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ORIGINAL ARTICLE

Multiple mating by both sexes in an invasive insect species, *Aethina tumida* (Coleoptera: Nitidulidae)

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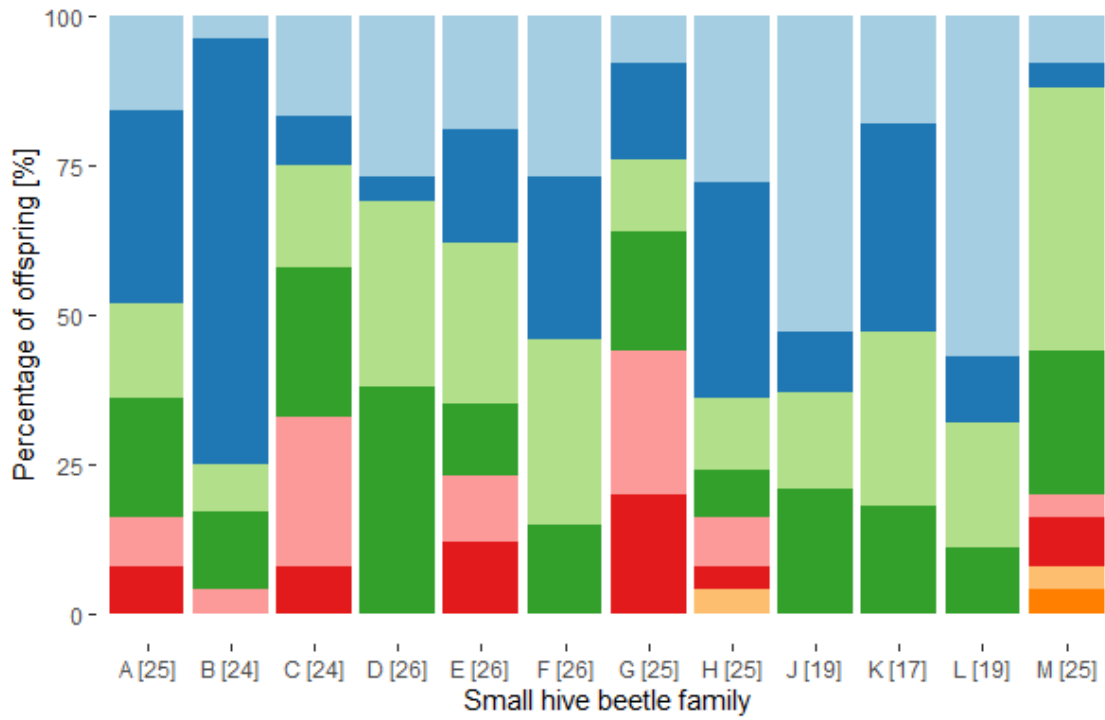
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Abstract

Multiple mating by both sexes is common among sexually reproducing animals. Small hive beetles (SHB), *Aethina tumida*, are parasites of bee nests endemic to sub-Saharan Africa and have become a widespread invasive species. Despite the considerable economic damages they can cause, their basic biology remains poorly understood. Here we show that male and female small hive beetles can mate multiple times, suggesting that costs for mating are low in this species. In an invasive *A. tumida* population in the USA, a combination of laboratory experiments for males and paternity analysis with eight polymorphic DNA microsatellite markers for field-caught females were used to estimate the number of mating by both sexes. The data show that females and males can mate multiple times – females mated with up to eight males, whereas males mated with at least seven females. The results also showed that *A. tumida* displayed a skewed paternity, although this was not consistent among the tested females. Thus, first or last male advantage seem to be unlikely in *A. tumida*. Our observations that individuals of both sexes of *A. tumida* can mate multiple times opens new research avenues for examining drivers of multiple mating and determining the role it may play in promoting biological invasions.

Graphical Abstract



Key words evolution of sex; invasive species; multiple partners; polygynandry; reproductive biology

Introduction

Sexual reproduction is the most prevalent form of reproduction in animals, but mating numbers considerably vary both between and within species (Shuster, 2009; Kokko *et al.*, 2014). Several types of mating systems can be defined based on the number of mates that males or females have within a defined period of time (e.g., breeding period or over their lifetime; Emlen & Oring, 1977; Krasnec *et al.*, 2012; Kokko *et al.*, 2014). Species that form pairs and mate with one partner only are monogamous, while species that mate with multiple partners are polygamous (Davies, 1991; Krasnec *et al.*, 2012). Furthermore, polygamy can be divided into polygyny, whereby a male mates and forms a bond with multiple females, and polyandry, whereby a female mates with multiple males. However, if both males and females mate multiple times and do not form any bonds, mating systems are then considered promiscuous. There are number of costs associated with multiple matings, such as increased risk of injury and predation, as well as disease transmission (Chapman *et al.*, 1995; Westendorp & Kirkwood, 1998; Arnqvist & Nilsson, 2000; Crudgington & Siva-Jothy, 2000; Kawagoe *et al.*, 2001). Nevertheless, multiple matings are a widespread phenomenon in insects (Ridely, 1988), and have been observed in several invasive species such as ladybugs, *Harmonia axyridis* (Coleoptera: Coccinellinae), grain beetles, *Tenebrio molitor* (Coleoptera: Tenebrionidae), red flour beetles, *Tribolium castaneum* (Coleoptera: Tenebrionidae) (Worden & Parker, 2001; Awad *et al.*, 2015; Rafter *et al.*, 2017). For instance, multiple matings by females may facilitate invasions by protecting against the effects of inbreeding depression (Cornell & Tregenza, 2007) or by reducing costs of mating with incompatible or infertile males (Watson, 1991). Thus, females can benefit from multiple mating (Kokko & Jennions, 2008). For males, it is generally considered that fitness increases with each additional mating (Bateman, 1948), and is hence adaptive (Trivers, 1972).

Small hive beetles (SHB), *Aethina tumida*, are long known parasites of western honey bee, *Apis mellifera*, colonies that are native to sub-Saharan Africa (Lundie, 1940; Schmolke, 1974; Hepburn & Radloff, 1998). In 1996, SHB became invasive. Since then, it has established itself nearly everywhere *A. mellifera* occurs (Neumann & Elzen, 2004; Neumann *et al.*, 2016; Al Toufailia *et al.*, 2017; Lee *et al.*, 2017; Muli *et al.*, 2018; Idrissou *et al.*, 2019; Liu *et al.*, 2020). In addition to the considerable damage SHB can cause to apiculture in the invasive ranges (Delaplane, 1998), research revealed the generalist nature of SHB. Indeed, SHB can infest nests of other social and solitary bee species (bumblebees: Ambrose *et al.*, 2000; Spiewok & Neumann, 2006; reviewed by Neumann *et al.*, 2016; solitary bees: Gonthier *et al.*, 2019; stingless bees: Nacko *et al.*, 2020; Pereira *et al.*, 2021). They can also feed on flowers (Gonthier *et al.*, 2019) and can successfully reproduce on fruits and other foods, including rotten meat (Buchholz *et al.*, 2008). Within its native range, SHB are usually considered to be a minor pest (Pirk & Yusuf, 2015) and display low levels of reproduction with only few larvae being present (Idrissou, *et al.*, 2019). Destructive mass reproduction with thousands of larvae resulting in the full structural collapse of the entire host nest (Hepburn & Radloff, 1998) can occur in Africa, but it is rare (Neumann, 2017). However, in its invasive ranges mass reproduction events are more frequent and even strong colonies of *A. mellifera* can quickly collapse (Neumann *et al.*, 2010).

Despite its known relevance for the beekeeping industry and possible impact on wild bees, very little is known about the reproductive biology and mating system of SHB. Given that SHB are known to be long-lived (>six months, Lundie, 1940; 16 months in laboratories, Somerville, 2003), they are more likely to have more chances to mate multiple times (Wiklund *et al.*, 2003; Taylor *et al.*, 2014). Moreover, the consistently female-biased operational sex ratio of SHB across several populations (Ellis *et al.*, 2002; Spiewok & Neumann, 2012; Papach *et al.*, 2019) suggests multiple mating by males. However, no study has yet confirmed that multiple matings occur. The only study

investigating SHB reproductive biology so far suggests that SHB becomes sexually mature at about two weeks of age, and reports that both male and female adult SHB were frequently observed mating (Mustafa *et al.*, 2015). However, mating does not necessarily imply successful fertilisation. For example, 15%–55% of red flour beetles, *Tribolium castaneum*, copulations do not result in fertilisation (Bloch Qazi *et al.*, 1996). Consequently, even though multiple matings were observed in SHB (Mustafa *et al.*, 2015), this does not necessarily imply that multiple paternities can be found (Coltman *et al.*, 1999). In fact, only egg laying by females after mating can be used as a reliable indicator of successful mating (Lewis, 2004; Tyler & Tregenza, 2012) when assessing the number of matings by males. Furthermore, DNA paternity analyses provide accurate estimates of the actual number of successful mating by females. In addition, mating with multiple partners may lead to sperm competition or cryptic female choice (Wedell *et al.*, 2002; Firman *et al.*, 2017) that may result in uneven offspring distribution among sires (skewed paternity; Boomsma & Sundström, 1998). Even though cryptic female choice has been suggested (Mustafa *et al.*, 2015), it is not clear if multiple and skewed paternities occur in SHB.

Here, we studied the mating system of SHB by combining experimental laboratory assays for males and DNA parentage analyses for field-sampled females. We predicted that both male and female SHB successfully mate multiple times, as suggested by preliminary observations (Mustafa *et al.*, 2015). To test this prediction, we estimated the number of females that started egg laying after exposure to a single male to assess multiple mating by males. Since multiple mating by females does not necessarily imply that multiple paternities can be found (Coltman *et al.*, 1999), we genotyped the offspring of field-sampled females and estimated the number of males contributing to the offspring of each female.

Materials and methods

Beetle rearing

All experiments were conducted at Auburn University, AL, USA in 2018 and 2019, between May to July. Adult SHB were sampled from three apiaries located within a 40 km radius from Auburn University with naturally-infested local honey bee colonies using an aspirator. Then, all collected individuals were sexed and used to initiate a standard laboratory rearing (Neumann *et al.*, 2013) as described below for each experiment.

Experiment 1: Estimation of mating numbers in females

Randomly chosen, field-collected adult SHB females ($N = 12$) were used to start the 12 families used in this study. In brief, they were individually placed in 473 mL mason jars with punctured lids, provided with honey-pollen paste (2 : 1) *ad libitum*, oviposition sites (two microscope slides separated with a half of a cover slip on each end and taped together), and then incubated at 25°C and 80% relative humidity (RH) (Neumann *et al.*, 2013). After two to three days, the SHB eggs hatched and the jars were checked daily to ensure that emerging larvae were fed *ad libitum* until they had reached the post-feeding larval stage (Lundie, 1940). Post-feeding larvae ($N = 25\text{--}30$ per mother) were transferred with a fine paintbrush into 12 mason jars (473 mL, one jar per offspring from one mother), filled 75% with autoclaved sandy soil (commercially available all-purpose sand; American Countryside) that was ~10% moisture by mass (Neumann *et al.*, 2013). All jars were incubated at 25°C and 80% RH until adult emergence (Neumann *et al.*, 2013), upon which all individuals were collected, freeze-killed at -20°C , then stored in 75% EtOH so that the 12 SHB

families (field-collected female + offspring) could be shipped to the Institute of Bee Health, University of Bern, Switzerland, for genotyping.

Genotyping and parentage analyses

Total DNA of the field-collected females and their offspring was extracted separately from each whole individual using the NucleoSpin® Tissue kit (Macherey-Nagel). At first, SHB were placed in 2 mL tubes with 75 μ L of PBS buffer and a metal bead, and homogenized for 1 min. Then, 50 μ L of the homogenized sample were transferred for lysis into a new tube with 180 μ L of buffer T1 and 25 μ L of proteinase K (22 mg/mL) and incubated overnight at 56°C. The extractions proceeded following the manufacturer's instructions. Finally, total DNA was obtained in 100 μ L of elution buffer. The DNA quality was estimated for each sample using a QuickDrop™ spectrophotometer (Molecular Devices, San Jose, California, USA).

To estimate the number of mates each field-collected female mated with, 19 DNA microsatellite markers were designed based on the SHB genome (Evans *et al.*, 2018) using the software Primer3 (Koressaar & Remm, 2007; Untergasser *et al.*, 2012) and tested for polymorphism in the study population together with ten previously published markers (Evans *et al.*, 2008). Overall, seven of the new markers and a single marker of the published ones (Table 1) proved to be polymorphic locally, resulting in eight markers for paternity testing.

All SHB ($N = 312$) were genotyped at these eight microsatellite markers. PCRs were run using a 2720 Thermal cycler (Applied Biosystems®) with Qiagen Multiplex PCR kits (Qiagen) and 2 μ L of DNA (with average concentration of 30ng/ μ L). PCR amplifications were performed as follows: denaturation step at 95°C for 15 min; 25 cycles of 94°C for 30 s and 60°C for 30 s and 72°C for 30 s; and final extension

step of 72°C for 5 min and kept at 4°C until use. PCR products were run on a Hitachi 3730 DNA Analyzer (Applied Biosystems®) sequencer. The genotypes were scored manually using Peak Scanner v 1.0 (Applied Biosystems®). Individuals possessing unique alleles were genotyped twice ($N = 4$). Individuals with information at less than 6 markers after the second run of genotyping were removed from the dataset ($N = 19$), resulting in 293 individuals remaining for the analyses.

Two approaches were used to infer the number of fathers siring the offspring of each field-collected female. First, paternity was assigned following a conservative approach using Mendelian rules of inheritance with direct allele counting (Simmons *et al.*, 2008). To do so, the scored genotypes of mothers and offspring were compared to infer the minimum number of males siring the offspring of a given female. The maternal alleles were retracted from the offspring genotypes and the diversity of the remaining alleles was assessed, thereby allowing for the quantification of the number of distinct male alleles. As genotypes of the sires were unknown, we used a conservative approach assuming that all males were heterozygous. Based on this assumption, the minimum number of males was estimated as half the number of non-maternal alleles at each locus. However, since this method only provides the minimum number of fathers, which is most likely an underestimation, the number of fathers was also estimated by using software COLONY v 2.0.6.6 (Jones & Wang, 2010) that estimates numbers of putative sires based on a maximum-likelihood approach commonly used in paternity studies (Awad *et al.*, 2015; Bórquez & Brante, 2017). The following settings were chosen for the COLONY runs: mating system with both male and female polygamy; inbreeding without clone; diploid dioecious species; medium length run with full-likelihood analysis method; no sib ship prior.

Experiment 2: Estimation of mating numbers in males

Five field-collected SHB males and five SHB females were placed in a plastic container ($\emptyset = 15$ cm; $h=30$ cm) provided with a honey bee brood frame and used to establish a laboratory rearing following standard protocols (Neumann *et al.*, 2013). Emerging larvae were fed *ad libitum* until they reached the post-feeding stage (Lundie, 1940). An individual pupation approach was used to ensure virginity of emerging SHB adults (Papach *et al.*, 2019), due to possible subterranean mating prior to emergence from the soil (P. Neumann personal observations). Using a fine paint brush, single post-feeding larvae were transferred into one standard Eppendorf tube (1.5 ml) each, which were filled with autoclaved sandy soil (75%) that had ~10% moisture by mass (Neumann *et al.*, 2013). Then, the tubes were incubated at 25°C and 80% RH until adult emergence (Neumann *et al.*, 2013). All emerging beetles were sexed and individually placed in standard Eppendorf reaction tubes (1.5 mL) with punctured lids to avoid suffocation (Neumann *et al.*, 2013). The tubes with SHB were then kept at 25°C, 80% RH, and constant darkness in an incubator and provided with water and food (honey-pollen paste (2 : 1) *ad libitum* (Neumann *et al.*, 2013) until becoming sexually mature and active after 10-18 days (Hood, 2004; Mustafa *et al.*, 2015). These sexually mature virgin males ($N = 10$) were then each placed with 14 random virgin females in a petri dish, provided with honey-pollen paste (2 : 1) and kept at 25°C and 80% RH in constant darkness for 72 hours. Afterwards, all females ($N = 140$) were individually placed in specimen cups (100 mL), provided *ad libitum* with honey-pollen paste (2 : 1) and oviposition sites (see above; Neumann *et al.*, 2013). Cups were checked daily for the presence of eggs, and the number of females that had started oviposition was recorded for each male to estimate the polygyny levels of SHB.

Statistical analyses

For each locus, observed and expected heterozygosity, number of alleles, and conformity to Hardy-Weinberg equilibrium, were calculated with GenAIX v 6.5 (Peakall & Smouse, 2006, 2012). Additionally, linkage disequilibrium between each pair of loci was performed with GenePop v 4.7 web version (Raymond & Rousset, 1995; Rousset, 2008) to test for marker independence. To investigate whether the contribution of fathers was different from equal, paternity skew values were calculated (Neff *et al.*, 2008). This index was calculated for each SHB family by first computing the effective number of males siring the offspring of each female (m_e):

$m_e = \frac{1}{\sum (\frac{rs_i}{total\ offspring\ genotyped})^2}$, where rs_i is the number of offspring sired by the i^{th} sire. Then, the

skew (s_{skew}) was calculated as: $s_{skew} = 1 - \frac{m_e}{actual\ number\ of\ genetic\ sires}$.

Paternity skew values can range from 0 to 1, with 0 corresponding to no skew among sires (equal contribution) and 1 implying a complete skew (all offspring are sired by one father).

Paternity skew data, number of sires contributing to the offspring, and number of females starting oviposition were visually checked for normality with Q-Q plots and with a Shapiro-Wilcox test. A one-sample *t*-test was performed to assess if the paternity skew differed from the null hypothesis of equal contributions between all sires (theoretical value of equal contribution was 0). All calculations were performed using R v 3.5.1 (R Core Team, Vienna, Austria).

Results

Experiment 1: Estimation of mating numbers in females

Out of 312 SHB samples used for the polyandry assay, a total of 293 (*i.e.*, 93.91%) successfully amplified at six or more loci after two rounds of PCRs and genotyping (209 individuals with data at eight markers, 66 individuals at seven markers and 18 at six markers). Overall, the level of polymorphism was two to six alleles per locus across all samples (Table 2).

Observed and expected heterozygosities across markers varied between 0.149–0.498 and 0.138–0.753, respectively (Table 2). Accordingly, six out of eight loci showed significant deviations from Hardy Weinberg equilibrium (Table 2). With the level of marker polymorphism and allelic frequencies, the overall estimated non-detection error was 0.5%. The majority of loci used in this study were at linkage disequilibrium (Supplementary material). For 10 out of 12 families, offspring were found to be sired by at least two fathers (Table 3) because at least three non-maternal alleles were observed at one or more loci. Furthermore, the relative distribution of the alleles could unambiguously be assigned to two distinct heterozygous sires. In addition, based on the COLONY estimates, the number of fathers across families were not normally distributed ($W = 0.849$, P -value = 0.04) and the median number of fathers siring the offspring in each family was 5.5 (IQR = 2, minimum = 4, maximum = 8, showing that 100% of females were multiple mated; Table 3, Fig. 2). The paternity skew values were normally distributed across families ($W = 0.9$, P -value = 0.16) and the mean paternity skew was 0.26 (SD = 0.18, minimum = 0.05, maximum = 0.62). This later parameter was significantly different from 0 ($t = 4.7845$, $df = 11$, P -value = 0.0006), thereby suggesting unequal sire contribution.

Experiment 2: Estimation of mating numbers in males

None of the virgin females (N=140) started oviposition prior to the experiments (18 days). Thirty-six females died during the experiment and three females did not oviposit after 2 months (Table 4). Each male successfully inseminated at least seven females, thereby showing that multiple mating occurred in 100% of observed cases. The number of ovipositing females across males was normally distributed ($W = 0.93$, P -value = 0.45), and the mean number of females starting oviposition was 10.10 (SD = 2.08, minimum = 7, maximum = 13).

Discussion

Despite the almost global spread and known impact of SHB, multiple mating in both sexes of this invasive species has never been clearly documented before. This study provides clear evidence that both female and male SHB commonly mate with multiple partners. The data further showed that the analysed SHB females mated with up to eight males and that paternity was skewed in some cases towards one of them. Furthermore, the studied male SHBs successfully mated with at least seven females.

Using DNA microsatellite markers, our results demonstrate for the first time multiple paternity in SHB. All markers displayed low number of alleles, and six out of eight loci indicated a low ratio of observed/expected heterozygosity, thereby significantly deviating from Hardy-Weinberg equilibrium (HWE). Moreover, most markers displayed a significant linkage disequilibrium (LD). These observations are a probable consequence of genetic bottlenecks associated with invasion (Freeland *et al.*, 2011). However, the methods used here to assess the levels of polyandry do not rely on HWE or LD assumptions, and these results therefore did not hinder our ability to detect multiple mating in the samples used. Another factor that can lead to deviations from HWE is the presence of null alleles

(Freeland *et al.*, 2011), which we could not test for due to high inbreeding levels in the population (Chakraborty *et al.*, 1992; Dakin & Avise, 2004). However, this possible bias would not have significantly impacted our estimates and conclusions; if null alleles were present in our dataset, the estimated levels of multiple mating would increase, not decrease. In addition, it is likely that many homozygous males carrying the same alleles as the females were siring the offspring, thereby remaining undifferentiated. Therefore, using the COLONY software allowed us to consider those factors mentioned above. Additionally, it complemented the conservative findings of multiple paternity obtained with estimations derived from Mendelian rules of inheritance, and suggested that each of the studied females mated with at least four, and up to eight, different males. The experimental design allowed estimating the natural mating frequency of female SHBs because all females had naturally mated in the field and only the offspring were reared in the laboratory. Even though the estimates of SHB male mating were obtained under laboratory conditions, the data nevertheless enable to estimate the maximum numbers under the given conditions (*i.e.*, no competition with other males).

Our data showed that SHB males can mate multiple times and can successfully inseminate up to 13 females within 72 hours. Since none of the virgin females started oviposition, these results show that mating is required, and further indicate that parthenogenesis is unlikely at least in this population. It is possible that multiple matings may explain why males are as long-lived as females in this species (> six months, Lundie, 1940; 16 months in the laboratory, Somerville, 2003). It further suggests that males might be able to replenish their sperm, as was documented in stinkbugs, *Podisus nigrispinus* (Rodrigues *et al.*, 2008) or polyandrous butterflies, *Pieris rapae* (Bissoondath & Wiklund, 1996).

Due to the known costs associated with mating (Fowler & Partridge, 1989; Chapman *et al.*, 1995; Hurst *et al.*, 1995), multiple matings by both SHB sexes suggests that the costs associated with SHB mating must be lower than the benefits. The abundance of mates in close spatial proximity in host nests, taken together with SHB clustering in specific host colonies in both native and invasive ranges (Neumann & Elzen, 2004; Spiewok *et al.*, 2008), indicate that costs associated with mate search may be generally low in this species. Furthermore, any increased risk of predation due to multiple matings (Rowe, 1994) seems highly unlikely for adult SHB in the sheltered and well-defended nests of any social host bees. Likewise, thanks to a range of defence behaviours (reviewed by Neumann & Elzen 2004; Neumann *et al.* 2016), the risk of injury due to attacks by host workers is generally low in this parasitic beetle (<1%, Neumann *et al.*, 2015). Another cost that is frequently associated with multiple mating is the spread of sexually transmitting diseases, which are often highly pathogenic in insects (Knell & Webberley, 2004). Due to the common enemy release in invasive species (Heger & Jeschke, 2014), it is possible that sexually transmitted diseases may be even less likely to occur in invasive SHB populations, thereby further reducing the costs of multiple mating. Furthermore, SHB may benefit from the efficient social immunity of their host bees (Simone-Finstrom, 2017), resulting in generally fewer pathogens in their environment (Simone *et al.*, 2009). However, even though mites, fungi, protists, bacteria and viruses have all been reported to be associated with SHB (Muerrle *et al.*, 2006; Eyer *et al.*, 2009a, 2009b; Schäfer *et al.*, 2010; Cilia *et al.*, 2018; Huwiler *et al.*, 2020; Nanetti *et al.*, 2021), there are no data available yet for any of those pathogens to be sexually transmitted or result in clinical symptoms post-mating. Moreover, those pathogens known to be transmitted via sex in *A. mellifera* (Deformed wing virus (Yañez *et al.*, 2012); *Nosema* spp. (Roberts *et al.*, 2015)) also occur in SHB in the invasive range (Eyer *et al.*, 2009; Cilia *et al.*, 2018).

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As in other species (Boomsma & Sundström, 1998; Awad *et al.*, 2015), the observed uneven SHB paternity might constitute one of the costs of mating for males, but there are no data yet on sperm competition and whether males can adjust the amount of sperm transferred, such as based on mating status or quality of a female or a number of rival males present (Wedell *et al.*, 2002; Joyce *et al.*, 2009; Kelly & Jennions, 2011). SHB may also be able to partition their sperm transfer as shown for *Drosophila pachea* males, based on the number of rival males in this fruit fly species with a female-biased sex ratio (Pitnick, 1993; Wedell *et al.*, 2002). Alternatively, uneven paternity distribution might also be a sign of a possible cryptic female choice, whereby females favour sperm of a male with particular traits. It is possible that it might be governed by sperm competition (Wedell *et al.*, 2002; Firman *et al.*, 2017), as well as possible first or last male advantage (Dziuk, 1996; Lewis *et al.*, 2005). However, there was no consistent skew among the tested females, thereby suggesting that first or last male advantage seem to be unlikely in *A. tumida*.

While it is generally accepted that multiple mating is advantageous for males (Bateman, 1948; Trivers, 1972), benefits that are gained by females are usually hard to explain. It may be that SHB females mate multiple times to ensure their spermatheca is filled as predicted by the sperm limitation hypothesis (Kraus *et al.*, 2004), which appears adaptive in light of mass reproduction with many offspring (Neumann & Elzen, 2004). However, our results clearly show multiple mating by a single male is sufficient for up to 13 females to start oviposition. Moreover, SHB females die quickly when ovipositing on a daily basis (Neumann *et al.*, 2016), thereby suggesting no need for sperm replenishment at an older age. Hence, sperm limitation seems rather unlikely to explain the observed multiple mating by female SHB. Nevertheless, since suitable time windows for successful SHB reproduction in host colonies are both unpredictable (“sit-and-wait” strategy; Neumann *et al.*, 2016) and rather short (*e.g.* only a few days after host bees have abandoned their nests; Neumann

et al., 2018), it seems adaptive for SHB females, regardless of the spermathecal filling, to start oviposition whenever food is available and their offspring are unlikely to die (*e.g.*, due to egg removal by host workers, (Neumann & Härtel, 2004)). Therefore, mating for more sperm cannot be safely excluded in SHB at this stage.

Since SHB is an invasive species with almost global distribution (Neumann *et al.*, 2016; Al Toufailia *et al.*, 2017; Lee *et al.*, 2017; Muli *et al.*, 2018; Idrissou, Huang, *et al.*, 2019; Liu *et al.*, 2020;) multiple mating by both sexes might constitute a preadaptation to foster invasion success. As it is generally accepted that populations of invasive species are often faced with inbreeding (Saccheri *et al.*, 1998) and genetic bottlenecks (Sakai *et al.*, 2001), Therefore, species with multiple mating may have a key advantage over monogamous species in colonizing new environments (Brown & Burdon, 1987). For example, female crickets, *Gryllus bimaculatus*, that mate with multiple partners, including both siblings and non-related males, have higher offspring viability compared to monandrous females mating with siblings only (Tregenza & Wedell, 2002). In addition, individuals with multiple mating often exhibit higher fecundity, egg viability and adult emergence compared to those mating with one partner only (*e.g.* *Coccinellidae* spp.; Srivastava & Omkar, 2005). For invasive species, higher fecundity and egg viability may be especially important when colonizing novel environments. This may also be the case for SHB because low level reproduction with few larvae seems to be common in its native range (Idrissou *et al.*, 2019; for rare exceptions see Neumann 2017), while in the invasive range mass reproduction with often thousands of larvae can more often be observed (Neumann & Elzen, 2004).

In conclusion, we here provide the first evidence of multiple mating by both sexes in small hive beetles, *A. tumida*. These results should be considered for sustainable management of this invasive pest (*e.g.*, the previously proposed sterile insect technique is almost certainly not a suitable strategy;

Neumann *et al.*, 2016). Further studies should be performed to better understand the drivers of multiple mating in *A. tumida* and what role polygyny and polyandry may play in promoting its invasion success.

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Disclosure

The authors declare no conflicts of interest.

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Supporting Information

Appendix 1. Linkage disequilibrium between tested loci used for the paternity tests in small hive beetle, *Aethina tumida*. Pairs of loci tested, p-values and standard errors are shown.

Table 1 DNA microsatellite markers for small hive beetle, *Aethina tumida*, used in this study. Marker names, primer names, forward and reverse primer sequences, sizes of the most common allele (bp) are shown. Annealing T° of all markers was 60 C. With the exception of B89 (Evans et al., 2008), all of the markers were designed in this study.

Marker	Primer name	Sequence 5'–3'	Size (bp)
Atu-4	Atu4-AGT30-F	TTGGTGAATGCTGATATGGTG	349
	Atu4-AGT30-R	TCCCGACTGTGAGGTTTCTT	
Atu-8	Atu8- AAC9-F	ATGCCAATGCAACAACGTAA	296
	Atu8-AAC9-R	TCCTGAACAGTTTCCTGACCT	
Atu-14	Atu14-CA17-F	CTTACACATTTGGGCCATT	217
	Atu14-CA17-R	CCCTTCTCTCAATAGATTGCTTT	
Atu-15a	Atu15a- AAAT6-F	TTTGTCCGACGCAATAAAAA	130
	Atu15a-AAAT6-R1	ATCGTCGACGTCCATTTCA	
Atu-16a	Atu16a-ACG8-F	CGCCGTTAACACGTAACACA	118
	Atu16a-ACG8-R1	AGCGACCGCAGTTACTGAAT	
Atu-17	Atu17-GCT8-F	CATGATGGTTCTGTGAGCAAC	168
	Atu17-GCT8-R	TGGCCACTATAACAGCCAAT	
Atu-18a	Atu18a-CT10-F	AATACGCATGTTTGCCACTTC	191
	Atu18a-CT10-R1	GGGTTTGCCATCTGAAAAT	
B89	AtumB89-F	CGTCGCTATTAGCCCTTTA	110

	AtumB89-R	CTGACCTTCTCGTGCAACAA	
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Table 2 Population genetic analysis of small hive beetle, *Aethina tumida*, offspring from field captured females for eight microsatellite loci. The loci names, number of alleles (Na), observed and expected heterozygosity (Ho and He respectively), df, chi-square values and P-values for Hardy-Weinberg equilibrium (HWE P-value, P-value<0.05 significant) are shown

Locus	Na	Ho	He	df	Chi-square value	HWE P-value
Atu-4	3	0.265	0.390	3	29.405	< 0.005
Atu-8	2	0.312	0.415	1	17.886	< 0.005
Atu-14	3	0.348	0.564	3	82.017	< 0.005
Atu-15a	3	0.404	0.500	3	8.991	0.029
Atu-16a	2	0.149	0.138	1	1.743	0.187
Atu-17	5	0.408	0.577	6	70.414	< 0.005
Atu-18a	6	0.498	0.753	15	468.947	< 0.005
B89	3	0.304	0.286	3	1.327	0.723

Table 3 Paternity of small hive beetle, *Aethina tumida*, in 12 families inferred from allele counts and the program COLONY. Family ID, number of genotyped offspring (N), number of loci, non-sampling error estimates (NSE), highest number of non-maternal alleles at one of the loci (N non-maternal alleles), number of sires using allele count, number of sires using COLONY and paternity skew in each family are shown.

Family	N	Loci	NSE	N non-maternal alleles	Paternity		Paternity skew
					N allele counts	N COLONY	
1	25	8	0.13	3	2	6	0.19
2	24	8	0.76	4	2	5	0.62
3	24	8	0.06	4	2	6	0.14
4	26	8	0.61	4	2	4	0.21
5	26	8	0.08	3	2	6	0.11
6	26	8	0.00	3	2	4	0.05
7	25	8	0.05	4	2	6	0.09
8	25	8	0.90	3	2	7	0.40
9	19	7	0.16	2	1	4	0.30
10	17	6	0.04	4	2	4	0.09
11	19	7	0.25	3	2	4	0.38
12	25	7	1.63	2	1	8	0.54

Table 4 Multiple mating by small hive beetle, *Aethina tumida*, males (N = 10) each placed with 14 random sexually mature virgin females in a petri dish for 72 hours. The male ID, the number of females that started oviposition, that died, and that did not oviposit after two months, are shown.

Male ID	Number of females		
	Ovipositing	Dead	No oviposition
1	9	4	1
2	13	1	0
3	11	3	0
4	13	1	0
5	8	6	0
6	10	4	0
7	11	3	0
8	7	7	0
9	11	1	2
10	8	6	0
Total	101	36	3

Figure 1



Figure 2

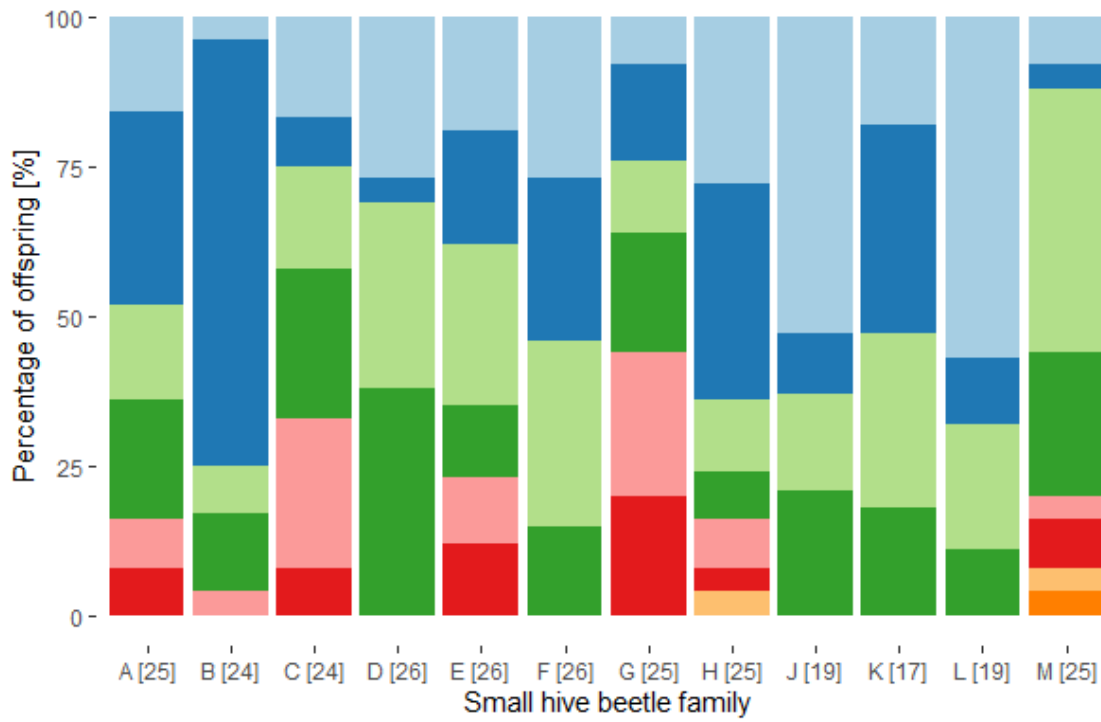


Figure 3

