Short and long-term costs of inbreeding in the lifelong-partnership in a termite

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Abstract

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ABSTRACT

Social life and lifelong partner commitments are expected to favor thorough partner choice, as an ill-suited partnership may have long-term consequences, adversely affecting the parents and spanning several cohorts of offspring. Here, we used ~1400 termite incipient colonies to estimate the short- and long-term costs of

inbreeding upon the survival of the parents over a 15-month period, their productivity, and the resistance of their offspring toward pathogen pressure. We observed that foundation success was not influenced by the relatedness of partners, but by their levels of microbial load. We showed faster growth in inbred colonies, revealing a potential tradeoff with pathogen susceptibility. Yet, inbreeding takes its toll later in colony development when incipient colonies face pathogen pressure. Although the consequences of choosing a lifetime partner is initially determined by the partner's health, the cost of inbreeding in incipient colonies favors outbred colonies reaching maturity.

SHORT ABSTRACT

We used termite colonies to study the short- and long-term costs of inbreeding on the survival of the parents, their productivity and their offspring survival toward pathogens. Colony founding success was not influenced by relatedness of partners, but by their microbial load, yet inbreeding may make small colonies more susceptible to pathogens. Lifelong partner commitment is first affected by the immediate benefit of a healthy partner, inbreeding depression restores outbreeding in mature colonies.

INTRODUCTION

The difference between the sexes in their gamete and offspring investment generally leads to females being considered the choosy sex and males the more promiscuous sex. However, in high fidelity species, epitomized by the social Hymenoptera where males *live* as stored sperm, a detrimental mating cannot be remedied by new reproductive events. Lifelong partner commitments are expected to favor extreme choosiness by both sexes (Shellman-Reeve 1999; Boomsma 2013). Additionally, the consequences of poor mate choice are higher for social species as the parents may be adversely affected, since they rely on their offspring for care, not only for themselves but also for rearing their future brood. Therefore, an ill-suited partnership may have long-term consequences, spanning several cohorts of offspring.

Mating with close relatives is commonly seen as detrimental due to the deleterious consequences of inbreeding, which logically suggests that evolution favors mechanisms preventing its occurrence (Nichols 2017). Particularly well-studied in social and/or monogamous groups, inbreeding avoidance may arise through increased dispersal, reducing the likelihood of encountering relatives (Clutton-Brock 1989), or through delayed reproduction via parental inhibition, preventing mating between the parents and their offspring (Wolff 1992; Abbott 1993). Remarkably, this sexual repression is lost when the opposite-sex parent is absent or replaced (Hanby & Bygott 1987; Koenig WD *et al.* 1998). Inbreeding may also be reduced through extra-group fertilizations, whereby offspring are not fathered by the males in their group, despite caring for the offspring (Brooked *et al.* 1990; Amos *et al.*1993; Sillero-Zubiri *et al.* 1996). Finally, inbreeding avoidance may occur through recognition and avoidance of kin matings (Blouin & Blouin 1988; Pusey & Wolf 1996; Gerlach & Lysiak 2006). In some cases, the scent of related males is unattractive and may even inhibit sexual behavior in their female relatives (Hurst *et al.* 2001).

Termites are eusocial insects that usually establish their colonies through the pairing of a winged queen and king (Vargo & Husseneder 2011). The royal couple spends their entire lives together secluded within colonies, therefore usually preventing extra-pair fertilizations (colony fusion may allow extra-pair fertilizations in rare cases). During colony foundation, the queen and king frequently engage in social interactions, such as grooming and trophallactic exchanges (Shellman-Reeve 1990), and founding success is directly tied to the health of each partner (Cole *et al.* 2018). The absence of workers prevents founding colonies from reaping the full benefits of social immunity, as workers collectively enhance disease resistance through the maintenance of nest hygiene, allogrooming and the exchange of antimicrobial substances (Traniello *et al.* 2002; Cremer *et al.* 2007; Rosengaus *et al.* 2011b). In incipient colonies, the parents' limited resources are drained by the production and care of the first brood, which is altricial for the two first instars which are more susceptible to pathogens than older workers (Rosengaus & Traniello 2001; Cole *et al.* 2018; Cole *et al.* 2020). Success of incipient colonies therefore increases with the body size of the founders and their contribution to biparental care (Cole *et al.*2018; Chouvenc 2019; Cole & Rosengaus 2019). However, as the colony grows, brood care, food foraging and immune maintenance are undertaken by older workers, whereas

the queen and king forego their parental duties to specialize in reproduction (Matsuura & Kobayashi 2010). These behavioral and physiological changes highlight the importance of both partners and their mutual compatibility in the success of incipient colonies. They also emphasize the changing roles queens and kings play within colonies, questioning whether these different pressures influence selection for distinct partner traits over the lifespan of a colony.

Several lines of evidence suggest that inbreeding hampers the development of termite colonies. In *Zootermopsis angusticollis* , inbred groups are more susceptible toward a fungal pathogen and exhibit higher cuticular microbial loads, potentially resulting from less effective allogrooming (Calleri *et al.* 2006). In*Reticulitermes flavipes,* a high proportion of reproductives pair up with nestmates during the nuptial flight (25%); yet this proportion is reduced among established colonies, suggesting that inbreeding negatively affects colony development (DeHeer & Vargo 2006). However, the susceptibility of mature colonies toward pathogens was not found to be associated with their level of inbreeding (Aguero *et al.*2021b); rather, specific genetic backgrounds seem to determine their survival to a greater extent than overall genetic diversity. Similarly, increased diversity from colony fusion was not found to improve survival toward pathogens. Merged colony survival was instead equal to that of either the more susceptible or the more resistant colony, highlighting the complementary roles of both colonies of origin (Aguero *et al.*2020). Similarly, inbreeding does not seem detrimental during colony establishment in *Z. angusticollis* and offspring production was reported to be similar between inbred and outbred pairings. However, the survival of incipient colonies was remarkably higher when initiated by inbred reproductives, which the authors suggested likely resulted from the immune priming of nestmate reproductives toward *familiar*pathogens due to prior exposure within their natal colony (Rosengaus & Traniello 1993). In contrast, high mortality in outbred pairings may stem from non-nestmates facing *naïve* pathogens carried by their partner, toward which they may be more vulnerable (Rosengaus & Traniello 1993).

Here, we sought to untangle the complex interaction between inbreeding and pathogen pressure on colony foundation in termites[,]. Using six stock colonies of *R. flavipes* (Perdereau *et al.* 2013; Eyer *et al.* 2021a), we set up inbred and outbred pairings. We first investigated the short-term cost of outbreeding by assessing the influence of genetic relatedness, microbial loads and microbial similarities on foundation success over the first 14 days. Second, we investigated the long-term cost of inbreeding by comparing inbred and outbred pairings over a 15-month period for their survival, their productivity (worker and soldier), and the resistance of their offspring toward entomopathogenic pressure. Overall, we show that inbreeding and outbreeding entail different costs at distinct stages of a colony's lifespan; identifying those costs can shed light on the evolutionary pressures influencing partner choice and inbreeding avoidance.

MATERIALS & METHODS

Termite collection and alate pairing

Six stock colonies (colonies A to F) of*Reticulitermes flavipes* were collected in Bryan, TX, USA in March 2020, a week before the swarming flight would have naturally occurred. Colonies were extracted from their wooden logs and transferred into 20cm plastic boxes. One worker per colony was sequenced at the mitochondrial 16S gene to confirm identity of the species, following methods from Aguero et al. (Aguero *et al.* 2020). For each colony, male and female alates were sexed and isolated with a group of workers. They were then paired in 3-cm petri dishes with sawdust and wood pieces (Eyer*et al.* 2021b). The incipient colonies were kept in high humidity chambers. Only dark-pigmented alates were used to ensure they were physiologically and motivationally ready to mate.

To investigate the short-term effect of inbreeding on founding success, we set up 40 inbred pairings for each colony (only 31 for colony$D$ due to a lack of available alates). We also prepared 40 outbred pairings for every combination of colonies, with an equal number of each sex per colony of origin (20 *queens*A x *king*B and 20*queens*B x *king*A); resulting in 231 inbred and 600 outbred incipient colonies. In addition, we estimated the long-term effect of outbreeding on incipient colony survival and productivity, as well as on pathogen resistance and microbial load of their offspring. To ensure robust sample sizes, we anticipated high mortality

during colony foundation and established an additional 290 inbred and 300 outbred pairings (100 inbred pairings for three colonies with enough alates available: colonies *A, B & F,* only 90 inbred pairings for *colony F* ; and 100 outbred pairings for all combinations of those colonies). Overall, we set up 1421 incipient colonies (521 inbred and 900 outbred), all of which were established on the same day.

Relatedness between colonies of origin

For each stock colony, DNA from 10 workers was extracted using a modified Gentra PureGene protocol and genotyped at nine microsatellite loci (Aguero *et al.* 2021b). Amplifications were carried out in a volume of 10µl including 1U of HS DNA polymerase, 2µl of 5x buffer (MyTaq, Bioline), 0.08µl of each primer, and 1.25µl of DNA template. PCR was performed using thermocycler T100 (Bio-Rad). Alleles were sized against a LIZ500 standard on an ABI 3500 genetic analyzer (Applied Biosystems) and called using Geneious v.9.1 (Kearse *et al.* 2012).

Relatedness coefficients (r) among nestmates and between workers from each pair of colonies were estimated using the Queller and Goodnight (Queller & Goodnight 1989) algorithm implemented in the program COANCESTRY v.1.0 (Wang 2011). Relatedness coefficients were weighted equally, and standard errors (SE) were obtained by jackknifing over colonies. A principal component analysis (PCA) was performed on the microsatellite markers using the *adegenet* package (Jombart 2008) in *R* (R Development Core Team 2016) to visualize and confirm genetic differentiation between sampled colonies (Figure S1).

Microbial load estimation

For each stock colony, microbial loads were estimated from the number of colony forming units (CFUs) cultured from individual cuticular washes of 12 alates (6 females and 6 males) and 6 workers per colony. Each alate was washed in a sterile 1.5ml tube with 300µl of a 0.1% Tween 80 solution, gently vortexed and centrifuged at 300×g at 4°C for 20 minutes (Rosengaus *et al.* 2003). For each sample, three 20µl replicates of the supernatant were plated on potato dextrose agar, while 20µl of the Tween 80 solution was used as a control. Plates were inverted and incubated at 37° C for three days. The number of CFUs at least 1mm in diameter was counted for each plate and averaged between triplicates. Microbial loads were quantified the same day as the alates were paired. Microbial loads were compared between colonies using a Mann-Whitney U-test. For each pairing combination, cumulative microbial load describes the sum of the microbial load across the two colonies of origin, while maximum microbial load only considers the colony of origin with the highest value.

Microbial diversity identification

Bacterial and fungal communities were identified for each colony by sequencing cuticular washes of three female alates, three male alates, and three workers per colony (N = 54). Individuals were collected using sterile tools and washed in 300µL of 0.1% Tween 80 solution. After 15 minutes of gentle rotation, the solution was removed for DNA extraction using a Phenol/Chloroform protocol. For the bacterial community, the v4 hypervariable region of 16S was amplified using the bacterial primers 515f and 806r (Kozich *et al.* 2013). For the fungal community, ITS was amplified using the primers CS1-ITS3 and CS2-ITS4 with Fluidigm CS1 and CS2 universal oligomers added to their 5' end (White *et al.*1990). PCR protocols are provided in Supplementary Information S1 (Aguero *et al.* 2021a). Pooled amplicons were loaded onto an Illumina MiSeq Standard v2 flow cell and sequenced in a 2x250bp paired end format using a MiSeq.v2.500 cycles reagent cartridge. Base calling was performed by Illumina Real Time Analysis v1.18.54 and output was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1. All analyses were performed using *QIIME 2* (Bolyen*et al.* 2019). Paired-end reads were filtered for quality control and combined using the *DADA2* pipeline (Callahan *et al.*2016). 16S and ITS sequences were joined at 250bp and identified as amplicon sequence variants. Samples with low coverage (<10,000 reads) were removed from further analyses; all samples were conserved for bacterial analyses, but 13 samples were discarded from fungal analyses. To estimate microbial difference within and between colonies, weighted and unweighted UniFrac distances between each individual were visualized using a principal coordinates analysis (PCoA) (Hamady *et al.* 2010). Unweighted distances only consider the presence or absence of observed microbes, while weighted

values also account for their abundance. Euclidean distances between pairs of individuals on the two PCs of the PCoA were used to build pairwise distance matrices and to compare differentiation among individuals within and between colonies using a Mann–Whitney U-test.

Short-term cost of outbreeding

The survival of the 231 inbred and 600 outbred colonies was assessed every two days for 14 days after pairing. The additional 590 colonies were not used for this experiment because they were only monitored once a month (see below). For each unsuccessful colony (*i.e.,* at least one reproductive died), the sex of the dead alate was assessed to determine its colony of origin. Survival distributions were compared between inbred and outbred pairings and between pairings using the*Coxph -proportional hazards* model implemented in the*survival* package (Therneau & Grambsch 2000) in R. This model was also used to calculate hazard ratios for each colony pairing. Linear and logarithmic regressions were performed to determine the relationships between the hazard ratio of each pairing and the effect of the relatedness between partners (microsatellite analysis), cumulative microbial load, maximum microbial load, as well as fungal and bacterial similarities.

Long-term cost of inbreeding

i. Survival and productivity of incipient colonies

The survival of the 1421 incipient colonies (521 inbred and 900 outbred) was assessed every month for 15 months. Survival distributions were compared between pairs of colonies of origin, as well as between inbred and outbred pairings using the *Coxph* model. The productivity of all surviving colonies was assessed monthly by counting the number of eggs, workers and soldiers. The difference in productivity between inbred and outbred pairings was determined using two generalized mixed models implemented in the *lme4* package (Bates *et al.* 2015) in R. The models tested the relationship between the numbers of workers and soldiers present in colonies as a function of time (fixed effect), with the type of pairing (inbred or outbred) tested as a random effect. The number of eggs present in a colony was not used because of its bimodal distribution (absence during winter) and non-cumulative nature (eggs *'disappear'* once they hatch).

ii. Survival and microbial load of the offspring produced

After 15 months, just 70 out of the 1421 incipient colonies survived, of which only 49 produced 10 or more workers. For each of the 49 colonies (24 inbred and 25 outbred colonies), a group of eight workers were isolated in 30mm petri dishes lined with filter paper (Whatman Grade 5, porosity 2.5µm). Groups were challenged with a pathogen solution containing three strains of *Metarhizium* fungus in equal proportions at the concentration of 1×10^7 conidia/ml in 0.1% Tween 80 (ITS sequences match accession numbers KU187187.1, MT374162.1 and LT220706.1, for *M. anisoplae* , *M. brunneum*and *M. guizhouense* , respectively). Offspring survival was monitored for 14 days following exposure by moistening the filter paper with 300µL of the fungal solution (Aguero *et al.* 2021b). Difference in survival between inbred and outbred offspring was determined using the *Coxph* model. In addition, 66 of the 70 incipient colonies had at least two workers (31 inbred and 35 outbred colonies), for which two workers (with three replicates each) were used to determine the microbial load of the offspring. Microbial loads were measured as described above, except that cuticular washes of workers were extracted in 100µL of a 0.1% Tween 80 solution.

RESULTS

Short-term survival of incipient colonies

Fourteen days after pairing, only 101 incipient colonies (202 alates) of the 831 established pairings survived; 35 out of the 231 inbred pairings (15.15%) and 66 out of the 600 outbred pairings (11.00%). No significant difference was observed between the survival of inbred and outbred pairings ($P = 0.212$; Figure 1a). However, strong differences in survival were observed between specific pairings ($P < 0.001$), ranging from a 47.5% survival for pairing AA to complete mortality for pairings AE and EE (the survival curve of each pairing is provided in Figure S2). Alates from colony A had the highest survival rate, with 74 out of the 202 surviving alates originating from this colony (Figure 2a). Parings including an alate from A showed good

survival overall (low hazard ratio), with the best survival observed for the inbred AA combination (Figure 2b). Notably, the opposite was also observed, with alates from colony E having the highest mortality rate. Consequently, pairings including an alate from this colony had low survival, with the lowest survival observed for the inbred pairing EE (Figures 2a&b). Overall, these results suggest that inbreeding has no effect on colony survival in the first several days after pairing; rather, the survival of the incipient colonies is strongly influenced by the colony of origin of the constituent partners.

The six colonies varied in their microbial loads, with colonies A, D and F exhibiting few CFUs (0.36, 0.39 and 0.58 for A, D and F, respectively; Figure 2d). In comparison, colonies B and C (and E to a lesser extent) displayed higher levels of microbial load with 14.86, 12.28 and 4.03 CFU, respectively. Interestingly, the susceptibility of a pairing was associated with the microbial load of the constituent colonies, both at the maximum microbial load ($P = 0.0009$) and the cumulative microbial load level ($P = 0.0002$; Figure 3a&b; Figure S3). The better fit of logarithmic regressions in both analyses suggests that hazard ratios only slightly increase after a certain threshold of microbial load (Table S1). In outbred pairings that included an alate from colonies B or C, the failure of the incipient colonies mostly resulted from the death of the alate from those colonies (Figure 2c), consistent with their elevated levels of microbial loads (the daily number and origin of dead alates are provided in Figure S2). In contrast, the opposite was found for outbred pairings including an alate from colonies A or D (low microbial loads), with the death of the partner originating from a different colony observed in most cases (Figure 2c,d and S2). Finally, the relationship between the relatedness of the partners and the hazard ratio of the colony pairing was not significant ($P = 0.666$), confirming the lack of effect of inbreeding on colony survival during the first 14 days after pairing (Figure 3c).

Bacterial communities were only slightly different between alates from different colonies on the PCoA (Figure 4a); weighted UniFrac values did not separate individuals from different colonies, while unweighted distances only moderately did (Figure 4a,b). Consequently, this results in similar levels of weighted bacterial differentiation observed within colonies and between different colonies ($P = 0.733$; Figure S4), and a lower, but non-significant, level unweighted differentiation within colonies than between colonies ($P = 0.381$). Fungal communities were also only moderately different between alates from different colonies (Figure 4a). The level of differentiation between nestmate and non-nestmate alates was significantly lower for weighted values ($P = 0.045$), but similar for unweighted values ($P = 0.677$; Figures 4a,b and S4). Overall, these results suggest that different colonies exhibit only slightly different bacterial and fungal communities. Consequently, only unweighted fungal dissimilarity between partners is marginally-significantly associated with an increase in hazard ratio of their pairing ($P = 0.092$; Figure 3g). However, the hazard ratio of a pairing was not associated with the weighted fungal similarity ($P = 0.261$), nor with the levels of either weighted or unweighted bacterial differences between partners (weighted:$P = 0.478$; unweighted: $P = 0.862$).

Long-term survival of incipient colonies

After a month, only 154 out of the 1421 incipient colonies survived (10.84%), and only 85 survived until the fourth month (when the altricial larvae developed into workers able to provide care to both the parents and the next brood). Most of these colonies, 70 out of 85, survived until the end of the experiment (450 days, month 15). Similar to the short-term survival, no significant difference was observed between the survival of inbred and outbred pairings over the course of the experiment ($P = 0.465$; Figure 1b), while strong differences in survival were observed between specific pairings (Figure S5). Notably, the hazard ratio of the different pairing combinations at 14 days was significantly correlated to that at 450 days ($P = 0.0009$; Figure S6). This means that certain colony combinations were more likely to survive to both time points and that the development of brood and workers did not alter the ratio of surviving pairings after 14 days.

Productivity of inbred and outbred colonies

Fifteen months after pairing, 68 of the 70 incipient colonies contained workers. The type of pairing significantly affects the number of workers present in colonies over time, with a higher production of workers in inbred colonies ($P < 0.001$; Figure S7b); the mean number of workers was 25.06 ($\pm$SD = 21.66) in inbred colonies compared to 19.70 ($\pm$SD = 21.16) in outbred colonies (Figure 5a). Similarly, 51 of the 70 colonies

contained at least one soldier, with an average of 1.33 (±SD = 1.17) and 1.13 (±SD = 0.93) soldiers in inbred and outbred colonies, respectively (Figure 5a). A similar effect of the type of pairing was found on the number of soldiers over time, with an increased production in inbred colonies ($P < 0.001$; Figure S7c).

Survival and microbial load of inbred and outbred offspring

Inbred and outbred offspring differentially survive when challenged with pathogens ($P = 0.001$), with inbred offspring exhibiting a higher mortality rate than those from outbred pairings (Figure 5b). However, no significant difference was found between the microbial load of inbred and outbred offspring ($P = 0.401$; Figure 5c), with the mean number of CFUs being 26.21 (±SD = 19.14) in inbred offspring and 30.93 (±SD = 25.30) in outbred offspring (Figure S8).

DISCUSSION

Our study shed light on the roles inbreeding and outbreeding play in the success of termite colonies over the course of their development. First, our results revealed comparable survival between inbred and outbred pairings during the first weeks of colony foundation, despite high survival differences between alates from different colonies. This suggests that inbreeding *per se* has no effect on survival at this stage of colony foundation; rather, the survival of incipient colonies is strongly influenced by the colony of origin of the constituent partners. The pairing with the highest survival was an inbred combination of alates from a low microbial-load colony, while the pairing with the lowest survival was also an inbred combination, but with alates from a high microbial-load colony. Our results show that the susceptibility of pairings increases with their cumulative and maximum levels of microbial load carried by the partners and only provide weak support for different colonies harboring distinct microbial communities; the susceptibility of a pairing was only marginally associated with the fungal dissimilarity between partners. Together with the failure of pairings typically caused by the death of the partner with the highest microbial load, our results highlight the risk of unhealthy mate pairings, regardless of their level of relatedness. Yet, our results suggest that inbreeding takes its toll later when incipient colonies face pathogen pressure, as inbred offspring exhibited higher mortality toward pathogens. These findings suggest that although the choosiness for a lifetime partner is initially influenced by the immediate benefit of a healthy partner rather than the long-term potential of fit offspring, inbreeding depression during colony development may restore the proportion of outbred mature colonies.

Avoidance of inbred or unhealthy partner

Although an equal number of pairings for every pair of colonies was experimentally set up, detection and avoidance of partners who are either susceptible (those with high microbial loads) or nestmates potentially occurs during nuptial flights, discouraging random pairing in the field and minimizing the chance of pairing with a weak partner. We originally planned to test whether the choice of alates in this study relies on their level of relatedness, microbial similarity and load (similar to (Li *et al.* 2013; Sinotte *et al.* 2021)). However, partner choice was inconsistent as alates either engaged in tri-tandem running or continuously changing partners (*pers. obs* .). To date, evidence of detection and avoidance of nestmate pairings are scarce and inconsistent in termites. Inbreeding avoidance can occur through a split sex-ratio between colonies, or differences between the sexes in their dispersal range or in their timing of emergence. However, these indirect mechanisms are rare among termites ((Aguilera-Olivares *et al.* 2015) but see (Husseneder *et al.* 2006; Miyaguni *et al.* 2021)). Long-range dispersal is probably the predominant mechanism preventing inbreeding in many species, as alates can disperse hundreds of meters (Mullins *et al.* 2015; Zhang *et al.* 2021), which leads to genetic differentiation between closely located populations/colonies (Shellman-Reeve 2001; Fougeyrollas *et al.* 2018). Alates of most species do not seem to discriminate against nestmates, although this mechanism has been poorly studied (Vargo & Husseneder 2011). Non-random matings despite long-range dispersal has been occasionally reported, with inbreeding avoidance in *R. chinensis* (Li *et al.*2013), but preference in *Coptotermes lacteus* (Thompson *et al.* 2007) and *R. flavipes* (DeHeer & Vargo 2006). Together with the large variation of relatedness between partners uncovered within and among species, and at different stages of the colony lifecycle (Vargo & Husseneder 2011; Vargo 2019), our findings also support the conclusion

that inbreeding avoidance is probably not a prime determinant of partner choice in termites during colony foundation (Sinotte *et al.*2021).

Similarly, there is little evidence of detection and avoidance of unhealthy alates in termites, despite the fact that pathogen avoidance is commonly documented in workers (Hussain *et al.* 2010; Tranter*et al.* 2014; Yanagawa *et al.* 2015). In *R. chinensis,* alates paired less frequently with an injured partner (Li*et al.* 2013), but females of *Z. angusticollis* showed no preference for healthy males rather than males infected with*Metarhizium* (Rosengaus *et al.* 2011a). Our results revealed that the high risk of pairing with a sick partner represents most of the mortality observed during colony foundation, which suggests that pathogen recognition and avoidance should act as a strong selective force. This selection should not only be based on the detection of the external presence of spores, but on an overall evaluation of partner health, such as changes in behavior or cuticular hydrocarbons (Beani*et al.* 2019) (Supplementary Information S2). However, the influence of other potential selective pressures associated with nuptial flights (*e.g.,* non-mating, predation and resource shortage) may instead lead partners to choose the first mate they encounter, regardless of their relatedness or health (Bengtsson 1978; Waser*et al.* 1986; Lehmann & Perrin 2003).

Offspring production

Our results revealed a higher and faster production of workers and soldiers in inbred colonies. This result may be driven by the prevalence of inbred AA pairings and their weak microbial load. The higher productivity of inbred colonies may therefore stem from a trade off in resource investment between pathogen defense and offspring production (Schwenke *et al.* 2016). In *Z. angusticollis* , pathogen pressure experienced by primary couples during colony foundation leads to a decrease in the likelihood of oviposition and the total number of eggs (Cole *et al.* 2018), and sibling pairs had higher survival than non-related couples when exposed to pathogens (Calleri *et al.* 2005). In *C. formosanus* , outbred pairings also suffered higher mortality than inbred pairings; but in this species, the decreased success of outbred pairings was offset by their increased productivity (Fei & Henderson 2003). Importantly, most studies investigating differences in survival or productivity between inbred and outbred colonies have not taken into account the colony of origin, nor used equal numbers of the various pairing combinations tested. These studies may have failed to provide deeper insight into this process due to potentially strong differences between alates originating from different colonies and the lack of proper control to account for these differences. In our study, the equal pairing of every combination accounted for differences between colonies and resulted in similar survival between inbred and outbred pairings. However, a bias toward inbred or outbred colonies could be observed in the case of an association of alates from different colonies in different proportions (more inbred pairings from the healthy colony A and less from the susceptible colony E would have resulted in better survival of inbred pairings compared to outbred pairings).

Offspring survival

Our results show that incipient colonies may suffer from inbreeding when facing pathogen pressure. Although cuticular microbial loads did not differ between inbred and outbred offspring in our study, the increased susceptibility of inbred offspring is consistent with higher microbial loads in inbred colonies of *Z. angusticollis* , potentially resulting from reduced grooming or a less diverse range of antimicrobials (Calleri *et al.* 2006). Notably, our results on incipient colonies contrast with those uncovered on mature field colonies of the same species, showing a weak influence of genetic diversity toward entomopathogens (Aguero *et al.* 2020; Aguero*et al.* 2021b). First, this difference may stem from a greater reduction in heterozygosity in the present study compared to those in mature colonies, where heterozygosity was only moderately reduced by neotenic reproduction (Rosengaus & Traniello 2001; Aguero *et al.*2021b). Similarly, offspring in the present study were probably younger and thus more susceptible to pathogen exposure []; they were also reared under lab conditions and did not face the same pathogen exposure as workers collected from the field, therefore removing the possibility that immune priming may potentially mask differences between inbred and outbred groups (Rosengaus *et al.* 1999; Rosengaus *et al.*2007). Despite these differences, the better survival of particular pairings also support the suggestion that the influence of a specific genetic background may be greater than the overall genetic diversity on colony survival (Aguero *et al.* 2020; Aguero *et al.* 2021b). Together with

previous findings, our results reveal that inbreeding is a negligible factor in the survival of both founding couples and mature colonies; but may have an important role in incipient colonies. These findings indicate that higher inbreeding depression during colony development, where incipient colonies may be more vulnerable, could increase the proportion of mature colonies headed by outbred reproductives (DeHeer & Vargo 2006) (illustrated in Figure 6).

Inbreeding is only a risk for small incipient colonies

Inbreeding acts differently upon colonies depending on their stage of development, and may therefore not play an important role in partner choice. Inbreeding depression only occurs in small colonies. In our study founding couples experienced drastic mortality in the first weeks, even though the risks associated with nuptial flights mentioned above were limited under laboratory conditions. The presence of strong selection against inbreeding during pairing is also discredited by the common occurrence of inbreeding through neotenic reproduction observed in mature colonies. Remarkably, while inbreeding is prevented in vertebrate social species via parental inhibition of sexual activity by the parent of the opposing sex, the opposite is found in termites. The removal of one of the parents triggers the development of neotenics of the opposite sex, therefore resulting in inbreeding to maintain the life of the colony. Neotenic inbreeding may be tolerated in populous colonies, when social immunity becomes more important than individual immunity in managing pathogen pressure (Cremer *et al.* 2007; Cotter & Kilner 2010; Cremer *et al.* 2018; Van Meyel *et al.* 2018; Liu *et al.* 2019). Social immunity in termites strongly relies on allogrooming, cannibalism, burial behavior and self-exclusion of infected individuals (Chouvenc & Su 2012; Davis *et al.* 2018). Although these behaviors may be adequate for mature colonies, they may be costly in incipient colonies, and cannot be applied to reproductive individuals. These behaviors may therefore be more prevalent and efficient in large groups (Rosengaus & Traniello 2001), accounting for the higher influence of individual immunity (related to individual genetic diversity as determined by inbreeding) in small incipient colonies. Interestingly, individual immunity is negatively correlated with colony-level immune behaviors in an ant, suggesting a trade-off between individual and social immunity in regulating overall parasite protection in this species (Cassidy *et al.* 2021). Similarly, the development of social immunity in shaping disease resistance in termites (also in social Hymenoptera (López-Uribe *et al.* 2016)) seems to occur at the expense of individual immunity, as the evolution of sociality is associated with a reduction in their immune gene repertoire (Viljakainen *et al.* 2009; Meusemann *et al.* 2020; He*et al.* 2021) (but see (Barribeau *et al.* 2015; Otani*et al.* 2016)).

Conclusion

Although inbreeding avoidance is an appealing concept in evolutionary biology, evidence is scarce for its widespread occurrence (de Boer*et al.* 2021), with mate choice ranging from inbreeding preference to tolerance to avoidance (Szulkin *et al.* 2013). This variability is observed both within and between species, and is related to the strength of inbreeding depression (Fox & Reed 2011). Individuals would not be selected to avoid mating with a related partner if the chance and costs of inbreeding are low and if the costs associated with nestmate discrimination are high (Kokko *et al.* 2006). For example, our findings may not apply to most social Hymenoptera, due to the extra cost of inbreeding resulting from their haplodiploid sex determination, in which a single founding queen cannot afford the burden of producing 50% workless and sterile diploid males (Ross & Fletcher 1986; Zayed & Packer 2005). In contrast, the common occurrence of inbreeding among neotenics in mature termite colonies suggests a lower level of inbreeding depression. Overall, our findings emphasize the varied and changing costs of outbreeding and inbreeding play out over the lifespan of termite colonies. Investigating this variation and its costs will surely provide insights into the evolutionary mechanisms driving inbreeding avoidance and preference in social insects.

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AUTHOR CONTRIBUTIONS

PAE and ELV designed the study. PAE collected the samples, performed the experiments and analyzed the data. PAE wrote the paper with contributions of ELV.

DATA AVAILABILITY STATEMENT

The data reported in this study will be deposited in the Open Science Framework database upon acceptance,*https://osf.io* (DOI XX).

SUPPLEMENTARY MATERIAL

Additional material may be found in the online version of this article.

Figure S1: PCA analysis based on microsatellite markers confirming that the nests sampled belong to distinct colonies.

Figure S2: Survival Kaplan-Meier curves for each pairing during the 14 first days of colony establishment (*i.e.,* short-term survival). Incipient colonies were monitored every two days. Hazard ratio for cox-proportional hazard model is reported in front of each pairing. At each census date, bar charts indicate colony of origin of the dead alate(s) in failed incipient colonies. Color bars denote dead alates originating from the studied colony, dark grey bars indicate the death of both alates (*i.e.,* studied colony and partner), and light grey bars represent death of the partner.

Figure S3: Hazard ratio for each pairing in the first 14 days after colony establishment. Additional matrices provide values of every variable tested for each pair of colonies (maximum pathogen load, cumulative pathogen load, relatedness, unweighted Unifrac bacterial difference, weighted Unifrac bacterial difference, unweighted Unifrac fungal difference and weighted Unifrac fungal difference. These values are used to test for the correlations presented in Figure 3).

Figure S4 : (a) Principal Coordinate Analyses (PCoA) of individuals based on their bacterial or fungal difference (weighted and unweighted Unifrac values). Each individual is colored according to its colony of origin, alates are indicated with circles and workers with squares. (b) Pairwise distance matrices between each pair of alates from the same or different colonies. Each pair is colored according to its microbial similarity (*i.e.,* bacterial and fungal similarity from weighted and unweighted Unifrac) obtained from Euclidean distances between the two individuals on the two first PCs of the PCoA. Darker values indicate low microbial differentiation between a pair of individuals (*i.e.,* close on the PCoA analysis). (c) Violin plots of bacterial and fungal differentiation (weighted and unweighted Unifrac) among individuals within and between colonies. Box plots represent median and 1st and 3rd quartile; whiskers include 95% of all observations; dots indicate individual values.

Figure S5: Survival Kaplan-Meier curves for each pairing during the overall length of the long-term survival experiment (450 days). Incipient colonies were monitored every two days. Hazard ratio for cox-proportional hazard model is reported in front of each pairing.

Figure S6: Correlation between hazard ratios of each pairing during the short-term experiment (14 days) and those of the long-term experiment (450 days). Hazard ratios of inbred pairings are highlighted in color.

Figure S7: Number of eggs, workers and soldiers present within inbred and outbred incipient colonies each month for 450 days (15 months) after pairing.

Figure S8: Microbial load (mean number of colony forming units, CFUs) for each pairing investigated.

Table S1: Linear and logarithmic correlations between the susceptibility of a pairing (*i.e.,* hazard ratio) and (*i*) the cumulative microbial load and (*ii*) the maximum microbial load of the constituent colonies. AIC model selection was used to assess whether linear or logarithmic regression better explains the data in both analyses.

Supporting Information S1: DNA extraction and PCR protocols for termite DNA, as well as bacterial and fungal DNA.

Supporting Information S2: Potential presence of internal parasites, not counted in the microbial load.

FIGURE CAPTIONS

Figure 1 : Kaplan-Meier survival distributions of inbred and outbred incipient colonies during the first 14 days after pairing (a) and along the overall length of the experiment (450 days; b).

Figure 2 : (a) Colony of origin of the 202 surviving alates 14 days after colony establishment (inner circle). For each colony of origin, pie charts represent the distribution of surviving inbred and outbred pairings; outbred pairings are divided and light-colored according to the colony of origin of the partner, inbred pairings are represented by bright colors. (b) Radar plot represents the hazard ratio of each inbred and outbred pairings in the first 14 days after colony establishment. Outbred pairings are marked with a circle, while outbred pairings are represented with a square

Figure 3 : Correlation between hazard ratio of a pairing and the maximum pathogen load (a), cumulative pathogen load (b), relatedness (c), unweighted Unifrac bacterial difference (d), weighted Unifrac bacterial difference (e), unweighted Unifrac fungal difference (f) and weighted Unifrac fungal difference (g). Trendlines represent logarithmic correlations for plots a and b, and denote linear correlations for all the other plots. In each plot, inbred pairings are colored according to their colony of origin.

Figure 4 : (a) Principal Coordinate Analyses (PCoA) of individuals based on their unweighted Unifrac values for bacterial similarity and weighted Unifrac values for fungal similarity. Each individual is colored according to its colony of origin, alates are indicated with circles and workers with squares. (b) Violin plots of bacterial (unweighted Unifrac) and fungal differentiation (weighted Unifrac) among individuals within and between colonies. Box plots represent median and 1st and 3rd quartile; whiskers include 95% of all observations; dots indicate individual values. Results for weighted Unifrac bacterial similarity and unweighted Unifrac fungal similarity are provided in Supplementary Figure S4.

Figure 5 : (a) Graphical representation of the productivity of incipient colonies over the overall duration of the experiment (450 days, 15 months). Productivity is measured as the number of workers (outer circle), soldiers (middle circle) and eggs (inner circle) for each pairing. Productivity of inbred pairings is reported on the upper half-circle, while the productivity of outbred pairings is reported on the bottom half-circle. Box plots represent median and 1st and 3rd quartile; whiskers include 95% of all observations; individual dots indicate outlier values. P values indicate significant effect of the type of pairing on the number of workers and soldiers in a colony over time, with an increased production in inbred colonies (see also Supplementary Figure S6). (b) Kaplan-Meier survival distributions of offspring from inbred and outbred colonies when challenged toward entomopathogens. (c) Violin plot of microbial loads (mean number of CFU) of offspring from inbred and outbred colonies. Box plots represent median and 1st and 3rd quartile; whiskers include 95% of all observations; dots indicate individual values.

Figure 6 : Schematic illustration of the inbreeding depression termite colonies face over the different stages of their lifespan. The black line represents the level of inbreeding depression. Inbreeding depression is low during colony foundation and offspring production, but is higher during colony development, when small colonies face pathogen pressure (this study; DeHeer & Vargo, 2006). The dotted lines represent colony size (*i.e.,* number of workers per colony). The red curve represents the efficiency of social immunity, which increases with colony size until it is expected to slightly decrease due to inbreeding from neotenic reproduction. The high efficiency of social immunity in large mature colonies releases inbreeding depression, allowing the development of inbred neotenic reproductives without suffering costs associated with pathogen pressure (Aguero et al. 2021).

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