens (Biedermann and Taborsky, 2011; Nuotclá et al., 2019). Furthermore, removal of beetles from galleries does lead to the immediate outgrowth of cultivars by non-mutualistic, weedy fungi that are present in a resting form in the fungus gardens of all ambrosia beetles studied (Schneider-Orelli, 1913; Francke-Grosmann, 1956; Norris, 1993).

My study species, *X. affinis,* is best known for its damages in sugar-cane plantations, it is reported to also attack more than 248 other woody plant species (Schedl K. E., 1963). Galleries are founded by single females within a stressed or recently dead plant and may be inhabited by one to several generations of beetle offspring if the nesting substrate is durable enough (Schneider, 1987). While boring an entrance tunnel perpendicular into the wood, foundresses inseminate tunnel walls with spores of *Raffaelea* ambrosia fungus mutualists [possibly *Raffaelea arxii*, which might be the primary food fungus (Saucedo-Carabez et al., 2018)] they carry in oral mycetangia [i.e., fungal-spore pockets (Francke-Grosmann and Schedl, 1960; Schneider, 1987)]. If successful, layers of conidiophores with nutritional conidiospores by the *Raffaelea* fungi line tunnel walls and the foundresses start to lay eggs. By feeding solely on the fungi, larvae pass through three instars followed by pupation (Biedermann et al., 2009). When reaching adulthood many daughters do not disperse immediately, but remain in the natal nest, where they may help in gallery expansion and hygiene, brood and fungus care, and possibly co-breed. Delayed dispersers have been found to loose reserves in the natal nest either by egg-laying and/or investments in helping (Biedermann et al., 2011). Thus, initial tunnel systems with a length of about 20–30 cm after one generation have been found to expand over 6 m (within 4 years) by the work of several consecutive overlapping generations (Schneider, 1987). Staying and consecutive breeding within one gallery is possible because *X. affinis* (i) produces its own fungal food and (ii) is an inbreeding species with regular brother-sister mating and a haplodiploid sex-determination system. Haplodiploidy enables founder females to assign optimal brood sex-ratios (by laying unfertilized eggs that develop into males and fertilized eggs that develop into females), which are strongly female biased (Schedl K. E., 1963; Biedermann et al., 2009). Eighty-five percent of laboratory galleries contain only a single male, which is hatching first and obviously capable to fertilize several dozens of sisters (Roeper et al., 1980). Males are flightless and may disperse only by foot, which they do after all female offspring matured (Biedermann, 2010).

Here I used artificial observation tubes that contained entire colonies of reproducing *X. affinis* beetles and their fungus gardens (a) to determine the social system of an ambrosia beetle breeding in a branching tunnel system and (b) to describe the fungal community of gardens in relation to the presence and the behaviors of beetles and their larvae. Furthermore, I asked whether (c) larvae and adult offspring engage in alloparental

brood care and fungus maintenance, (d) decisions of adult females to help, to breed and to disperse relate to the number of potential beneficiaries, the number of potential competitors and depend on the location within the nest (old vs. freshly excavated gallery parts), and (e) ovary status affects the propensity to engage in cooperative behaviors.

# MATERIALS AND METHODS

## Beetle Collection, Laboratory Breeding and Phenology

Females used for breeding in this study were directly collected from oak logs in Pineville, LA, United States (123 ft asl; 31°20′, 92°24′) in June 2007. Carried in sterile glass vials, they were immediately brought to the laboratory and used for artificial rearing: Females were surface-sterilized (by washing them first for a few seconds with 95% ethanol and then with distilled water) and afterward singly placed on prepared sterile artificial medium in separate glass tubes (18 mm diameter × 150 mm length; Bellco Glass, Vineland, NJ, United States) that were covered by sterile plastic caps (Bellco Glass kap-uts, Vineland, NJ, United States). Artificial medium had been drying for 5 days before the placement of beetles and consisted of an autoclaved mixture of 0.35 g streptomycin, 1 g Wesson's salt mixture, 5 g yeast, 5 g casein, 5 g starch, 10 g sucrose, 20 g agar, 75 g oak tree sawdust (freshly grounded and oven dried), 2.5 ml wheat germ oil, 5 ml 95% ethanol and 500 ml deionized water (for details on the preparation see Biedermann et al., 2009). After introduction of the beetles, tubes were stored at room temperature (∼23°C) in darkness (wrapped in paper, but light could shine on the entrance). This way, females start digging tunnels as if in wood and often build them adjacent to the glass of the tube. Thus, this technique allows to observe behavior of adults and larvae in the tunnels when the paper is removed (Biedermann et al., 2009). At 23°C a fungal layer and first eggs appear in successfully founded galleries around 10 days after gallery foundation. The first adults hatch about a month later and females remain in the natal nest for at least a week before they disperse by crawling onto the surface of the media, where they try to fly off (and where they can be collected for consecutive rearing). Peak productivity is around 60 days after gallery foundation. At that time, offspring of all stages are present together and the first adult daughters start to disperse. In the laboratory, more offspring will develop typically for another 20–30 days until the medium dries out and all individuals leave the gallery (Roeper et al., 1980; Biedermann et al., 2011).

## Behavioral Observations, Dissections of Galleries and of Female Ovaries

Here I used a pool of 23 successfully founded laboratory galleries. Tunnels were visible in 16 galleries, which were used for behavioral observations. Fungi were isolated from 15 galleries. Both behavior and fungal communities were determined for eight galleries among those (for details on these galleries see **Supplementary Table S1**). I started the treatment at about the peak of gallery productivity, when study galleries ranged between 51 and 60 days of age (after gallery foundation) and adult daughters started to disperse. In the 3 days before the treatment, I conducted daily behavioral observations of all visible individuals ($N = 16$ galleries). For each individual I noted the developmental stage and sex, its location within the gallery (main tunnel, side tunnel, brood tunnel; see **Figure 1**) and its behavior at the time of observation [i.e., "scan observation" technique (Biedermann and Taborsky, 2011)]. Larvae were classified by size in either 1st or 2nd/3rd instars. After pupation, females first have a brownish coloration and turn black when fully sclerotized. Light brown females were termed *tenerals* and dark brown females *adults*. Males are discernable from females by their smaller body size small horns on the pronotum. I differentiated between the larval behaviors *allogrooming*, *fungus cropping*, *cannibalism*, *locomotion*, *being pushed* by an adult female and *inactivity*, and the teneral/adult behaviors *shuffling frass*, *blocking*, *digging*, *allogrooming*, *self-grooming*, *fungus cropping*, *cannibalism*, *locomotion*, *pushing larva*, *inactivity* and the male *mating (attempt)* (for details see **Table 1**). For a video of *shuffling frass* by adults see **Supplementary Material**.

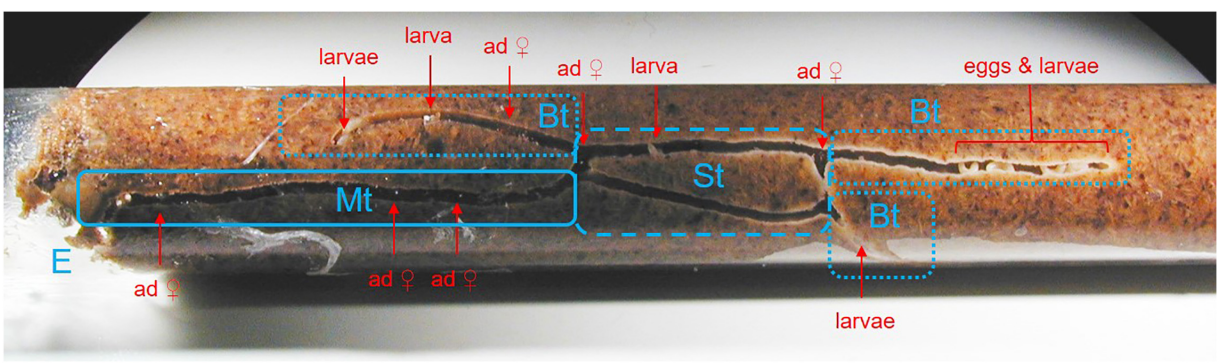


**FIGURE 1 |** Morphology of a *X. affinis* laboratory gallery in artificial medium at about 60 days after its foundation. Note the species-characteristic tunnel system with the four compartments: E, entrance; Mt, main tunnel; St, side tunnel; Bt, brood tunnel. A white fungal layer and white larvae are visible within the Sts and the Bts. Several adult beetles are sitting in the Mt and a few in the Sts and the Bts.

**TABLE 1 |** Ethogram of the observed behaviors of *X. affinis* larvae (L), females (F), and males (M) (modified from Biedermann and Taborsky, 2011).

| Behavior | Shown by | Definition | Mutual benefit |
|---|---|---|---|
| *Digging* | F | Excavating new tunnels | Gallery extension |
| *Fungus cropping* | L, F, M | Grazing and feeding on the fungal layer covering gallery walls with the maxillae and/or mandibles | Fungus care |
| *Shuffling frass* | F, M | Moving frass and sawdust with the legs and elytra | Hygiene |
| *Cannibalism* | L, F, M | Feeding on a larva, pupa or adult beetle that is usually dead | Potentially hygiene |
| *Allogrooming* | L, F, M | Grooming an egg, larva, pupa or adult beetle with the mouthparts (i.e., maxillae, labium) | Brood care, hygiene |
| *Blocking* | F | Staying inactive in the entrance tunnel and plugging it with the body (abdomen directed to the outside) | Protection |
| *Pushing others* | F | Shifting a larva or male within a tunnel | Protection |
| *Self-grooming* | F, M | Grooming oneself with the legs | – |
| *Inactive* | L, F, M | Being inactive without moving | – |
| *Locomotion* | L, F, M | Creeping (L), or walking on the tibia with back-folded tarsi (F, M) | – |
| *Being pushed* | L, M | Getting shifted by an adult female | – |
| *Mating (attempt)* | M | Mounting a female or copulating with her | – |

*An update of mutually beneficial behaviors has been published by Nuotclá et al. (2019).*

Most galleries are not fully visible from the outside. Therefore, to assess the number of individuals within a gallery and the reproductive status of adult females, I broke the glass of eight galleries at an age between 55 and 61 days, when the first generation of offspring matured and just started to disperse (**Supplementary Table S1**). All developmental stages (eggs, 1st instar larvae, 2nd/3rd instar larvae, pupae and adults) from within the nests were counted and all 49 females from within nests as well as 14 dispersing females were preserved in 95% ethanol until dissection. For all the 49 females from within nests, I noted their location within the nest and for 21 of these females it was even possible to record their behavior immediately before the glass of the tube was destroyed. I dissected female ovaries on microscope slides within some drops of phosphate-buffered saline (PBS) buffer solution under a binocular (6.4×–40× magnification). Females were decapitated, their wings removed and ovaries were exposed by dorsal opening of the abdomen with high precision tweezers and a scissor. I discriminated between *non-developed* (the four ovarioles are all thin or not present), *developed* (four ovarioles present, but not thickened and no oocytes visible) and *egg-carrying ovaries* (one to two of the four ovarioles are thickened and contain one to two oocytes); numbers of oocytes (i.e., developing eggs) were noted (for figure of ovaries see Fischer, 1954).

## Fungal Isolations and Identification of Filamentous Fungal Isolates

On the fourth day of the treatment (i.e., 55 to 64 days after gallery foundation), I brought some of the galleries ($N = 15$) to a sterile bench, carefully broke the glass of the tubes, and took 12 fungus samples each from the main tunnel, the side tunnel and the brood tunnel with a forceps that was flame-sterilized after each sample. Four samples were placed on malt agar plates (MA: 25 g malt extract, 20 g agar, 1 L deionized $H_2O$), four samples on cycloheximide-streptomycin malt agar plates (CSMA: 10 g malt extract, 15 g agar, 20 ml filter sterilized CSMA stock solution containing 2 mg of cycloheximide and 1 mg streptomycin, 1 L deionized $H_2O$) and the last four on benomyl malt agar plates (BMA: 10 g malt extract, 15 g agar, 1 ml filter sterilized BMA stock solution containing 1 mg benomyl dissolved in dimethylsulfoxid, 1 L deionized $H_2O$). MA is unselective, CSMA is selective for Ophiostomatoid fungi (e.g., *Raffaelea* species; Harrington, 1981) and BMA for Basidiomycetes (Ross et al., 1992). Samples were spotted in the center of the plates. Afterward, I fully dissected the whole gallery system and counted all eggs, larvae, pupae, adult females and males (see above).

For obtaining fungal isolates from female cadavers ($N = 4$), I put diseased adult females (from within nests) singly in 1.5 ml Eppendorf tubes with 1 ml PBS buffer. Bodies were crushed with a pestle and vortexed within the PBS buffer for 5 s. Afterward 1:1000 and 1:10000 dilution series were generated and 100 ul of both dilutions were plated out on the three media above. Presence/absence of fungal isolates on these plates was recorded 10 days later.

Isolated fungi were first sorted into groups based on cultural growth and morphological colony characteristics (i.e., morphology and color of the mycelium) and these were compared with a database of fungal isolates from bark and ambrosia beetles available at the Southern Research Station of the USDA Forest Service. Afterward, I brought pieces of fungal tissue on microscope slides with drops of PBS buffer and examined their spore structures. Confirmation and final determination of four of the five fungal isolates was done using internet sources and the illustrated key to identify genera of imperfect fungi by Barnett and Hunter (1998). Representative samples were stored for DNA sequencing, but material got lost through a failure of a refrigerator and could neither be revived nor successfully sequenced. Therefore, only morphological, genus-level identifications are available.

## Statistical Analyses

If not stated differently, all statistical analyses were done using generalized estimating equations (GEEs) in R [lmer; Version 2.12.1 (R-Development-Core-Team, 2008)]. GEEs are an extension of generalized linear models with an exchangeable correlation structure of the response variable within a cluster, which allows for controlling the variation between observations from a single gallery. This was necessary

because of variation in sample sizes between galleries (as for the fungal community analyses a few plates without microbial growth had to be excluded).

Using all 12 samples taken from each location (main, side, brood tunnel) of each gallery (independent variable), I estimated the frequency and presence (yes/no) of each fungal isolate (dependent variables) by controlling for medium (MA, CSMA, BMA; fixed factors) and gallery of origin (random factor). I also analyzed how number of eggs, larvae and adult females (dependent variable) were affected by the location within the gallery (fixed factor) and the presence of the different fungi (fixed factor) by controlling for gallery of origin (random factor). Differences in the frequency of male observations between locations within the gallery were tested using Fisher's exact test, because of the small number of males observed. In a third series of models, I analyzed whether frequencies of larval and adult behaviors (dependent variable) were influenced by the location within the gallery (fixed factor).

By correlating behavior with fungal communities, I first determined whether the frequency of larval and adult behaviors (dependent variable) were affected by the presence of the different fungi (fixed factor), by controlling for location within the gallery (fixed factor) and gallery of origin (random factor). Second, I modeled whether the frequency of larval and adult *fungus cropping* and *shuffling frass* (fixed factors) and gallery of origin (random factor) affected isolation frequency of each fungus (dependent variable). Finally, I used logistic regression models (GLMs with binary response variable) to model if the proportion of egg-laying and dispersing females and females with developed ovaries (dependent variables) were affected by the proportion of immatures relative to adults in the nest, the proportion of different female reproductive groups relative to all females inside the nest, and the proportion of dispersing relative to staying females (fixed factors).

# RESULTS

## Overlapping Generations, Factors Influencing Reproductive Division of Labor and Alloparental Care by Adult Daughters

Between day 55 and 61, when the first generation of offspring matured and started to disperse, galleries ($N = 8$) contained on average 33.6 (±10 SE) eggs, 7.1 (±2.2) larvae, 5.4 (±3) pupae, 0.3 (±0.3) immature females, 6.8 (±0.9) adult females and 0.9 (±0.1) adult males. Of the adult females a mean of 26.8% (±9.7) had non-developed ovaries, 19.2% (±7.9) had developed ovaries, and 54.1% (±13.3) were laying eggs (**Supplementary Table S1**). All 14 dispersing adult females that were dissected had either non-developed ($N = 9$) or developed ovaries ($N = 5$).

Egg-laying was unequally distributed among females and there was no fixed proportion of females laying eggs per gallery: First, egg numbers were independent of the number of potential egg-layers (i.e., females with developed ovaries and egg-laying females) (GLM: $p > 0.05$) and second the proportion of egg-laying females were negatively affected by the proportion of females inside the nest with non-developed ovaries (GLM: $p = 0.016$; **Supplementary Table S2**) and developed ovaries ($p = 0.031$). The proportion of females with developed ovaries correlated negatively with the proportion of females with non-developed ovaries ($p = 0.013$) and with the proportion of egg-layers ($p = 0.042$), which might suggest that development of ovaries is triggered by the opportunity to breed. The propensity of females to disperse was reduced with increasing numbers of larvae dependent on brood care ($p = 0.019$), but independent of the relative proportion of immature offspring to adult females inside the nest as well as the proportion of female egg-layers and females with non-developed ovaries. In contrast, proportion of female dispersers correlated negatively with proportion of females with developed ovaries, possibly suggesting that ovary development typically leads to either dispersal or egg-laying in the natal nest (see above).

Both potential egg-layers and females with non-developed ovaries engaged in alloparental care (i.e., *allogrooming*, *fungus cropping* and *shuffling frass*) at the natal nest. However, despite the small sample size, there was a trend for adult females with non-developed ovaries ($N = 11$) to engage less in these mutually beneficial behaviors immediately before galleries were dissected, than females potentially laying eggs (Fisher's exact test: $p = 0.085$, $N = 21$; **Figure 2**). Additionally, potential egg-layers tended to be found more frequently close to the brood (i.e., in the side-tunnel) than in the main tunnel compared to females with non-developed ovaries (Chi$^2$-test: $\chi^2 = 2.94$, df $= 1$, $p = 0.087$, $N = 93$).

## Offspring and Adult Numbers and Their Behaviors in Relation to the Three Gallery Compartments

Eggs and pupae were only found in the brood tunnels of the galleries. Larvae were most commonly found in the brood tunnels, followed by the side tunnels (GEE: $p = 0.03$; **Supplementary Table S3**, **Figure 3**), and the main tunnel ($p < 0.001$). When present in the main tunnel they only showed *locomotion*, which was also often observed in the side tunnel, but only rarely in the brood tunnel ($p < 0.001$; **Supplementary Table S4**). Larval *allogrooming* and *fungus cropping* were most common in the brood tunnel ($p < 0.05$). Larval *cannibalism* was only present at low rates in the side tunnel.

Adult females and males were least common in the brood tunnels ($p < 0.05$; **Supplementary Table S3**, **Figure 4**). Males tended to stay in the side tunnels ($p = 0.064$). The most common adult female behaviors were *shuffling frass* and *fungus cropping*, with the last most common in the brood tunnel ($p = 0.001$; **Supplementary Table S5**, **Figure 4**). *Inactivity* tended to be expressed most often in the main tunnel ($p < 0.066$). *Blocking* was, by definition, only found in the main tunnel, whereas *digging*, *cannibalism* and *pushing larva* was never expressed there. All other female behaviors were equally common in all three gallery compartments ($p > 0.05$). Adult males spent their time mainly with *fungus cropping* (27% of their time), *locomotion* (25%), *inactivity* (16%), followed by *attempting to*

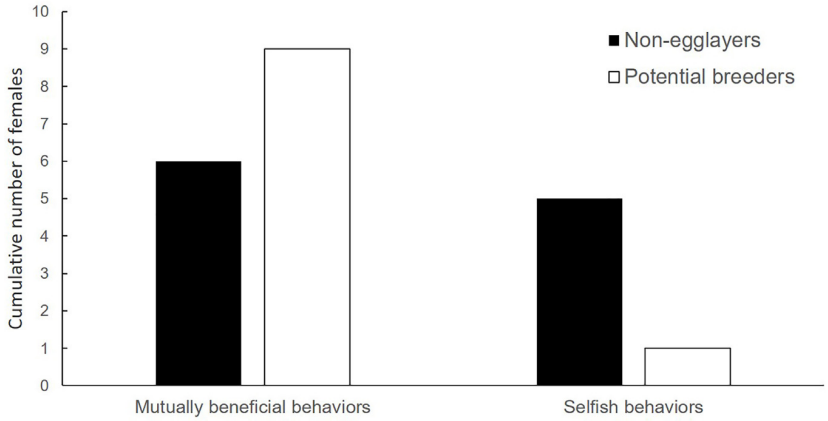


**FIGURE 2 |** Reproductive status in relation to adult female behavior. All females engage in mutually beneficial behaviors. However, relative to selfish behaviors, females with fully developed or egg-carrying ovaries (*potential breeders*) tended to engage more often in mutually beneficial behaviors (see **Table 1**) than females with non-developed ovaries (*non-egg-layers*) (Fisher's exact test: $p = 0.085$, $N = 21$).

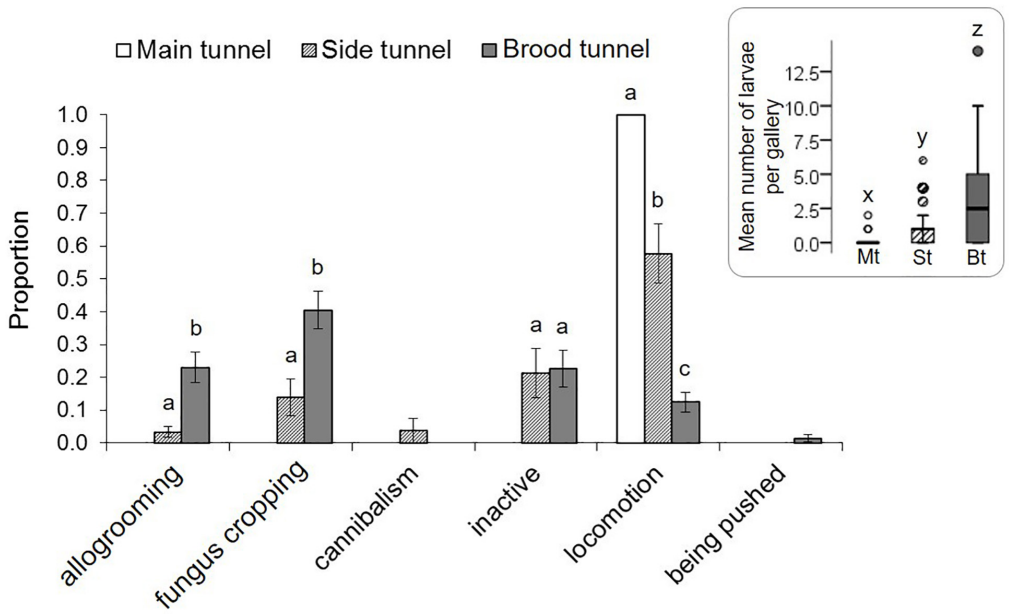


**FIGURE 3 |** Behaviors of *X. affinis* larvae in the three gallery compartments. Mean ± SE; different letters denote significant differences ($p < 0.05$; for exact values see **Supplementary Table S4**) in pairwise comparisons between compartments. The Mean (±SE) numbers of larvae observed in the three compartments are given in the box on the right.

*mate* (11%), and *allogrooming* (11%). *Self-grooming*, *mating*, *being pushed* and *shuffling frass* were only rarely shown by males (<4%).

## Fungal Isolations

Our isolations revealed five isolates of filamentous fungi associated with galleries of *X. affinis*: a *Fusarium*, a *Mucor*, a *Phaeoacremonium*, a *Raffaelea* and an unidentified filamentous fungus ("*Unknown*") isolate. The *Mucor* isolate was identified by its fluffy aerial mycelium and the black sporangiophores. The *Raffaelea* isolate was identified by its characteristic growth on plates (filamentous on the outside of the culture, yeast-like in the center) and the morphology of conidiophores and the budding conidiospores. The *Fusarium* isolate was identified by its characteristic sickle-shaped conidia. The identity of the *Phaeoacremonium* isolate was confirmed by macro- and micro-comparisons with a morphologically nearly identical *Phaeoacremonium rubrigenum* isolate in the fungal database of the USDA Forest Service. The *Raffaelea* isolate was the most common fungus isolated.

Overall, *Raffaelea*, *Phaeoacremonium* and *Unknown* were more commonly detected on CSMA than MA

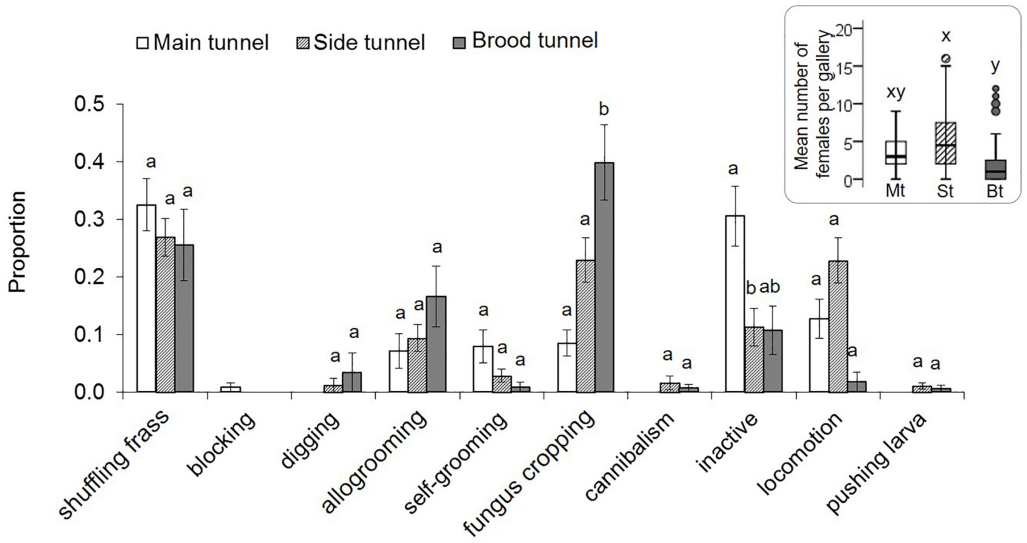


**FIGURE 4 |** Behaviors of *X. affinis* adult females in the three gallery compartments. Mean ± SE; different letters denote significant differences ($p < 0.05$; for exact values see **Supplementary Table S5**) in pairwise comparisons between compartments. The Mean (±SE) numbers of adult females observed in the three compartments are given in the box on the right.

(GEEs: $p = 0.017$–$0.001$; **Supplementary Table S6**), which shows that these three isolates are resistant to the antibiotic cycloheximide. Interestingly they grew also better on BMA than MA ($p = 0.007$–$0.001$). The opposite was found for *Mucor* and *Fusarium*, which were most frequently isolated from MA followed by BMA and CSMA ($p < 0.01$).

## Fungal Diversity in Relation to Gallery Compartment and Age of Gallery

The *Raffaelea* was isolated from all compartments of all galleries, and was most commonly detected in the brood tunnels (GEEs: $p = 0.003$–$0.001$; **Supplementary Table S6**, **Figure 5** and **Supplementary Figure S1**), which strongly suggests its nutritional role for *X. affinis*. *Fusarium*, *Mucor*, and *Unknown* were present in about 60% of all galleries. *Unknown* and *Fusarium* were isolated equally often from all compartments ($p > 0.05$), whereas *Mucor* and *Phaeoacremonium* occurred more frequently near the entrance than the brood ($p = 0.003$–$0.006$). *Raffaelea*, *Fusarium* and *Mucor* were strongly associated with old galleries (i.e., isolation rate > 60%; **Supplementary Figure S1**). The other two fungi were detected in <20% within these samples. Also dead females harbored *Raffaelea*, *Fusarium* and *Mucor*; the latter in >60% of diseased individuals.

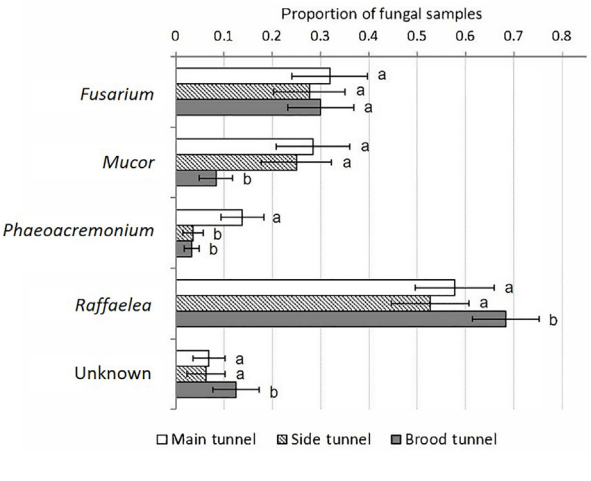


**FIGURE 5 |** Percentage of fungal morphospecies isolated relative to the total number of samples taken per gallery compartment. Mean ± SE; $N = 12$ samples per compartment; main tunnel – eight galleries, side tunnel – six galleries, brood tunnel – seven galleries. Different letters denote significant differences ($p < 0.05$; for exact values see **Supplementary Table S6**) in pairwise comparisons between compartments.

## Influence of Fungal Species on Beetle Productivity and Their Farming Behaviors

The regular presence of *Raffaelea* in samples from all compartments of all galleries supports its essential function for the beetles, but inhibited testing the influence of its presence on beetle productivity and behaviors. It is possibly the only fungus with a nutritional role for the brood, because egg and larval numbers were both not affected by any other isolate (GEEs: $p > 0.05$; **Supplementary Table S3**). In contrast, adult female numbers were negatively affected by the presence of *Phaeoacremonium* ($p = 0.005$). Adult females increased *fungus cropping* frequency in the presence of *Unknown* ($p = 0.038$; **Supplementary Table S7**, **Figure 6**), increased *shuffling frass* ($p = 0.048$) and general activity levels in the presence of *Fusarium* ($p = 0.02$) and decreased activity levels in the presence of *Phaeoacremonium* ($p = 0.002$). None of the fungal isolates

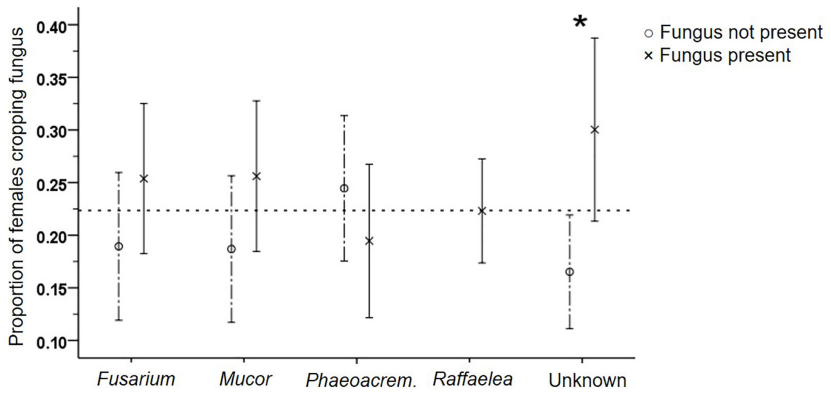


**FIGURE 6 |** Effect of the presence of the five different fungi on the frequency of adult female *fungus cropping*. Mean (±SE) frequencies, for fungus present or not per gallery, are given. *Raffaelea* was always present. *$p < 0.05$ (for exact values see **Supplementary Table S7**).

influenced the activity of larvae (i.e., their *fungus cropping* and *shuffling frass* behaviors; $p > 0.05$; data not shown).

## Potential Influence of Larval and Adult Farming Behaviors on the Isolation Frequency of the Fungi

*Raffaelea* was less commonly isolated from those galleries with higher activity levels of larvae in the 3 days before the fungal isolations ($p < 0.05$; **Supplementary Table S8**). Isolation frequency of *Unknown* was positively correlated with larval *fungus cropping* ($p = 0.004$). Adult *fungus cropping*, *shuffling frass* or activity levels showed no significant correlation with the isolation frequency of the five fungi. There was, however, a very weak trend for a higher isolation frequency of *Mucor* and *Phaeoacremonium* from those galleries with lower activity levels of adults in the days before ($p < 0.12$).

# DISCUSSION

This study is the first to correlate ambrosia beetle behavior with egg-laying, location within the gallery and frequency of different fungal symbionts. Ambrosia beetle behavior normally cannot be observed within solid wood, so here I reared beetles in artificial medium in glass tubes (Biedermann et al., 2009). That gave the chance for individual behavioral observations of females immediately before dissection of ovaries and targeted fungal samplings, in combination with behavioral observations. So for each of the three *X. affinis* gallery compartments – brood tunnel, side tunnel, and main tunnel – I was able to give an overview of the number of larval and adult inhabitants, their respective behaviors (including egg-laying) and the diversity of filamentous fungi. The social system was similar to that of other Xyleborini ambrosia beetles (**Table 2**) and there was correlative evidence for active management of fungus gardens by the beetles, i.e., behavioral changes in response to the fungal composition of their ambrosia gardens.

Observations and female dissections revealed that in *X. affinis* some adult daughters delay dispersal, help in hygienic tasks, brood and fungus care (**Figure 2**; see **Supplementary Video**) and on average half of them reproduce in the natal gallery together with their mother (**Supplementary Table S1**). This social system is termed cooperative breeding or facultative eusociality (Boomsma and Gawne, 2017) and has already been described from the ambrosia beetle *Xyleborinus saxesenii* (Biedermann and Taborsky, 2011) (**Table 2**). Development, offspring numbers, number of egg-layers and dispersed females after 60 days almost equals the numbers reported from Biedermann et al. (2012) that used also the same rearing media. Even though we currently lack behavioral studies on other ambrosia beetle species, data on development and delayed dispersal of adult offspring indicate that cooperatively breeding can be possibly found also in some other *Xyleborinus* and *Xyleborus* species, even if it is probably not widespread (Schedl W., 1963; Kingsolver and Norris, 1977; Gagne and Kearby, 1979; Maner et al., 2013).

*Xyleborus affinis* galleries contained high numbers of individuals of all age groups at around day 60, which is shortly after adult daughters start to disperse from galleries (Biedermann et al., 2011). Multiple egg-layers were already present and on average, about half of the adult daughters that had remained in the natal nest had started to lay eggs. Similar numbers been reported for *X. saxesenii* (Biedermann et al., 2012). In contrast to *X. saxesenii,* the propensity of females to disperse did not correlate with brood depending on care (i.e., eggs, larvae, pupae). Although all adult females participate in social tasks in the natal nest, egg-laying females were more inclined to do so. This higher investment of egg-laying individuals than of non-reproducing helpers into brood care is typical for social groups that have not taken the transition to superorganismality (i.e., obligate eusociality; Boomsma and Gawne, 2017). Reproducing females in the equally cooperatively breeding *X. saxesenii*, for example, typically protect the gallery entrance against the introduction of predators, which is the most risky task in an ambrosia beetle society (Biedermann and Taborsky, 2011). By contrast, in superorganismal societies sterile workers are not only overtaking the most dangerous tasks, but usually all non-reproductive tasks that need to be done (Wilson, 1971; Bourke, 2011; Boomsma and Gawne, 2017).

**TABLE 2 |** Overview of social traits found in the ambrosia beetle tribe Xyleborini (Curculionidae: Scolytinae).

| Social characteristic | Prevalence in Xyleborini | References |
|---|---|---|
| Sibling mating, haplodiploidy | All species | Kirkendall et al. (2015) |
| **Subsocial behavior** | | |
| Maternal care (brood care, nest hygiene and protection, fungus farming) | All species | Jordal et al. (2011), Kirkendall et al. (2015) |
| **Overlapping generations** | | |
| Delayed dispersal of adult daughters* | Widespread; shown for *Xyleborus affinis, X. ferrugineus*, *X. glabratus*, *X. monographus, Xylosandrus germanus* and *Xyleborinus saxesenii* | Schedl W. (1963), Kingsolver and Norris (1977), Peer and Taborsky (2007), Biedermann et al. (2011), Maner et al. (2013), this study |
| **Reproductive division of labor** | | |
| Not present: Daughters stay but do neither breed nor help | Probably widespread; present in *Xylosandrus germanus* | Personal observation |
| Not present: All daughters co-breed with mother | Rare; shown only for *Ambrosiophilus* spp. | Kasson et al. (2013) |
| Present: Some, but not all staying daughters co-breed with mother* | Prevalence unknown; shown only for *Xyleborus affinis* and *Xyleborinus saxesenii* | Biedermann et al. (2012), this study (**Supplementary Table S1**) |
| **Help by offspring in the natal nest** | | |
| Cooperative larvae (brood care, nest hygiene, fungus farming) | Probably widespread; shown for *Xyleborus affinis* and *Xyleborinus saxesenii* | Biedermann and Taborsky (2011), this study (**Figure 3**) |
| Alloparental care by adult daughters that do not breed (brood care, nest hygiene and protection, fungus farming)* | Prevalence unknown; shown only for *Xyleborus affinis* and *Xyleborinus saxesenii* | Biedermann and Taborsky (2011), this study (**Figures 2**, **4** and **Supplementary Video**) |

**Overlapping generations, reproductive division of labor and alloparental brood care are characterizing cooperatively breeding animal societies, as present in the Xyleborini ambrosia beetles Xyleborus affinis and Xyleborinus saxesenii.*

Observations of the three gallery compartments (**Figure 1**) revealed a spatial organization of developmental stages and behavioral tasks. Brood tunnels were surrounded by thick layers of fungal growth and inhabited mostly eggs, pupae and larvae, whereas males and females capable of laying eggs (i.e., developed ovaries or ones containing eggs) tended to be found in the side tunnels. Males were waiting in the side tunnels for freshly emerging females to mate. There was a trend for females with undeveloped ovaries to be found in the main tunnels. This compartmentalization of developmental stages reflects the spatial fidelity found in ants, where brood and adults are also unequally distributed within the nest (Mersch et al., 2013). Not surprisingly, this compartmentalization is also reflected by a spatial division of labor in the sense that most *fungus cropping* and *allogrooming* are found in the brood tunnels, whereas *shuffling frass*, *inactivity* and *locomotion* are found most often in the food-deficient side and main tunnels (**Figures 2**, **3** and **Supplementary Video**). This compartmentalization of age groups and the resulting spatial division of labor has not been reported for any other ambrosia beetle and is probably caused by breeding in tunnels instead of brood chambers (where the fungal food resource and all age groups occur together; Biedermann and Taborsky, 2011; Biedermann et al., 2012). Given that all *Xyleborus* species make tunnel systems (Roeper, 1995; Kirkendall et al., 2015), it is likely that compartmentalization is present in other species of this genus. In the future, some more comparative analyses of brood-chamber- and branching-tunnel-building species need to be done, to check if gallery morphology allows inferences on the social structures of the societies.

This is the first study to look at fungal communities of *X. affinis* using artificial laboratory cultures. Five different morphospecies were identified: a *Raffaelea*, a *Fusarium*, a *Phaeoacremonium*, a *Mucor* and an *Unknown* fungus. This diversity of fungi is much lower than culture independent studies from *X. affinis* in the field reported (Kostovcik et al., 2014; Saucedo-Carabez et al., 2018), but also not surprising as many environmental microbes are excluded by the lab rearing. This study was not successful to determine a primary mutualistic fungus. Here I present correlative evidence that *Raffaelea* and *Unknown* are putative nutritional mutualists of *X. affinis*. *Raffaelea* formed thick ambrosia layers on the walls of the side and brood tunnels, which were fed upon by larvae and adults. *Raffaelea* species produce nutritional conidiospores and have been proven to be primary cultivars in other *Xyleborus* and *Xyleborinus* species (Francke-Grosmann, 1967; Roeper, 1996; Biedermann et al., 2013; Hulcr and Stelinski, 2017; Saucedo et al., 2017; Saucedo-Carabez et al., 2018). Saucedo-Carabez et al. (2018) predominately isolated *Raffaelea arxii* from field galleries of *X. affinis,* next to unfrequent *R. lauricola* and *R. subfusca*. *R. arxii* might therefore be the primary mutualist of *X. affinis* that I here could not determine to the species level. A recent metabarcoding study also confirmed the ubiquitous presence and nutritional role of a *Raffaelea* species for *X. affinis* (Ibarra-Juarez et al., 2020). *Unknown* may function as a secondary food source because it was particularly common in brood tunnels and its presence had a positive influence on adult female feeding.

It is unlikely that the other three fungal isolates, *Mucor*, *Fusarium*, and *Phaeoacremonium*, have a mutualistic role for the beetles. All three were predominantly found in the main tunnels, old galleries and associated with dead individuals. Presence of *Fusarium* also correlated with more active adult females and hygienic *shuffling* activity. *Phaeoacremonium* had negative correlative effects on the number of adult females

present. *Mucor* species are typical saprotrophs (Ritz, 1995), whereas *Fusarium* and *Phaeoacremonium* species are often plant pathogens (Larignon and Dubos, 2000; Ma et al., 2013) and might profit from transmission by ambrosia beetles. One clade of *Fusarium* species is known as primary mutualists of *Euwallacea* ambrosia beetles, however (Kasson et al., 2013; O'Donnell et al., 2015; Freeman et al., 2016; Aoki et al., 2019). Fungi in this clade have typically thickened conidiospores, which I did not see in the isolate found in this study. Clearly, molecular identifications and experimental manipulations are needed to determine the roles suggested here.

I found some significant correlations between ambrosia beetle behavior and presence of specific fungi. Whether these behavioral changes are actually caused by the fungi is unclear, however. Ambrosia beetles are termed "farmers," but currently there is very little data proving that the beetles indeed actively manage fungal communities in the sense that they have means to promote beneficial fungi over antagonistic fungi. The strongest evidence comes from a recent study on *X. saxesenii* by Nuotclá et al. (2019), who found that injection of *Aspergillus* pathogens in laboratory galleries may induce adult (allo-)grooming and - over time - reduced *Aspergillus* spore loads (Nuotclá et al., 2019). Previously it had been shown that *X. saxesenii* larvae are able to suppress the growth of not-identified fungal pathogens (Biedermann and Taborsky, 2011). Mechanistically, beetles may suppress fungal pathogens by application of antibiotic producing bacteria. Application of oral secretions containing bacteria have been observed (Cardoza et al., 2006) and bacteria selectively inhibiting fungal pathogens by producing cycloheximide (which does not harm *Raffaelea* species) have been isolated from *X. affinis* and *X. saxesenii* laboratory galleries (Grubbs et al., 2011, 2019).

In summary, *X. affinis* lives in a branching gallery in a cooperatively breeding social system with multiple egg-layers and division of labor between larvae and adults. Adult daughters have been shown previously to lose reserves during the delayed dispersal period (Biedermann et al., 2011). Future studies have to determine if some daughters lay eggs before dispersal, which may be possible as we found that one third of the dispersing females had developed ovaries. A similar social system has been described for *X. saxesenii*, which builds brood chambers, so interactions between adults and larvae are more common there (Biedermann and Taborsky, 2011; Biedermann et al., 2012). *X. affinis* is predominately associated with one or two fungal mutualists, of which the predominating one is a *Raffaelea* fungus (maybe *R. arxii*). This species has been reported by Saucedo-Carabez et al. (2018) and confirms the mutualism between the genus *Xyleborus* and *Raffaelea* species. Even though two recent articles suggest that yeasts (e.g., *Ambrosiozyma*) are additional mutualists of *X. affinis* and other *Xyleborus* species (Saucedo-Carabez et al., 2018; Ibarra-Juarez et al., 2020), their mutualisms appear less promiscuous then the first metabarcoding study on *Xyleborus* field galleries suggested (Kostovcik et al., 2014). More experimental work on the fitness effects of single fungal species on the beetles is clearly needed to test how specific the ambrosia beetle-fungus symbioses are (see e.g., Skelton et al., 2019). Finally, it is important to mention that our findings from the Xyleborini lineage should not be extrapolated to other independently evolved ambrosia beetle-fungus symbioses (Kirkendall et al., 2015; Hulcr and Stelinski, 2017). There is some evidence that the many independent origins of the ambrosia symbiosis in beetles differ considerably in their behaviors, social systems and ways of farming (Biedermann and Vega, 2020). We are just at the beginning of getting a small glimpse in the diversity of these fascinating beetle farmers.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## AUTHOR CONTRIBUTIONS

PB conceived the study, performed all analyses, and wrote the manuscript.

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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.2020.518954/full#supplementary-material

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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 $y = f(ax + bx\hat{}2 + cx\hat{}3 + d)$, 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 $0 = a + 2bx + cx\hat{}2 + d$ 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

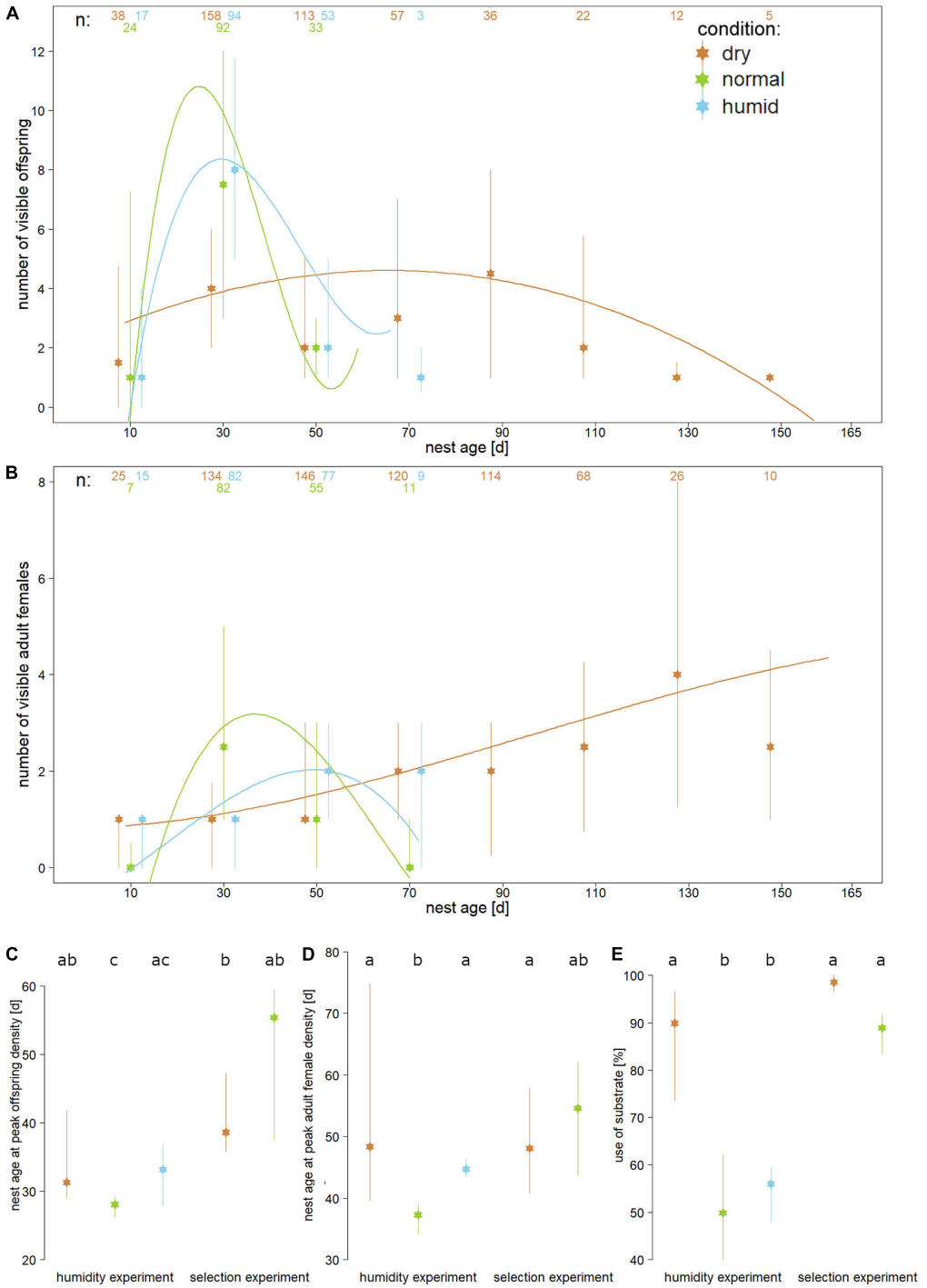


**FIGURE 1 |** Medians and interquartile ranges of relevant nest development parameters are displayed for three substrate humidities ("dry" = brown, "normal" = green, and "humid" = blue) measured during either the *humidity experiment* **(A–E)** or during the *selection experiment* **(C–E)**. The *humidity 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; $P < 0.05$).

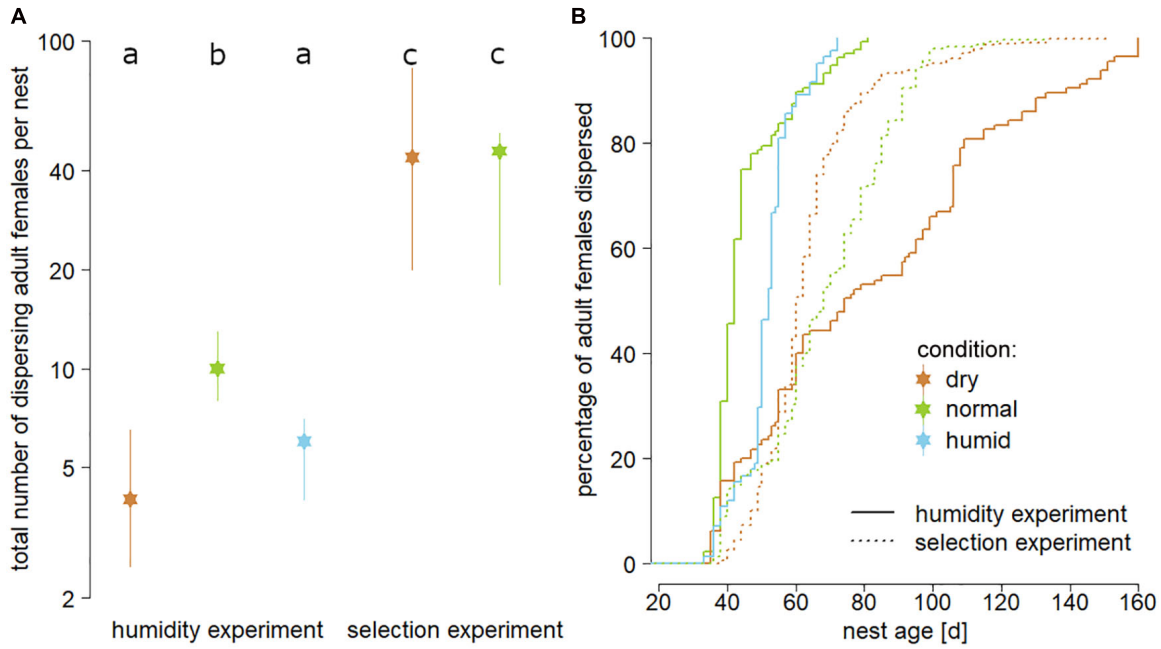


**FIGURE 2 |** Panel **(A)** illustrates the overall fitness impact of the three tested substrate humidities, indicated by medians and interquartile ranges of the total number of dispersing females per nest (logarithmic scale; "dry" = brown, "normal" = green, "humid" = blue). Different lower-case letters 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).

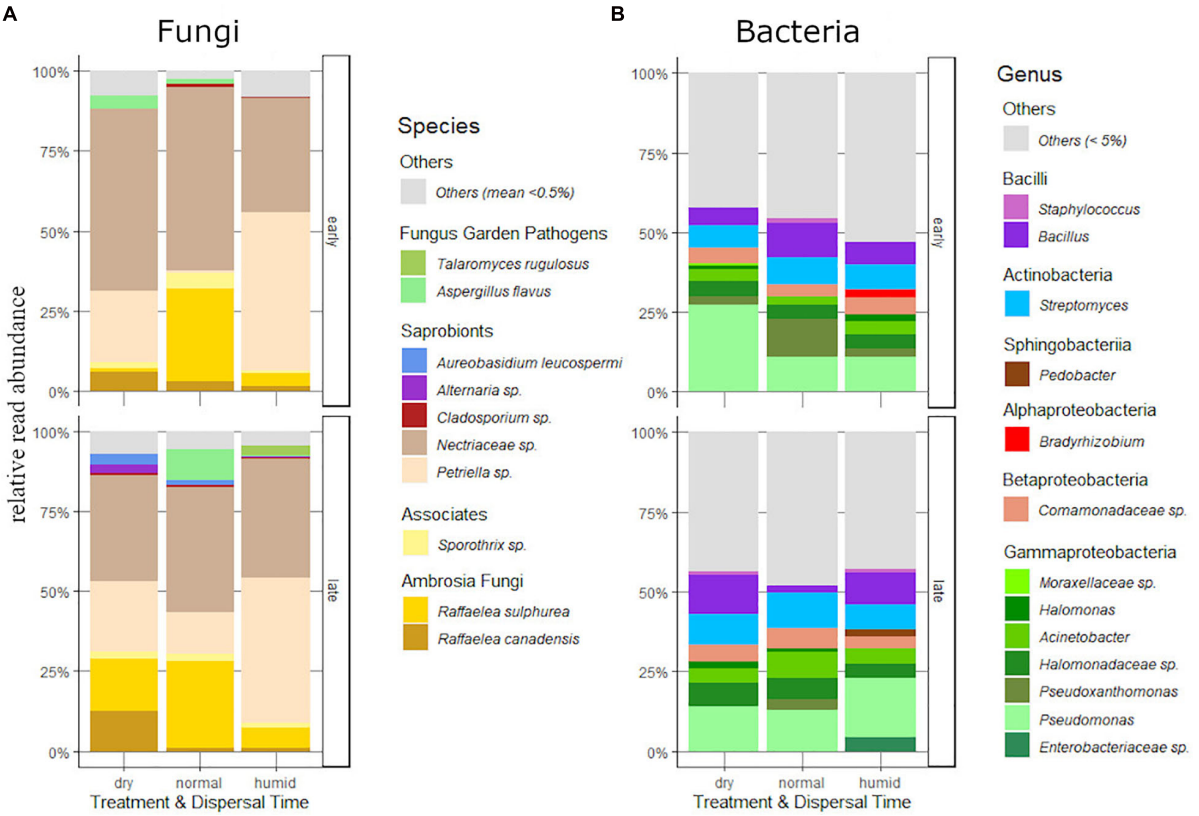


**FIGURE 3 |** Relative read abundance of the major fungus **(A)** and **(B)** bacteria taxa carried by dispersing females of the *humidity experiment*, differentiated by dispersal time ("early" or "late") and humidity treatment ("dry," "normal" or "humid").

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

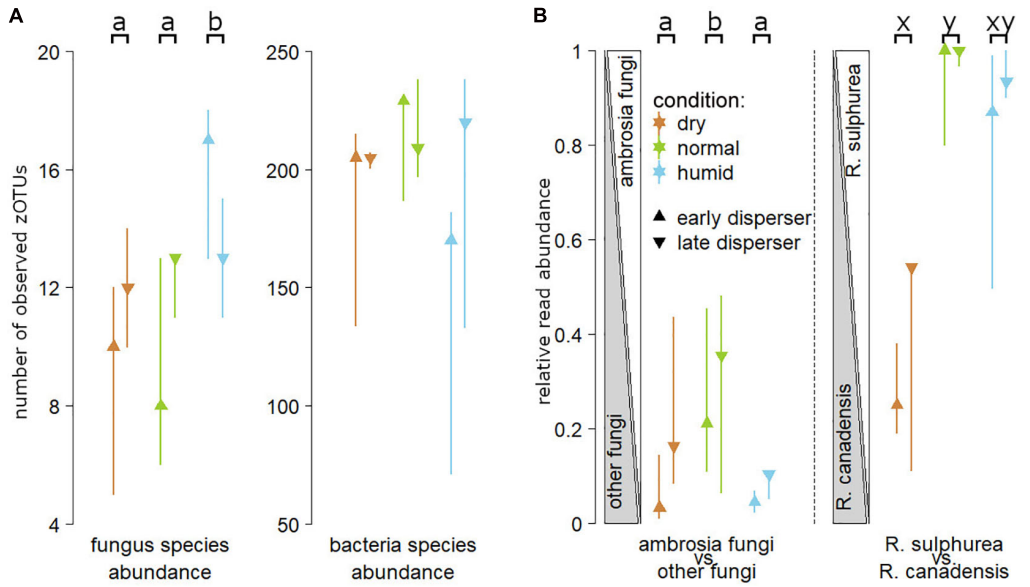


**FIGURE 4 |** Panel **(A)** illustrates the numbers of zero radius Operational Taxonomic Units (zOTU, ≈ number of clearly distinguishable species) for fungus and bacteria species that are carried by first ("early") or last ("late") dispersing females from nests reared in "normal," "dry" and "humid" 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

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