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Three-dimensional gallery system reconstruction reveals more frequent intraspecific than interspecific interactions in ambrosia beetles

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Ambrosia beetle gallery systems are typically excavated into the xylem of host trees by a single mated female and are generally considered to function as independent units. However, field observations suggest that interactions among gallery systems may also occur. Using X-ray tomography to obtain three-dimensional reconstructions of ambrosia beetle galleries in flood-stressed and ethanol-injected trees, we found that intersections, where two or more galleries excavated by different females merge at one or more points, and intrusions, where a female begins excavating her gallery from within a gallery previously abandoned by another female of a larger species, are recurrent phenomena. We also observed that intraspecific intersections were generally more frequent than interspecific ones, regardless of tree treatment. These intraspecific intersections may represent a potential mechanism for cross-fertilization among the offspring of different founding females, thereby potentially increasing opportunities for outbreeding in these otherwise inbreeding species. Interspecific intersections, which could potentially facilitate lateral transfer of symbionts, occurred less frequently than expected, suggesting that ambrosia beetles may actively avoid such interactions. Overall, our study highlights that interactions among gallery systems may play a key role in shaping ambrosia beetle communities and their symbiotic networks, warranting further investigation.

1. Introduction

Wood is a complex and dynamic microhabitat, characterized by limited nutrient availability, the presence of plant defence compounds, such as lignin, terpenoids and phenolics that impede insect feeding and hinder microbial degradation [1,2], fluctuating microclimatic conditions [3,4] and intricate interactions among diverse and rapidly changing microbial communities [5]. Despite these apparent constraints, it supports remarkable biodiversity, with fungi and insects being the most prevalent taxa [6,7]. Consequently, conspecific and heterospecific individuals living within wood interact in various

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ways, often with significant implications for the species involved [8,9]. While some of these interactions, such as competition and the mechanisms to avoid it [10–12], have been well studied in certain taxa, many others remain largely unexplored. This is mainly because observing processes within wood without destroying the sample has required technologies that were either unavailable or prohibitively expensive until recent years.

Ambrosia beetles in the tribe Xyleborini (Coleoptera: Curculionidae: Scolytinae) are a group of wood-boring insects that have gained prominence in invasion science owing to their economic and ecological damage resulting from an increasing number of introductions worldwide [13,14]. In addition, xyleborine ambrosia beetles have been extensively studied for their unique ecological traits, including inbreeding via sib-mating, social behaviour, and symbiosis with both nutritional and non-nutritional fungi [15–17]. Their gallery systems are typically excavated into the xylem of host trees by a single mated female [15,18]. The resulting family group, consisting of diploid female and haploid male offspring, cooperates in brood care, gallery maintenance and cleaning, and fungal cultivation, exhibiting a clear division of labour between larval and adult stages [19–21]. Each gallery system is generally considered an independent family unit, from which females emerge after mating with their brothers and acquiring the nutritional fungi growing on the gallery walls [18,22]. However, sporadic field observations have suggested that interactions among gallery systems may also occur [23–25].

Two main forms of gallery system interactions have been observed: (i) intersections, where two or more galleries excavated by different females merge at least at one point, and (ii) intrusions, where a female begins excavating her gallery from an abandoned gallery excavated by another female of a larger species [23,25–27]. These interactions can give rise to significant ecological processes. For instance, intersections between galleries excavated by females of the same species may promote cross-fertilization and increase outbreeding [23]. Intersections involving females of different species could lead to lateral fungal transfer and the acquisition of novel symbionts [28–30], a phenomenon that further accelerates economic and ecological problems caused by alien ambrosia beetle–fungus associations [31]. The tendency of many xyleborine species to select host trees based on ethanol [32–35], which is released in response to abiotic or biotic stress [36–38], can lead to the simultaneous attacks on and colonization of the same tree by multiple females [39–42]. Consequently, gallery intersections and intrusions may be more frequent than currently documented. Nevertheless, to date, no study has quantified the frequency or extent of these interactions.

In this study, we used X-ray tomography to investigate interactions among ambrosia beetle gallery systems, specifically focusing on intraspecific intersections, interspecific intersections, and intrusions. These interactions were analysed in trees subjected to either ethanol injection or flooding. Ethanol injection was used to maximize the likelihood of gallery system interactions, creating an artificially favourable environment. In contrast, flooding was applied to simulate a more realistic stress condition, allowing us to assess whether trends observed under the highly favourable ethanol-induced conditions also occur in natural settings. Our aims were to: (i) investigate the overall frequency of the aforementioned interactions under the two different stress scenarios and (ii) test whether they constitute random events or may be instead driven by more defined ecological mechanisms.

2. Methods

(a) Experimental design and tree treatments

Bolts used for investigating gallery systems via X-ray tomography were collected from 80 container-grown trees (base diameter 3.0–4.5 cm, approximate height 3.5 m) representing eight species (n = 10 per species): *Carpinus betulus* L., *Corylus avellana* L., *Malus sylvestris* L., *Prunus armeniaca* L., *Prunus avium* L., *Pyrus pyraster* L., *Quercus ilex* L. and *Quercus robur* L. These container-grown trees were purchased in March 2022 from a nursery located in Ferrara, Emilia Romagna region, Italy (44°39′52″ N, 12°07′38″ E), and subsequently moved to a broadleaf-dominated forest in northeastern Italy (Riserva Naturale Integrale Bosco Nordio, Veneto region, 45°07′30″ N, 12°15′47″ E). The trees were arranged across five clearings, with two individuals of each species per clearing. One tree from each pair was subjected to flooding, while the other underwent ethanol injection, simulating realistic stress conditions versus a treatment aimed to maximize attacks and colonization. These two techniques have been shown to induce attacks on and colonization of container-grown trees by ambrosia beetles in a number of previous studies [43–46]. All container-grown trees were secured to the nearest tree with metal wires to prevent them from falling.

To induce flood stress, a pot was first lined with a plastic bag, into which a second pot containing a single tree was placed [47]. The plastic-lined inner pot was then irrigated with tap water until standing water accumulated around the tree base. Water levels were replenished weekly to compensate for evapotranspiration losses and maintain the flooding condition. The flooding conditions were maintained throughout the whole duration of the experiment (i.e. 28 days) by refilling the water lost by evapotranspiration. Ethanol injection was carried out using the BITE tool [48]. Specifically, a 75% (v/v) ethanol–water solution was injected twice, once at the start of the experiment and again after 15 days, by inserting the BITE into the trunk 20 cm from the tree base. Further details about the experimental setup can be found in Cambronero-Heinrichs *et al.* [40,49], where other data from the same experiment were analysed to address different research questions.

(b) Classification and counting of interacting galleries

Twenty-eight days after the start of the experiment (26 April 2022), the potted trees were harvested, and the stem section located between 20 cm and 1 m above the tree base was cut into two 40 cm bolts. These bolts were placed individually into rearing chambers, where emerging beetles were collected, counted and identified morphologically to species level.

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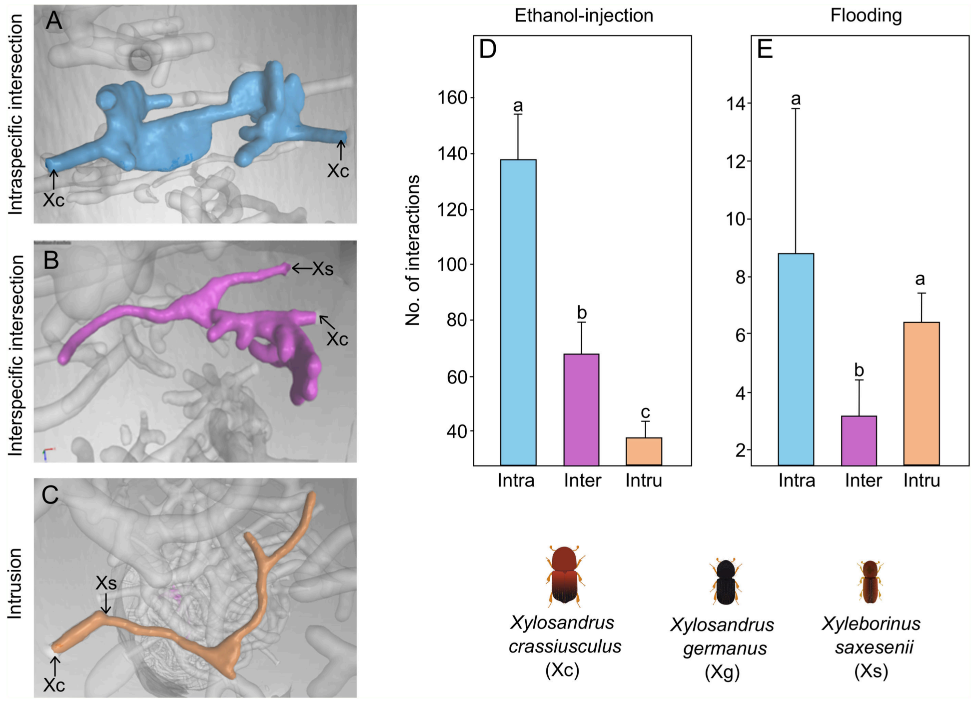

Figure 1. Mean number (+standard error) of intraspecific intersections (A), interspecific intersections (B) and intrusions (C) per tree, in ethanol-injected trees (D) and flood-stressed trees (E). Black arrows indicate entry holes. Different lowercase letters above the error bars in (D,E) indicate significant differences in pairwise comparisons ($p < 0.05$) among interaction types within each treatment based on the generalized linear mixed model.

Each bolt showing signs of ambrosia beetle activity (e.g. entry holes) was heat-treated at 105°C for 5 days to eliminate any remaining beetles within the galleries, which could have otherwise damaged the tomography machine. The logs were then scanned using X-ray computed tomography with a ZEISS METROTOM MT 1500 industrial computerized tomography (CT) system at the Carl Zeiss S.p.A.—Zeiss Quality Excellence Centre in Reggio Emilia, Italy.

For each scanning session, five or six logs were individually wrapped in polystyrene packaging material and placed vertically on the machine's sample holder. They were kept separate from one another. Scans were conducted over a 10 min period using an acceleration voltage of 160 kV and a resolution of 32.6 µm. Image reconstruction was performed using ZEISS METROTOM OS 3.10, and image analysis was carried out with VG Studio MAX version 2022.1 (Volume Graphics, Heidelberg, Germany).

The three-dimensional (3D) reconstruction of the gallery systems within the wood was visually analysed. Owing to the large amount of galleries (i.e. 18 100 for ethanol-injected trees and 2648 for flood-stressed trees) and the substantial amount of time required to reliably assign each gallery to an ambrosia beetle species, only galleries that interacted with at least one other gallery (hereafter referred to as 'interacting galleries') were considered. For all interacting galleries, we first identified the ambrosia beetle species responsible for excavating them and then determined the interaction type, either intraspecific intersection (figure 1A), interspecific intersection (figure 1B) or intrusion (figure 1C). To assign each interacting gallery to a specific ambrosia beetle species among those emerged from the logs, we measured the entry hole diameter by taking 5–10 evenly spaced measurements along the initial section of the interacting gallery (electronic supplementary material, figure S1). These measurements were performed using the 'wall thickness analysis (sphere method)' tool in the myVGL program, a free viewer app for projects created with Volume Graphics software (VGSTUDIO and VGSTUDIO MAX formats). Each interacting gallery was then assigned to one of the four diameter classes corresponding to the four ambrosia beetle species colonizing the trees, namely *Anisandrus dispar* (Fabricius), *Xyleborinus saxesenii* (Ratzeburg), *Xylosandrus crassiusculus* (Motschulsky) and *Xylosandrus germanus* (Blandford) (electronic supplementary material, figure S1). Diameter classes were determined by measuring the width of 40 beetles of each species that emerged from the logs (electronic supplementary material, figure S1). Data from the two logs obtained from each individual tree were pooled for subsequent analysis. Only a few interacting galleries ($n = 30$) were attributed to *A. dispar*, and because of this low number, that species was excluded from further analysis. Intersections were thus classified into six types: three intraspecific (i.e. *X. crassiusculus*–*X. crassiusculus* (Xc–Xc), *X. germanus*–*X. germanus* (Xg–Xg) and *X. saxesenii*–*X. saxesenii* (Xs–Xs)) and three interspecific (i.e. *X. crassiusculus*–*X. germanus* (Xc–Xg), *X. crassiusculus*–*X. saxesenii* (Xc–Xs) and *X. germanus*–*X. saxesenii* (Xg–Xs)).

(c) Statistical analysis

Generalized linear mixed models (GLMMs) with a negative binomial distribution and an ln link function were used for most analyses. The first model included the total number of interacting galleries per tree as response variable, the beetle species (three levels: *X. crassiusculus* (Xc), *X. germanus* (Xg) and *X. saxesenii* (Xs)), the tree treatment (two levels: ethanol-injection and flooding) and their interaction (beetle species × tree treatment) as explanatory variables, and the tree identity as random factor.

The second model included the number of interactions per tree, regardless of the beetle species involved, as response variable, the interaction type (three levels: intraspecific intersection, interspecific intersection and intrusion), the tree treatment

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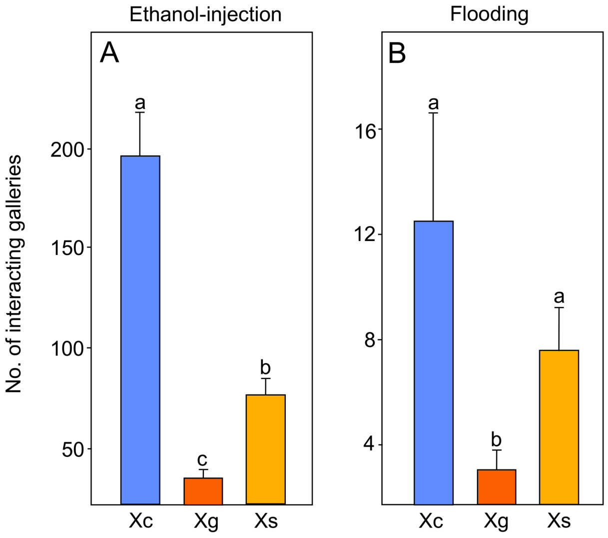

Figure 2. Mean number (+ standard error) of interacting galleries per tree excavated by *Xylosandrus crassiusculus* (Xc), *Xylosandrus germanus* (Xg) and *Xyleborinus saxesenii* (Xs), in ethanol-injected trees (A) and flood-stressed trees (B). Different lowercase letters above the error bars indicate significant differences ($p < 0.05$) in pairwise comparisons among beetle species within each treatment based on the generalized linear mixed model.

(two levels: ethanol injection and flooding) and their interaction (interaction type × tree treatment) as explanatory variables, and the tree identity as random factor.

The third model included the number of intersections per tree as response variable, the intersection type (six levels: Xc–Xc, Xc–Xg, Xc–Xs, Xg–Xg, Xg–Xs and Xs–Xs), the tree treatment (two levels: ethanol injection and flooding) and their interaction (intersection type × tree treatment) as explanatory variables, and the tree identity as random factor.

For all models, pairwise comparisons among the levels of the tested variables within each tree treatment were carried out with Tukey-adjusted p-values to account for multiple testing. For models 2 and 3, only trees containing at least two galleries for each ambrosia beetle species were included in the analysis, as this was the minimum number of galleries required to allow the formation of any interaction.

To evaluate whether intersections constitute random events or are instead driven by more defined ecological mechanisms, we compared expected versus observed numbers of each intersection type. Expected numbers of intersections were calculated separately for each tree, based on the observed number of interacting galleries. Given the total number of interacting galleries in a tree (n_t), the total number of observed intersections (i_t) and the number of interacting galleries of each beetle species (n_x, where $x \in \{\mathrm{Xc}, \mathrm{Xg}, \mathrm{Xs}\}$), the expected number of intraspecific intersections for species x (denoted i_{xx}) was computed as:

$$i_{xx} = \frac{\binom{n_x}{2}}{\binom{n_\mathrm{t}}{2}} i_\mathrm{t}\,.$$

Similarly, the expected number of interspecific intersections between species x and y (denoted i_{xy}, where $\mathrm{x} \neq \mathrm{y}$) was computed as:

$$i_{xy} = \frac{n_x n_y}{\binom{n_\mathrm{t}}{2}} i_\mathrm{t}\,.$$

In both cases, the numerator represents the number of possible pairwise intersections for that specific pair (either intraspecific or interspecific), while the denominator represents the total number of possible pairwise intersections among all interacting galleries in the tree. The result is then scaled by the total number of observed intersections in the tree (i_t) to obtain the expected value. Notably, the sum of all six expected intersection types exactly equals i_t:

$$i_{\mathrm{XcXc}} + i_{\mathrm{XcXg}} + i_{\mathrm{XcXs}} + i_{\mathrm{XgXg}} + i_{\mathrm{XgXs}} + i_{\mathrm{XsXs}} = i_\mathrm{t}\,.$$

Once the expected number of intersections was calculated for each tree, six separate paired Wilcoxon signed-rank tests (one for each intersection type) were performed, treating each tree as a replicate and comparing, within each tree, the observed and expected number of intersections. Non-parametric tests were chosen because the assumption of normality was not met.

All the analyses were carried out in R [50]. GLMMs were fitted using the ‘glmmTMB’ package [51] and checked for overdispersion and residual distribution using the ‘DHARMa’ package [52]. Pairwise comparisons were calculated using the ‘emmeans’ package [53].

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Table 1. Total number of interacting galleries, intraspecific intersections, interspecific intersections and intrusions for each ambrosia beetle species in ethanol-injected trees and flood-stressed trees. Intersections refer to the case when two or more galleries excavated by different females of the same (intraspecific intersection) or different (interspecific intersection) species merge at least at one point. Intrusions refer to the case when a female begins excavating her gallery from an abandoned gallery excavated by another female of a larger species. *X. crassiusculus*, *Xylosandrus crassiusculus*; *X. germanus*, *Xylosandrus germanus*; *X. saxesenii*, *Xyleborinus saxesenii*.

	interacting galleries	intraspecific intersections	interspecific intersections	intrusions
ethanol-injected				
X. crassiusculus	7853	4225	2329	—
X. germanus	1373	235	1069	31
X. saxesenii	3036	893	1964	1429
flood-stressed				
X. crassiusculus	240	95	34	—
X. germanus	56	2	17	2
X. saxesenii	144	13	25	100

3. Results

(a) Interacting galleries

A total of 12 702 galleries that interacted with at least one other gallery were recorded. Of these, 12 262 occurred in ethanol-injected trees, while 440 were found in flood-stressed trees, representing 67.7 and 16.6% of the total number of galleries, respectively. The majority of galleries were excavated by *X. crassiusculus*, followed by *X. saxesenii* and *X. germanus* (table 1).

The number of interacting galleries was significantly affected by beetle species, tree treatment and their interaction (table 2). Overall, significantly more interacting galleries were observed in ethanol-injected trees compared with flood-stressed trees (figure 2A,B). Within ethanol-injected trees, *X. crassiusculus* excavated significantly more interacting galleries than both *X. germanus* and *X. saxesenii* (figure 2A). In flood-stressed trees, *X. crassiusculus* also produced significantly more interacting galleries than *X. germanus*, but not significantly more than *X. saxesenii* (figure 2B).

(b) Interaction types

All three interaction types (i.e. intraspecific intersections, interspecific intersections and intrusions, figure 1A,B,C) were well represented in both ethanol-injected and flood-stressed trees (table 1). Irrespective of the beetle species, the number of interactions was significantly affected by the interaction type, tree treatment and their interaction (table 2). Overall, significantly more interactions were observed in ethanol-injected trees compared with flood-stressed trees (figure 1D,E). Within ethanol-injected trees, significantly more intraspecific intersections were present compared with both interspecific intersections and intrusions (figure 1D). In flood-stressed trees, intraspecific intersections were also higher than interspecific intersections, but not significantly higher than intrusions (figure 1E).

When considering the beetle species, *X. crassiusculus* exhibited the highest number of intraspecific intersections, followed by interspecific intersections and intrusions (table 1). *Xylosandrus germanus* showed more interspecific intersections, followed by intraspecific intersections and intrusions (table 1). In contrast, *X. saxesenii* displayed more interspecific intersections, followed by intrusions and then intraspecific intersections (table 1).

Considering intrusions, *X. saxesenii* initiated its own gallery more often within already existing galleries of *X. crassiusculus* (n = 1046 in ethanol-injected trees and n = 62 in flood-stressed trees) than did *X. germanus* (n = 383 in ethanol-injected trees and n = 38 in flood-stressed trees). In addition, *X. germanus* was recorded to initiate a gallery within an already existing gallery of *X. crassiusculus* 31 times in ethanol-injected trees and twice in flood-stressed trees (table 1).

(c) Intraspecific and interspecific intersection types

The number of intersections was significantly affected by the intersection type and tree treatment but not their interaction (table 2). Overall, significantly more intersections were observed in ethanol-injected trees compared with flood-stressed trees (figure 3A,B). For both tree treatments, the number of intraspecific intersections between *X. crassiusculus* galleries (Xc–Xc) was the most common intersection type (figure 3A,B). In ethanol-injected trees, this intersection type was significantly more frequent than both the others, although significant differences also emerged among the remaining types. In particular, the number of interspecific intersections between *X. crassiusculus* and *X. saxesenii* galleries (Xc–Xs) was significantly higher than all the other intersection types (figure 3A). Similarly, the number of intraspecific intersections between *X. saxesenii* galleries (Xs–Xs) and the interspecific intersections between *X. crassiusculus* and *X. germanus* galleries (Xc–Xg) were significantly higher than both the intraspecific intersections between *X. germanus* galleries (Xg–Xg) and the interspecific intersections between *X. germanus* and *X. saxesenii* galleries (Xg–Xs) (figure 3A). In flood-stressed trees, the number of intraspecific intersections between *X. crassiusculus* galleries (Xc–Xc) was significantly higher than all the other intersection types, except for the interspecific intersections between

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Table 2. Analysis of deviance tables from generalized linear mixed models. Response variables are indicated in italic type. Type II Wald χ^2-tests, degrees of freedom (d.f.) and *p*-values are provided for all models.

	χ^2	d.f.	*p*-value
no. interacting galleries			
beetle species	272.344	2	<0.001
tree treatment	108.322	1	<0.001
beetle species × tree treatment	7.908	2	0.019
no. interactions			
interaction type	93.777	2	<0.001
tree treatment	58.334	1	<0.001
interaction type × tree treatment	23.670	2	<0.001
no. intersections			
intersection type	508.163	5	<0.001
tree treatment	55.930	1	<0.001
intersection type × tree treatment	0.342	5	0.997

X. crassiusculus and *X. saxesenii* galleries (Xc–Xs) (figure 3B). Moreover, no significant differences were found among the latter and the remaining intersection types.

When comparing observed and expected intersection types in ethanol-injected trees, all intraspecific intersections (Xc–Xc, Xg–Xg, Xs–Xs) occurred significantly more frequently than expected (figure 4A). In contrast, all interspecific intersection types (Xc–Xg, Xc–Xs, Xg–Xs) occurred significantly less frequently than expected (figure 4A). In flood-stressed trees, only Xc–Xc occurred significantly more frequently than expected among intraspecific intersection types (figure 4B). In addition, only Xc–Xs occurred significantly less frequently than expected among interspecific intersection types (figure 4B).

4. Discussion

All three types of interactions (i.e. intraspecific intersections, interspecific intersections and intrusions) earlier observed [23,25–27] occurred in our artificially stressed trees. Despite the very high number of interactions found on ethanol-injected trees, which might be considered an overestimation caused by the unrealistic quantity of ethanol present in the wood tissues [49], our results for flood-stressed trees support that gallery systems of ambrosia beetles may regularly interact within the wood. This ecological process may be facilitated by the tendency of multiple females, either from the same or different ambrosia beetle species, to excavate galleries within the same woody substrate, driven by shared or overlapping olfactory and visual cues [32,54,55]. Besides intrusions, which are likely the result of a facilitation process whereby it is easier for a founder female to start digging galleries within a previously abandoned gallery of a larger species, intraspecific and interspecific intersections may give rise to important ecological processes.

Intraspecific intersections were generally more frequent than interspecific ones, regardless of the tree treatment. While this overall pattern can be partly influenced by the highly uneven number of galleries excavated by the three ambrosia beetle species, the expected versus observed number of intersections in our artificially stressed trees supports the occurrence of a mechanism whereby xyleborine ambrosia beetle females might be more likely to intersect galleries of conspecifics than those of other species. Intraspecific intersections have been proposed as a potential mechanism for cross-fertilization among the offspring of different founding females, thereby potentially increasing opportunities for outbreeding [23]. The existence of such a mechanism might also explain the lack of any antagonistic behaviour observed among beetle females originating from different nests despite the existing differences in their cuticular chemical profile [56]. The only other mechanism that could potentially lead to outbreeding in xyleborini ambrosia beetles involves flightless haploid males leaving their natal nest and entering nests founded by unrelated females [57,58]. However, this strategy may carry greater risks: once outside the nest, males are more vulnerable to predation [59,60], and successful entry into another nest depends on the founder female, which typically blocks the entrance hole [21,61]. Under this scenario, it is evident that there should be mechanisms allowing females to locate galleries of conspecifics within the wood. Excluding species-specific stridulatory sounds, which are apparently not produced in xyleborine ambrosia beetles [62,63], recognizing vibrations or sounds produced by conspecifics when moving or chewing within the nests or aggregating on the woody substrate and thus establishing galleries close to those of conspecifics might be potential strategies. The attractiveness towards fungal volatiles and the attraction of beetle females to substrate pre-colonized by conspecifics, or to volatiles emitted by nutritional symbiotic fungi, might support the occurrence of this mechanism [64–66]. Irrespective of the mechanism, whether outbreeding is regularly performed by xyleborine ambrosia beetles remains unclear. The lower-than-expected inbreeding coefficients observed in *X. crassiusculus* [67] may suggest that outbreeding is relatively common. In contrast, the low genetic variation reported in *X. germanus* [68], along with the presence of outbreeding depression but not inbreeding depression [69], seems to indicate the opposite. Besides promoting outbreeding, intraspecific intersections may also lead to the exchange of fungal symbiont strains among conspecifics [70], a mechanism that can be

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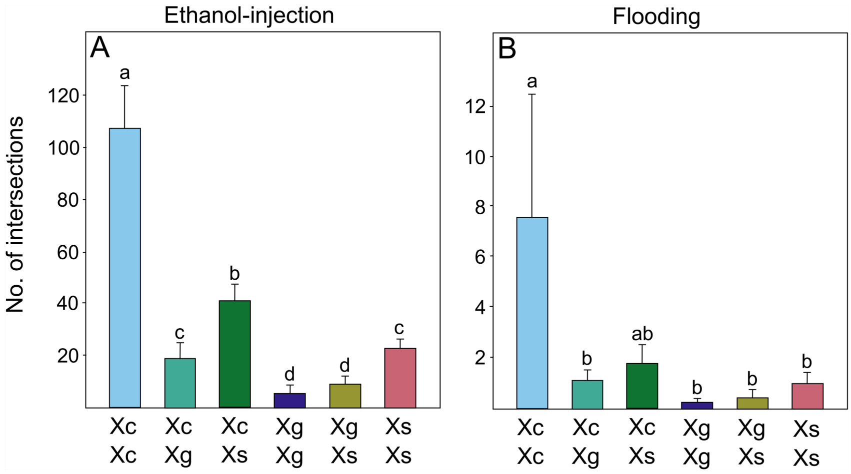

Figure 3. Mean number (+ standard error) of intersections for intersection types (intraspecific: Xc–Xc, Xg–Xg, Xs–Xs; interspecific: Xc–Xg, Xc–Xs, Xg–Xs) per tree, in ethanol-injected trees (A) and flood-stressed trees (B). Different lowercase letters above the error bars indicate significant differences ($p < 0.05$) in pairwise comparisons between intersection types within each tree treatment based on the generalized linear mixed model. Xc, *Xylosandrus crassiusculus*; Xg, *Xylosandrus germanus*; Xs, *Xyleborinus saxesenii*.

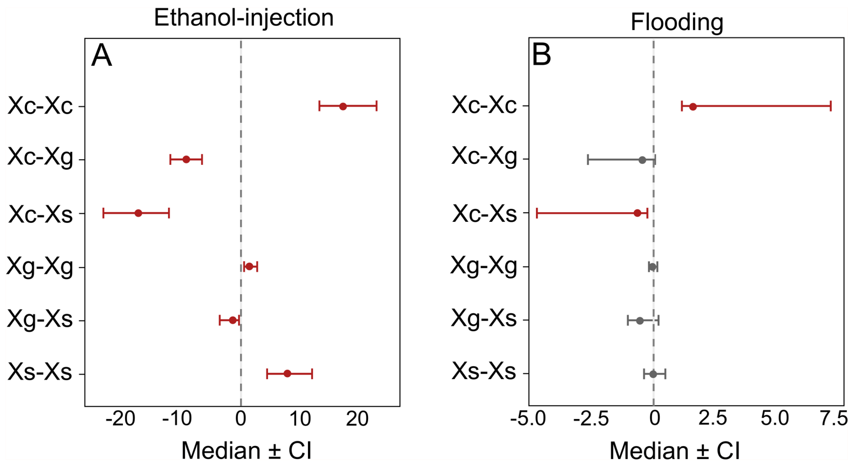

Figure 4. Median (± non-parametric confidence intervals) of the differences between observed and expected number of intersection types (intraspecific: Xc–Xc, Xg–Xg, Xs–Xs; interspecific: Xc–Xg, Xc–Xs, Xg–Xs) per tree, in ethanol-injected trees (A) and flood-stressed trees (B). Red dots and bars indicate significant deviations from zero ($p < 0.05$) based on paired Wilcoxon signed-rank tests. Xc, *Xylosandrus crassiusculus*; Xg, *Xylosandrus germanus*; Xs, *Xyleborinus saxesenii*.

particularly interesting when individuals from distant populations are involved [71]. More studies based on more complete genetic approaches [72] are needed to investigate these processes.

Although less frequent than intraspecific intersections, interspecific intersections were also regularly observed in our study, occurring on both ethanol-injected and flood-stressed trees. The potential implications of this interaction type could be significant, leading to the lateral transfer of microorganisms among different ambrosia beetle species with positive or negative effects on beetle fitness [73,74]. Nonetheless, our results also indicated that these interspecific intersections occurred less frequently than expected. This means that ambrosia beetles may tend to avoid such interactions. Dispersing females likely avoid wood pre-colonized by fungi, because their fungal symbionts need uncolonized medium to grow. A simple mechanism could be the avoidance of foreign fungal volatiles, just as these beetles recognize and avoid antagonistic fungi [75] because vicinity to those fungi most likely results in fitness costs. During laboratory-based behavioural studies, for example, *X. crassiusculus* and *X. saxesenii* were repelled or not responsive to volatiles from fungal symbionts of other beetle species [64]. Apart from repulsive volatiles, aggressive behaviour directed towards heterospecific non-nestmates may be an additional mechanism, as observed in other social insects [76,77], even though tests between *X. germanus* and *X. saxesenii* could not identify any [56]. In any case, considering that mycetangia, i.e. organs responsible for transporting the spores of primary symbiotic fungi [22], appear to be selective at the genus but not at the species level [78], and the existing evidence for new fungal acquisitions or symbiont shifts among ambrosia beetles [28,79–83], it is likely that the aforementioned mechanisms are not always fully effective, allowing interspecific intersections to occur nonetheless. This is particularly likely when native species interact with non-native ones, since they may not have evolved the necessary mechanisms to avoid such encounters. From an ecological point, such fungal exchanges can be dramatic if native species acquire non-native plant-pathogens because native ambrosia beetles may turn invasive owing to their newly acquired symbionts [31]. The currently most detrimental example for this process is Dutch-elm disease, which led to the almost eradication of European elm trees because the native elm bark beetles acquired the invasive fungal pathogen *Ophiostoma novo-ulmi* [84].

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5. Conclusions

Investigating the processes occurring within wood offers valuable insights into ecological mechanisms that would otherwise remain inaccessible. X-ray tomography has increasingly been employed for this purpose [40,85,86], although it has not before been used to examine interactions among gallery systems of wood-boring beetles. Focusing on xyleborini ambrosia beetles, this study reveals that such interactions are more frequent than previously assumed and may not be random events, but rather the possible result of evolutionary processes within this beetle group. Confirming these findings, however, requires more targeted laboratory and field trials capable of isolating the various mechanisms we have proposed and addressing the main limitations of our study. First, our analysis was limited to only three ambrosia beetle species, one native and two non-native species. Given that Xyleborini species differ in their ecological characteristics, the mechanisms observed in our study may not be generalizable to the entire tribe. Second, the comparison between expected and observed numbers of intersections relied on simplified assumptions that did not account for differences in gallery size, orientation, depth and shape, nor for the temporal dynamics of beetle attacks and timing of excavation. These simplifications may have biased the results of the analysis testing whether certain interactions occurred more frequently than expected by chance. For example, females of one species may have attacked trees earlier than females of the other two ambrosia beetle species, thereby favouring intraspecific over interspecific interactions. Third, some of the observed trends are based on trees exposed to highly artificial conditions, characterized by extreme ethanol concentrations, which likely inflated gallery density and interaction frequency. Confirming the occurrence of similar gallery interactions in field-collected material would be important to assess the extent to which these interactions occur under natural conditions. Deeper investigations into interactions among galleries could offer valuable insights into ecological mechanisms that remain largely unexplored, yet may play a crucial role in structuring ambrosia beetle communities and their symbiotic networks. Finally, given that invasive ambrosia beetles are becoming more prevalent worldwide, our study suggests that we should take a closer look at the interactions between these species and consider how native species may be affected by them.

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

Data accessibility. All the data used in this study are available in the online supplementary material (appendix S1).

Supplementary material is available online [87].

Declaration of AI use. We used AI tools exclusively to check spelling and grammar.

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

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

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

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