

## Research



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# Pathogen defence is a potential driver of social evolution in ambrosia beetles

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Social immunity—the collective behavioural defences against pathogens—is considered a crucial evolutionary force for the maintenance of insect societies. It has been described and investigated primarily in eusocial insects, but its role in the evolutionary trajectory from parental care to eusociality is little understood. Here, we report on the existence, plasticity, effectiveness and consequences of social pathogen defence in experimental nests of cooperatively breeding ambrosia beetles. After an *Aspergillus* spore buffer solution or a control buffer solution had been injected in laboratory nests, totipotent adult female workers increased their activity and hygienic behaviours like allogrooming and cannibalism. Such social immune responses had not been described for a non-eusocial, cooperatively breeding insect before. Removal of beetles from *Aspergillus*-treated nests in a paired experimental design revealed that the hygienic behaviours of beetles significantly reduced pathogen prevalence in the nest. Furthermore, in response to pathogen injections, female helpers delayed dispersal and thus prolonged their cooperative phase within their mother's nest. Our findings of appropriate social responses to an experimental immune challenge in a cooperatively breeding beetle corroborate the view that social immunity is not an exclusive attribute of eusocial insects, but rather a concomitant and presumably important feature in the evolutionary transitions towards complex social organization.

## 1. Introduction

Pathogens pose a major risk to highly social animals. Insect societies, for instance, provide ideal conditions for their dissemination [1,2], because a large number of closely related individuals with potentially very similar immune defences live together in intimate contact and under homogeneous, environmentally buffered conditions. Low genetic variance has been shown to reduce the chances of successfully resisting severe fungus infections in honeybees, and in ants it reduces the effectiveness of anti-pathogen behaviours [3,4]. To counter pathogen risk, social insects evolved various physiological and behavioural strategies to inhibit the spread of diseases [5].

The innate immune system, pathogen avoidance and self-cleaning behaviours are probably the most common anti-pathogen strategies in insects. In addition to such traits that might be termed 'non-social', many social insects were found to express social immunity, which refers to cooperative sanitation involving the joint mechanical and chemical removal of bacterial and fungal pathogens. Originally, social immunity was regarded as a nest-wide parasite and pathogen defence mechanism that evolved in eusocial insects to counter the beforementioned inherent risks of infection caused by the social lifestyle and genetic homogeneity [5]. Importantly, this concept has highlighted the parallels between the innate non-social immune system of a single multicellular organism and a nest-wide 'social immune system' of a complex insect society. This idea relates to the concept of superorganismality, where a whole nest of social insects is regarded as a single reproducing entity (the 'super organism')

[6,7]). Groups of nest members take on specialized roles, which corresponds to the differentiated cell tissues of a multicellular organism [8,9]. In the best-studied societies of ants and bees, for example, this is proposed to have led to the evolution of sophisticated group-level social immune defences by workers, including application of antimicrobial substances onto contaminated areas, removal of corpses and diseased brood, social fever and allogrooming [10–12]. Such sanitation behaviour is not restricted to eusocial insects, however, as its precursors are already present in subsocial insects with parental care (e.g. [13–15]), although empirical data from such systems are scarce. This sparked a debate about whether the concept of social immunity should be extended to include cooperative sanitation tasks performed in non-eusocial group living species, to better understand the evolutionary origins of social immunity [11,16].

This recent debate highlights that the evolution of social immunity is hitherto unclear. Either social immunity evolved as a result of increased pathogen transmission in eusocial organisms (termed *the eusocial framework* [5,11,16]) or sociality and social immunity co-evolved in a close feedback interaction (termed *the group living framework* [15–17]). In some taxa, the suppression of pathogens is a very important social task, not only exhibited by parents towards offspring, but sometimes even between all individuals in a nest or aggregation. Hence, it is conceivable that under certain circumstances, pathogens themselves may be important drivers of sociality. This might be true especially in taxa that live in permanent close contact to a decaying food source and are thus frequently in contact with various microbes (e.g. involving parental care in burying beetles, larval aggregations in *Drosophila* or worker specialization in attine ants [17]). Our study explored this possibility further by introducing fungus-farming bark beetles as a system for the experimental study of social immunity.

These so-called ambrosia beetles offer a unique opportunity for studying the evolution of social traits because closely related species express various social structures ranging from uniparental care to eusociality [18]. Cooperative breeders are of particular interest for experimentally studying social evolution, as here adult females delay dispersal and act as helpers or temporary workers. The length of dispersal delay is affected by the presence and quantity of dependent offspring in the colony and the level of nutrition [19,20], but it might also be affected by the presence and load of pathogens.

All ambrosia beetles live in close mutualistic relationships with different fungi and bacteria, which they farm as their sole source of nutrition within tunnel systems in the heartwood of trees. The main mutualists are so-called ambrosia fungi, primarily from the ascomycete orders Microascales and Ophiostomatales [21–23]. These fungi are taken up from the natal nest by dispersing adult females in special spore carrying organs called mycetangia and subsequently spread on the walls of newly excavated tunnel systems. Finally, they are cultivated and possibly protected from other (fungal) pathogens or competitors [18,23,24]. In addition to these fungal mutualists, several other fungi have been isolated from beetle nests, many of which are pathogens for the beetles or at least competitors of the beetles' fungal mutualists [21,23]. The genera *Aspergillus* and *Beauveria*, for example, can directly infect and kill adults and brood of ambrosia beetles [23,25–28]. Other

fungi compete with the ambrosia fungi and thus deplete the food source of the beetles (e.g. *Penicillium* sp., *Chaetomium* sp., *Nectria* sp. [29]). Such pathogens and competitors are probably the primary threat for the beetles because within the wood, they are well protected from most other natural enemies.

Morphological castes such as those in eusocial insects do not exist in ambrosia beetles. Instead, many species show division of labour among totipotent adult and larval offspring, with adults overtaking nest protection and sanitation, and larvae engaging in nest enlargement and packing of frass (i.e. sawdust, faeces and possibly pathogens) [19]. Larvae and adults join forces to pack and expel pellets of waste through the nest entrance. One of the most common behaviours in both adults and larvae is allogrooming of each other and the brood, probably against pathogens. Diseased individuals are either cannibalized or removed from the nest [19]. Currently, it is unknown, however, if ambrosia beetle larvae and/or adults can detect pathogens and actively suppress their load within nests. Some bark beetles have been shown to exude secretions from their mouth to kill pathogenic fungi [30]. Others, like the species *Dendroctonus frontalis*, are associated with bacteria that produce antibiotics which selectively kill antagonistic microorganisms threatening their fungal associates [31]. Indications for such a bacterial defence mechanism that specifically targets fungal pathogens and not the fungal cultivars have been recently also found in our model species *Xyleborinus saxesenii* [32].

Recent advancements in laboratory rearing, observation and *in situ* manipulation techniques [24,33,34] allow studies of social pathogen defence in ambrosia beetles. Previous studies revealed vigorous cleaning behaviours by adult offspring and even larvae. Since all ambrosia beetles live in close contact to a rich microbial environment, similar to some of the best-described models for social immunity in eusocial insects, we expect to find convergent behavioural adaptations to increased pathogen exposure. In addition, the naturally very high inbreeding rate found in cooperatively breeding ambrosia beetles is assumed to create a condition similar to eusocial insects, where the genetic homogeneity of nestmates renders group members highly vulnerable to microbial attack.

To test this idea, we used the cooperatively breeding and naturally highly inbred species *X. saxesenii* Ratzeburg to determine the effect of *Aspergillus* fungal pathogens on beetle social behaviours and potential social immunity. This pathogen was chosen because it has been repeatedly isolated from diseased individuals from *X. saxesenii* nests (see electronic supplementary material, figure S1) and it is well known for its pathogenicity for many insects (including other bark beetles [26,27]), which is a result of produced aflatoxins [35,36]. *Aspergillus* spores were experimentally injected in laboratory nests, and effects were determined on (i) the social behaviours displayed by larvae and adults and (ii) the timing of dispersal of adult offspring from the natal nest. In addition, (iii) we assessed the effectiveness of the beetles' hygienic behaviours on pathogen spore loads, by comparing pathogen spore loads of nest parts with beetles present against parts where beetles had been experimentally removed after injection of the pathogen. We predict that the group members increase nest sanitation in response to the introduced pathogen and that this behaviour reduces pathogen spore loads. Furthermore, daughters will either delay their dispersal to help with nest hygiene and thus increase

their indirect fitness benefits or disperse earlier to protect their individual health and direct fitness gains.

## 2. Material and methods

### (a) The study species

The fruit-tree pinhole borer, *X. saxesenii*, is a polyphagous, inbreeding and haplodiploid ambrosia beetle species native to Eurasia, which has a highly biased male:female sex ratio (about 1:20) and is known for delayed dispersal and cooperative brood care, similar to the Asian ambrosia beetle, *Xylosandrus germanus*, that is now invasive in Europe and North America [37]. Recent studies have shown that even the larvae in *X. saxesenii* are involved in the division of labour, which is unique for holometabolous insects [19]. The haploid male offspring live side by side with their diploid sisters, but in contrast with females, males show little brood care except for allogrooming, which is often followed by copulation [19]. Their major role is to fertilize their sisters and only a small number of males ever leaves the nest. After having dispersed, *X. saxesenii* females initiate new nests by boring a tunnel with a single side branch ('gallery') into weakened or freshly dead trees, which will later be extended mainly by wood-chewing larvae into a larger nesting chamber. Here, they establish a fungus garden dominated by the mutualistic fungus *Raffaella sulphurea* Batra (Ophiostomatales, Ascomycota) [23]. Spores of this fungus are transmitted vertically from a natal nest within the beetles' guts [38]. Other pathogenic and competitor fungi, like *Aspergillus*, *Penicillium*, *Nectria* and *Ophiostoma* spp., may co-occur at low abundance in the fungal gardens [23]. For the present study, we used laboratory-bred nests of *X. saxesenii* (see the electronic supplementary material for a detailed rearing protocol).

### (b) Pathogen injection

In the first experiment, we tested whether beetles show plastic behavioural responses when either an *Aspergillus* spore buffer solution or a sterile buffer solution was injected into the nest. We started the experiment when more than five adult females were present within a laboratory nest, which is usually the case at a nest age of 30–45 days. We randomly assigned nests to two groups: in the treatment group, we injected spores of a pathogenic *Aspergillus* sp. which were dissolved in a PBS buffer solution ( $n=16$  nests). In the control group, we only injected sterile PBS buffer solution ( $n=15$ ). The *Aspergillus* strain had been repeatedly isolated from diseased *X. saxesenii* individuals both in the field and laboratory (for further details, see the electronic supplementary material).

### (c) Behavioural observations

Behavioural observations were performed daily for 2 days before and 2 days after the injections. On the day of the manipulation, we additionally observed the behaviours immediately before and 2 h after the injections. During a behavioural observation, every individual was either classified as larva, pupa, adult female or male, and then the behaviour an individual displayed at the moment of first sight while gradually scanning through the nest was recorded (i.e. scan sampling; for details on all observed behaviours, see electronic supplementary material, table S4; cf. [19,39]). Every nest was scanned five times per observation and the counted behaviours were then pooled for every category (larvae, adult female, male). Females emerging through the entrance tunnel and found on the surface of the breeding substrate were considered dispersers. They were collected and counted daily. These collections of dispersers were continued until 3 days after the last injections had occurred. After that, all

nests were opened and if present, undispersed beetles and larvae were counted.

### (d) Effectiveness of the social immune response

In the second experiment, we tested the effectiveness of the beetles' social behaviours in suppressing the spread of *Aspergillus* sp. The beetles were reared and treated like in the first experiment. Spore–PBS solution was injected into the treatment group nests ( $n=21$ ), whereas the control group nests received pure PBS solution ( $n=9$ ). Two hours after the injections, all nests were opened and the solid substrate with the nest was gently shaken out of the plastic tube. Then, the substrate was cut crosswise into two parts exactly through the middle of the breeding chamber (where the solutions had been applied 2 h before) using a sterile scalpel. The outer half of the nest with the entrance tunnel was immediately pushed into a new, sterile tube to prevent the loss of beetles. The inner half was cleared of all inhabitants (adults and brood) and put into another sterile tube without any beetles. Both tubes containing the two halves of the same original nest were closed with caps and stored under standard rearing conditions as described above. Three days later, a  $2 \times 2$  mm piece of tunnel wall was sampled from each tube under sterile conditions. These wall samples were immediately crushed in 0.5 ml of PBS buffer (+0.05% Tween 20) solution. For the next step, these samples were further diluted with PBS buffer to get 1/10 and 1/100 solutions. Colony-forming units (cfu) were then determined from culturing both solutions on yeast-malt agar plates (see the electronic supplementary material for further details).

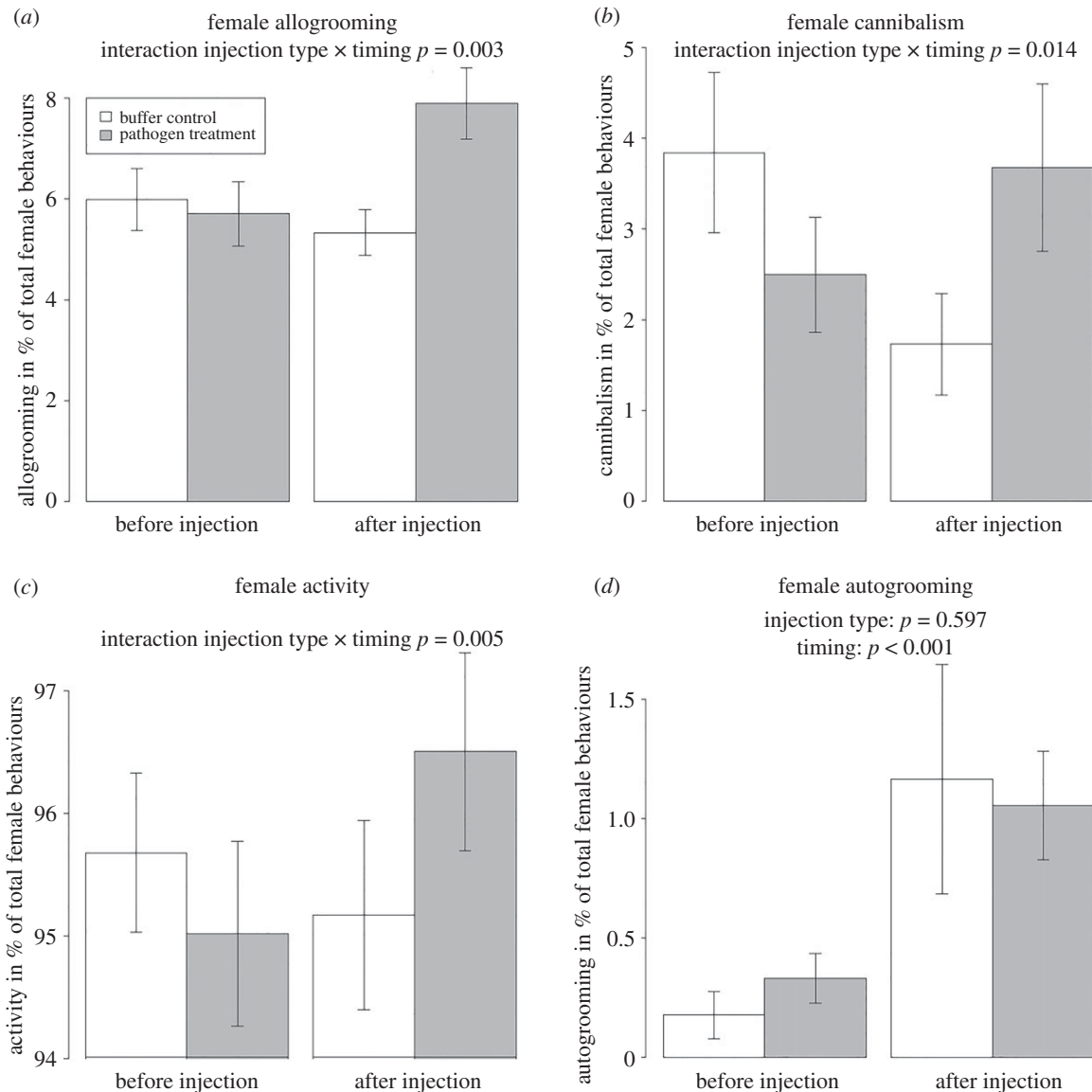
### (e) Data analysis

A series of generalized linear mixed models (GLMMs) with binomial error distribution and logit-link function was used for the analyses of the behavioural data. To examine the effects of the injection treatments (blank versus pathogen spore solution, and before versus after the injection) on the different behaviours of the colony members, the frequency of the respective behaviour was set as response variable and the treatments and their interactions as explanatory variables. To control for potential influences of nest age and numbers of larvae, pupae and adults, these variables were included in the models as covariates. As nests were measured over multiple days, we included the nest ID as a random variable [40,41]. Stepwise backwards elimination of non-significant terms was used to simplify maximal models containing all of the above variables [42,43]. Whenever we found overdispersion in a model [43], we corrected for it by incorporating an additional observation-level random variable [44]. We performed log-likelihood tests to examine the significance of the explanatory variables.

To compare the relative amount of hygienic behaviours between larvae, males and females, we ran an additional GLMM with pooled hygienic behaviours as a response variable and sex/age class as an explanatory variable. A post hoc Tukey test was performed to account for multiple comparisons.

Differences in dispersal behaviour were analysed using a Cox model, likelihood ratio test [45]. We accounted for the beetles remaining in the nest after the end of the experiment by including them as censorings to the survival analysis. In the rare cases, where the dispersing beetles could not be counted daily, we estimated the day of dispersal to lay in between the two nearest days where counting did occur ( $n=18$  beetles of blank solution treated nests and 16 beetles of pathogen-treated nests).

Colony-forming unit data were analysed with GLMMs similar to the behaviour analysis, but here we used only the two treatments and their interaction as explanatory variables (blank versus pathogen spore solution, beetles present versus removed),



**Figure 1.** Proportions of different hygienic behaviours displayed by adult females (arithmetic mean  $\pm$  s.e.) before and after the injection of an *Aspergillus* spore solution or the control (blank buffer) into the main breeding chamber. (a) Allogrooming rates were enhanced after the injection of *Aspergillus* spores. (b) Pathogen injections increased the rate by which females cannibalized larvae. (c) Female activity was increased as a response to the injected pathogen. (d) Injections overall increased the autogrooming frequency, regardless if pathogenic spores were added to the buffer solution or not.

and thus, no reduction of the maximal models was performed. For these models, we assumed a Poisson error structure.

All statistical analyses were performed in R v. 3.4.3 32 bit [46] with additional packages 'lme4' [47], 'survival' [45], 'lmerTest' [48], 'multcomp' [49] and 'blmenco' [50].

### 3. Results

#### (a) Behavioural response to pathogen injection

Female allogrooming frequency within nests was significantly enhanced in the treatment group after injection of the *Aspergillus* spores compared to the control group with only buffer added (treatment  $\times$  timing:  $p = 0.003$ ). The model controlled for the significant positive influences of the number of pupae ( $p = 0.003$ ) and females ( $p < 0.001$ ) on female allogrooming, and the negative influence of gallery age ( $p < 0.001$ ; figure 1a; electronic supplementary material, table S1).

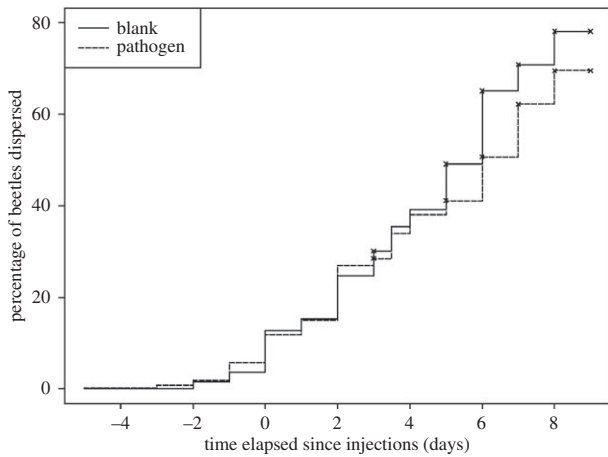
Female cannibalism significantly increased after injection of *Aspergillus* spores (treatment  $\times$  timing:  $p = 0.014$ ). The

number of larvae (GLMM:  $p = 0.214$ ) had no significant effects on the model and the age of the nest showed a non-significant trend ( $p = 0.056$ ), but were both kept in the model as mainly larvae were cannibalized and considering nest age significantly enhanced model fit (ANOVA of model with versus without nest age:  $p < 0.001$ ; figure 1b; electronic supplementary material, table S1).

We found a significant positive interaction effect of treatment (blank versus spore solution) and timing (before versus after the injections) on the activity (all behaviours minus inactivity) of the beetles (treatment  $\times$  timing:  $p = 0.005$ ). This indicates that nests treated with pathogenic fungi showed increased beetle activity compared to nests only treated with a blank buffer solution. We accounted in the model for the influences of nest age (GLMM:  $p < 0.001$ ) and the number of present larvae ( $p < 0.001$ ), which both correlated positively with activity (figure 1c; electronic supplementary material, table S1).

Injections generally increased female autogrooming frequency (GLMM:  $p < 0.001$ ), independent of whether *Aspergillus* spores or only buffer solution were added





**Figure 2.** Proportion of females dispersed over time in nests injected with a control buffer solution or an *Aspergillus*-spore solution. Female dispersal was significantly delayed in nests with pathogenic spores added compared to controls ( $p = 0.020$ ,  $n = 714$  beetles from 31 nests). Crosses indicate censoring (cf. methods). Time point 0 indicates the day of injection.

(treatment  $\times$  timing was not significant and removed from the final model). This model controlled for the significant negative effect of nest age on autogrooming ( $p = 0.008$ ; figure 1*d*; electronic supplementary material, table S1).

Male and larvae behaviours were recorded during this study as well but are not reported here in detail since for males, observations were rare due to the strongly female-biased sex ratio, and for larvae none of the hygienic behaviours (i.e. cannibalism, allogrooming, balling) were significantly affected by our manipulations (all interactions in GLMMs  $p > 0.1$ ). A GLMM using combined hygienic behaviours as response variable revealed that females dedicated overall more of their time to nest hygiene than males ( $p < 0.001$ ) or larvae did ( $p < 0.001$ ). Larvae showed some hygienic behaviours as well (larvae versus males  $p = 0.009$ ), whereas the contribution by males was negligible (electronic supplementary material, figure S2).

### (b) Female dispersal in response to pathogen injection

Females significantly delayed their dispersal from nests that were injected with the *Aspergillus* spores compared to nests in which only the buffer solution was injected (Cox model, likelihood ratio test: ratio = 5.41,  $p = 0.020$   $n = 714$  dispersing beetles in 31 nests; figure 2).

### (c) Effectiveness of the social immune response

Overall, gallery wall samples from nests in which pathogens were injected showed a higher amount of *Aspergillus* sp. cfus compared to blank buffer-injected nests (figure 3*a*; electronic supplementary material, table S2). There was a significant positive interaction between the type of injection and the beetle removal treatment (injection  $\times$  treatment:  $p = 0.038$ ), indicating that the gallery halves with beetles had a significantly reduced *Aspergillus*-spore load compared to gallery halves without beetles (figure 3*a*; electronic supplementary material, table S2). *Penicillium* sp. cfus tended to be higher when pathogens were injected in the gallery parts where beetles were present (injection  $\times$  treatment;  $p = 0.077$ ; figure 3*b*; electronic supplementary material, table S2). *Ophiostoma* sp. cfus were not influenced by the treatments (figure 3*c*;

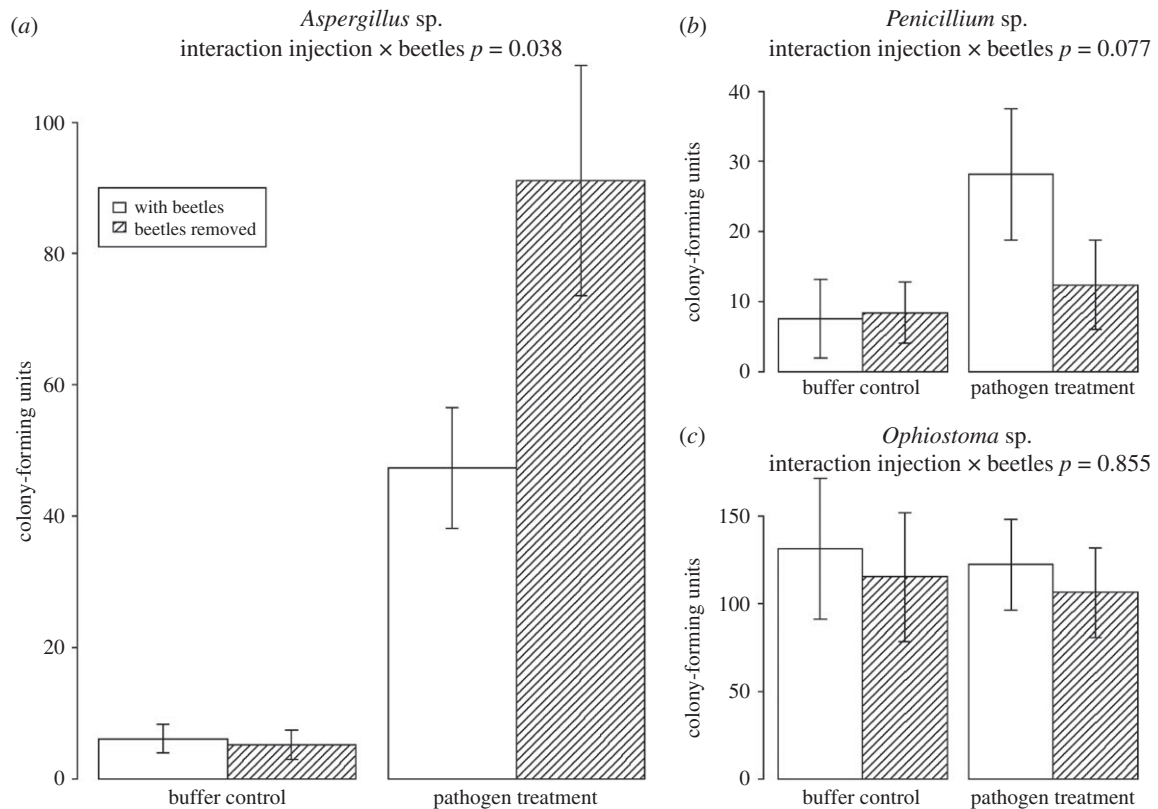
electronic supplementary material, table S2). Note that for our suspensions made from a 4 mm<sup>2</sup> gallery wall sample, *Ophiostoma* sp. had by far the highest cfu counts (mean = 234 407 cfu ml<sup>-1</sup>) followed by *Aspergillus* sp. (9607 cfu ml<sup>-1</sup>) and *Penicillium* sp. (3200 cfu ml<sup>-1</sup>).

## 4. Discussion

This study provides first experimental evidence for social immunity (i.e. adult allogrooming and cannibalism) in a cooperatively breeding insect. Our results demonstrate that adult female *X. saxesenii* ambrosia beetles can detect and plastically respond to spores of a pathogenic *Aspergillus* fungus with various hygienic behaviours like allogrooming, cannibalism and general activity. Males and larvae also showed hygienic behaviours towards the fungus, but they did not plastically adjust behavioural frequencies to the experimental manipulation of fungal spore loads. Beetle presence significantly suppressed spore loads of the *Aspergillus* fungus, which is a common natural associate of *X. saxesenii* in the field [29]. Thus, social immunity in these fungus-farming beetles comprises traits similar to those observed in other fungus-farming societies in attine ants and macrotermites [51].

Our experimental treatment affected particularly behaviours that are beneficial to the whole nest. Autogrooming increased in both, the pathogen treatment and the PBS buffer control group, which indicates that it was triggered by the injection itself rather than by the pathogen. By contrast, both allogrooming and cannibalism of larvae were induced in the adult females by the injection of pathogenic spores. Our *Aspergillus* strain infests insects (even though it can also grow within the nest) and so it is likely that the enhanced allogrooming particularly of the vulnerable larvae reflects an adaptive response of females to the experimental injection of spores. Cannibalism of brood is known to be an effective way of eliminating infected individuals in other insects [15]. Therefore, it is likely to constitute an important component of the social immune system of *X. saxesenii* as well. Generally, adult female activity (i.e. which includes all kinds of selfish and cooperative behaviours; electronic supplementary material, table S1) increased significantly in response to our pathogen treatment, and with both the age of nests and the amount of larvae present. In fact, most of the anti-pathogen behaviours we found in this study were not only affected by the pathogen treatment, but they increased also with the numbers of dependent offspring (pupae and/or larvae) and decreased with nest age. Therefore, we controlled in our models for these two factors (electronic supplementary material, table S1). Our data corroborate observations of previous studies suggesting that females plastically adjust both their behaviour within nests and their dispersal timing to the needs of the brood [19,20]. Furthermore, the frequency of social behaviours seems to decline over time as females prepare for dispersal. Interestingly, allogrooming frequencies of adult females increased with the number of females in the colony, which might indicate that this behaviour is not only an important component of brood care but also between adults.

Compared with other social insects with foraging individuals, ambrosia beetles are largely protected from environmental microbes within their colonies, as they do not leave the nest for foraging [18]. Nest entrances are blocked by an adult female beetle most of the time [39], which affords



**Figure 3.** Colony-forming units (arithmetic mean  $\pm$  s.e.) of fungi from samples of the breeding chamber collected 2 days after either an *Aspergillus*-spore solution or a blank buffer control were injected into the nest. Each nest was split into halves at the injected site (paired design) and subsequently, all beetles were removed from one half. (a) Beetle presence significantly reduced the severity of the artificially induced *Aspergillus* sp. infection. (b) *Penicillium* sp. colony-forming units tended to be higher in nest halves with beetles where *Aspergillus* had been injected. (c) *Ophiostoma* sp. colony-forming units were not significantly influenced by the treatments.

protection of the nest against predators and possibly also microbial threats. We found a tendency for the blocking frequency to increase when the nest was challenged with *Aspergillus* sp. (injection  $\times$  timing:  $p = 0.063$ ). However, we did not specifically select nests with visible entrance tunnels in this study, so relative blocking frequencies could be analysed only in few nests. Also, it is not completely clear how blocking frequency should be expected to change relative to pathogen levels inside the nest. Either blocking frequencies by the nest guarding mother should be upregulated in order to coerce the daughters into fighting the infection instead of dispersing [39], or the mother should stop blocking the entrance to facilitate the dispersal of daughters in order to reduce infection probability of the daughters' future nests.

The direct and indirect fitness benefits of staying, feeding and/or helping in the natal nest, namely, energy uptake for independent breeding and enhancing the reproduction of the mother, are closely linked to the quality of the fungus substrate [52]. Our data show that adult daughters delay their dispersal in response to enhanced pathogen levels. This might reflect selfish or altruistic responses to pathogen prevalence. For instance, females may simply need more time to build up reserves for independent reproduction if pathogens abound in their natal gallery. This is an unlikely explanation for the delay of dispersal in response to the pathogen load, however, as in *Xyleborus affinis* Eichhoff, a closely related species with the same social system, females were shown to suffer a reduction in their own reproductive potential by a dispersal delay rather than gaining reserves during prolonged philopatry [52]. Instead, females might be constrained to disperse by being infected themselves and needing to build up an

immune response before they can sustain the demanding dispersal phase. Alternatively (and in our view most likely), adult daughters may stay longer in pathogen-infested nests because of inclusive fitness benefits, rendering the dispersal delay an adaptive response of infection. As our results reveal, philopatric group members can effectively suppress pathogens, which can improve the reproductive success of the foundress (i.e. mother). Positive effects of larval numbers on the inhibition of *Aspergillus* growth have been previously suggested for *X. saxesenii* [19], and the present study demonstrates that this is also true for adults. Importantly, our results show that brood care, social hygiene and dispersal delays are triggered by pathogens in ambrosia beetles, which indicates that pathogens may be important evolutionary drivers of sociality in ambrosia beetles [17].

Social immunity in ambrosia beetles resembles that of eusocial insects in many ways [5,15]. The entrance tunnel is regularly blocked by adult females, which impedes the intrusion of predators and parasites (e.g. parasitoids, mites and nematodes), and possibly also pathogens (e.g. fungal spores). Blocking behaviour would thus constitute the first layer of prophylactic protection against the threat of pathogen infection, which resembles nest guarding in bees ([53]; table 1; electronic supplementary material, table S4). A change in the general activity level of nest members can be part of a social immune response in insects [15,54,55] and was also a significant response to our pathogen injections. Changes in activity levels may thus constitute an important part of the social immune response of ambrosia beetles. A recent study in ants found that the social network structures can change depending on whether an individual was infected

**Table 1.** Social immunity traits and responses found in this study compared to similar behaviours in eusocial insects. Challenging beetle nests with *Aspergillus* sp. either significantly increased ( $\uparrow$ ,  $p < 0.05$ ), decreased ( $\downarrow$ ) or had no effect ( $\rightarrow$ ,  $p > 0.1$ ) on the observed frequency of traits (\*trend  $p < 0.1$ ). Responses to pathogens/parasites can be either prophylactic (pro) or induced upon contact (ind). The table was compiled using the information listed in [5,15].

ambrosia beetle trait	effect	equivalent in eusocial insects	taxa	response
blocking the entrance tunnel	$\uparrow^*$	guarding of nest entrance	bees	pro, ind
larvae form frass and faeces balls that are removed from the nest by adults	$\rightarrow$	garbage removal, corpse removal	ants, bees	pro
allogrooming	$\uparrow$	allogrooming	ants, wasps, termites	ind
cannibalism of potentially infected larvae	$\uparrow$	removal/cannibalism of infected individuals/brood from the group	bees, termites	pro, ind
activity <sup>a</sup>	$\uparrow$	decrease in activity to reduce pathogen spread or increase in activity to relocate resources to social immune traits	theoretical model; ants?, bees?	ind
dispersal	$\downarrow$	abandon infected nests/nest relocation	ants, bees	ind

<sup>a</sup>Modulating the frequency of general activity (all behaviours – inactivity) was not specifically treated in these publications as a social immune trait. However, potential advantages and disadvantages of activity and inactivity were considered in other studies, both theoretically [54] and empirically [55].

or not. This leads to infected individuals being segregated from the brood and lower interactions between infected and healthy individuals [56]. It is possible that infected and healthy individuals take on different roles also in ambrosia beetles, or that they show differences in their dispersal patterns, but this cannot be tested with the present data. This question would be a worthwhile challenge for future studies.

The most important similarity between social immune responses of *X. saxesenii* and those of eusocial Hymenoptera is connected with hygienic behaviour, which is crucial because both pathogens and parasites would otherwise accumulate over time and cause severe damage to the colony [15,19]. Interestingly, in ambrosia beetles, also larvae contribute to nest hygiene and waste management. By ‘balling’, they form pellets out of sawdust remains and faeces (i.e. ‘frass’), which can be transferred to the nest entrance and removed by the adult females. The participation of larvae in such colony hygiene is a unique trait shown only in ambrosia beetles [19], but general garbage disposal is widely found in ants and bees, albeit performed by adults [15]. Nevertheless, due to their soft body tissue, larvae seem to get infected by pathogens more easily than adults. The observed cannibalism of infected larvae by adults may thus be a mechanism to lower pathogen virulence within nests similar to destructive disinfection recently described in ants [57]. Such brood cannibalism occurs also in bees [58] and termites [59]. It requires special adaptations for disinfecting and killing fungal spores to avoid self-infection and transmission of the pathogens [60]. Fungus gardening ants, for instance, kill ingested spores of pathogenic fungi within their infrabuccal pockets with the assistance of fungicidal actinomycete bacteria [61]. *Cryptocercus* woodroaches spread antifungal  $\beta$ -1,3-glucanase enzymes from their salivary glands by allogrooming. Similar endo- $\beta$ -1,3-1,4-glucanases have been described from *X. saxesenii* [62], which may also have antifungal properties. In the bark beetle *Dendroctonus rufipennis*, oral secretions were shown to contain bacteria producing such antifungal substances [30]. *Streptomyces griseus* bacteria have been recently isolated from nests of *X. saxesenii*

and shown to produce antifungal cycloheximidine, which specifically targets non-ambrosia fungi (i.e. Ophiostomatoid fungi are insensitive to cycloheximidine) [32]. Such bacteria could be used as defensive symbionts in this system. Independent of the exact mechanism of *Aspergillus* spore killing, the spread of antifungal substances by allogrooming would partly explain why this behaviour has been upregulated after our experimental injection of pathogens. Such anti-pathogen effects of grooming may have originally evolved as part of an individual immune response (i.e. maximizing the effect of autogrooming), and later been modified to be socially beneficial (i.e. via allogrooming).

Interestingly, we found a trend for the *Penicillium* sp. fungus to be more numerous in the pathogen-treated nest halves with beetles than without beetles or in the buffer control. *Penicillium* sp. is often found at the entrance of *X. saxesenii* galleries, and the ability of this fungus genus to produce antibiotics and fungicides is long known [63–65]. Further, another *Penicillium* sp. is used as a defensive fungal mutualists in a leaf-rolling weevil [66]. We have observed that *Penicillium* sp. (i) is always present in *X. saxesenii* galleries, (ii) seems to harm neither the larvae nor the adult beetles, and (iii) seems to be able to outcompete *A. flavus* under certain conditions. These observations suggest that *Penicillium* fungi may serve a defensive role in *X. saxesenii*. Future experiments specifically designed to test this defensive role of *Penicillium* sp. seem worthwhile.

We conclude that cooperatively breeding ambrosia beetles possess a social immune system and the ability to fight off pathogens by increasing overall activity and the frequency of hygienic behaviours, which resembles antimicrobial behaviours of eusocial Hymenoptera and termites (table 1). Moreover, adult female offspring delay dispersal when their natal gallery is infected with pathogens, which can provide inclusive fitness benefits. The major upregulated hygienic behaviours were allogrooming and cannibalism, which are tools against pathogens that might be particularly effective in combination with antimicrobial compounds. Future



research will show whether defensive microbial symbionts play a role in this as in other fungus-farming and eusocial insects [67]. The ambrosia fungal cultivars are insensitive to the antibiotic cycloheximidine, which may be a possible candidate produced by a putative defensive symbiont that has been recently isolated from laboratory nests [32]. Our study highlights that social immunity is not a trait confined to eusocial insects, but it might rather be a crucial prerequisite for the evolution of insect grouping and higher levels of sociality. As hygienic services are the dominant cooperative behaviours in ambrosia beetles, it is conceivable that pathogens are a crucial driver of sociality in these weevils.

**Data accessibility.** Raw data can be accessed in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.np5hqbzpb> [68]. SSU rDNA

sequence information of the different used fungal isolates is accessible at NCBI GenBank (MG995854, MG913203, MG905755, MG995023).

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**Competing interests.** We declare we have no competing interests.

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