



'Late' male sperm precedence in polyandrous wool-carder bees and the evolution of male resource defence in Hymenoptera



Kathrin P. Lampert, Vanessa Pasternak, Philipp Brand, Ralph Tollrian, Florian Leese, Thomas Eltz*

Department of Animal Ecology, Evolution and Biodiversity, Ruhr-Universität Bochum, Bochum, Germany

ARTICLE INFO

Article history:

Received 2 October 2013
Initial acceptance 4 November 2013
Final acceptance 9 January 2014
Published online
MS. number: 13-00824R

Keywords:

Anthidium manicatum
interspecific territoriality
last male sperm precedence
microsatellites
multiple mating
paternity
polyandry
polygyny
promiscuity
resource defence

The mating system of European wool-carder bees, *Anthidium manicatum*, differs from that of most bees in three important aspects: females (1) are polyandrous and (2) mate continuously over the course of their reproductive life, while males (3) exhibit resource defence polygyny, that is, defend patches of food plants where copulations occur. To shed light on the evolution of this mating system we investigated male paternity using a combination of cage experiments and microsatellite genotyping of brood. We found that, although females possess a spermatheca for long-term sperm storage, most brood was fathered by males that had very recently mated with the breeding female, indicating pronounced last (or at least 'late') male sperm precedence. In the absence of males (male exclusion experiment) a large proportion of eggs remained unfertilized (resulting in haploid male offspring), but some diploid daughters arose from fathers that had been removed at least 11 days prior to egg laying. It appears that most *A. manicatum* eggs are fertilized with sperm from the bursa copulatrix, while the spermatheca serves only as a backup reservoir. This is the first demonstration of last male sperm precedence in aculeate Hymenoptera (bees, wasps, ants). We suggest that it has coevolved with 'resource defence' or 'patrolling'-like male mating strategies in Hymenoptera, and with polyandry in anthidiine bees.

© 2014 The Association for the Study of Animal Behaviour. Published by Elsevier Ltd. All rights reserved.

Mating behaviours and mating systems of bees have received considerable attention from evolutionary biologists owing to their diversity and potential for comparative analysis (Paxton, 2005; Thornhill & Alcock, 1983), but also because of their relevance to the evolution of sociality (Brown & Schmid-Hempel, 2003; Crozier & Fjerdingstad, 2001; Strassmann, 2001). In the majority of bee species, both solitary and social, the females are sexually receptive only at the beginning of adult life, mate only once with a single male, and store sperm from this copulation for use throughout their reproductive life. Such single mating (monandry) in females appears to be the ancestral state in aculeate Hymenoptera, whereas polyandry is a derived exception (Hughes, Oldroyd, Beekman, & Ratnieks, 2008; Strassmann, 2001). Eusocial honeybees (*Apis* spp.) are a particularly notable and well-studied exception (Franck et al., 2002; Haberl & Tautz, 1998; Oldroyd et al., 1996; Schlüns, Moritz, Neumann, Kryger, & Koeniger, 2005). A less prominent exception are bees within the Anthidiini, of which few species have been

studied in detail, but those that have all appear to be polyandrous (Alcock, Eickwort, & Eickwort, 1977). Among them, the European wool-carder bee, *Anthidium manicatum*, was unintentionally introduced to the Americas and New Zealand in the late 1960s and is now the most widely distributed unmanaged bee species of the world (Strange, Koch, Gonzalez, Nemelka, & Griswold, 2011). *Anthidium manicatum* represents an extreme case of polyandry that is combined with male territoriality and floral resource defence (Severinghaus, Kurtak, & Eickwort, 1981). Females forage on pollen and nectar mostly from Lamiaceae and Fabaceae, which often grow in aggregated patches. Males establish territories centred on such patches, fend off conspecific males and other flower visitors, and mate with females that forage within the territory (Severinghaus et al., 1981; Wirtz, Kopka, & Schmoll, 1992; Wirtz, Szabados, Pethig, & Plant, 1988). Copulations are an extremely common sight at a given territory and repeated copulations of the same individual regularly occur within a few minutes in both sexes (Severinghaus et al., 1981). The number of female visits and copulations that a male obtains was found to be positively correlated with territory quality, that is, the number of flowers in the territory, and with male size (Severinghaus et al., 1981). Males that cannot defend a territory, often the smaller males, adopt an alternative

* Correspondence: T. Eltz, Department of Animal Ecology, Evolution and Biodiversity, Ruhr-Universität Bochum, Universitätsstraße 150, 44780 Bochum, Germany.
E-mail address: thomas.eltz@rub.de (T. Eltz).

'sneaking' tactic, but receive fewer copulation opportunities than territory owners (Severinghaus et al., 1981).

The territorial mating behaviour of *A. manicatum*, and that of the similar *Anthidium maculosum* (Alcock et al., 1977), is probably derived from behaviours where mate-seeking males patrol at floral resources, which is relatively common in bees, especially in species with nonaggregated nesting sites (Paxton, 2005). In fact, male territoriality at flower patches can be considered a 'localized' case of patrolling which happens when floral resources are sufficiently aggregated to allow monopolization and defence by individual males.

Floral resource defence by males may benefit females because males fend off heterospecific flower visitors such as honeybees and bumblebees, which would otherwise compete for pollen and nectar (Wirtz et al., 1988). If females prefer to forage in defended sites, and the costs of additional matings are low, then male resource defence and polyandry might coevolve. However, for males the benefits from resource defence will depend strongly on patterns of sperm use by females. Female anthidiine bees, as is typical for solitary bees, lay their eggs more or less continuously throughout the breeding season, ovipositing briefly before closing each consecutive brood cell (see e.g. Westrich, 1989; this study). If only sperm from the first copulation(s) is used by a female to fertilize eggs throughout the season, then a 'sit-and-wait' strategy such as resource defence is unlikely to be favoured by selection over more 'pre-emptive' male strategies such as patrolling at nest sites. If, however, delayed mating has a high probability of siring offspring, then resource defence is more beneficial. Clearly, establishing patterns of sperm use is important for understanding the evolution of male resource defence.

The term 'last male sperm precedence' (henceforth LMSP) usually refers to experimental situations in which two males are allowed to mate in sequence and the second mate sires more than 50% of the offspring. It has been found in many species and groups of insects, and might be caused by a range of mechanisms including stratification of sperm from different males in the sperm-storing organ of the female (last in, first out), sperm digestion or removal by the female, or sperm removal by the second male (Simmons, 2001). In bees, the contribution of sequential mates to paternity has only been studied in honeybees, *Apis mellifera*, with mostly negative results, that is, sperm from sequential copulations appears to be mixed in the queen spermatheca to an extent that no particular position in the male sequence is favoured (Franck et al., 2002; Schlüns, Koeniger, Koeniger, & Moritz, 2004). Generally, in many insect taxa with LMSP, the extent of bias in favour of the last copulating male is reduced with increasing numbers of matings per female and with increasing time between the last and a preceding copulation (Simmons, 2001).

In the present study, we examined paternity dynamics in polyandrous wool-carder bees, *A. manicatum*, using a combination of flight cage mating experiments and genotyping of brood, for which we developed novel microsatellite markers. We tested the hypothesis that 'late' copulations lead to an above-average chance of siring 'late' eggs compared to the alternative hypotheses of first male precedence or random sperm mixing (the latter being the null hypothesis).

METHODS

Development of Microsatellite Markers

A genomic library of *A. manicatum* enriched for microsatellites was created using the reporter genome protocol (Nolte, Stemshorn, & Tautz, 2005) as described in Leese, Mayer, and Held (2008). For this, pooled DNA from four females of *A. manicatum* was hybridized

on a chip (Hybond N+, GE Healthcare) with DNA from *Mus musculus*. As a modification to Leese et al. (2008), 0.03 U/μl Hotmaster Taq (5Prime) were used in PCR reactions. Also, nick repair and PCR were carried out in one reaction tube by incubating for 10 min at 70 °C prior to PCR (94 °C for 2 min followed by 25 cycles of 15 s at 94 °C, 30 s at 52 °C, 60 s at 65 °C and 15 min final elongation at 65 °C). For elution, hybridization chips were transferred into 500 μl TE buffer (pH 8.0, 80 °C) for 10 min. DNA was precipitated using a standard isopropanol–sodium acetate protocol. The enriched fragments were PCR amplified in 50 μl reactions and cloned into pGEM-T easy (Promega) vectors and transformed into competent JM109 *E. coli* (Promega).

Plasmid preparation of 48 colonies and shotgun sequencing using a standard M13-forward primer was conducted by GATC-Biotech (Konstanz, Germany). Analysis of electropherograms, vector clipping, assembly of contigs, redundancy filtering and primer design were performed with the software Geneious 5.6 (Drummond, Ashton, Buxton, & Cooper, 2011). Microsatellites in the final contigs were searched using the search tool Phobos version 3.12 (Mayer, 2011). Primers were designed for six loci (see Table 1) using Primer3 (Rozen & Skaletsky, 2000). To enable fluorescent marking for fragment size analyses an M13 tail was added to either the forward or reverse primer of each primer pair (Table 1). The decision was based on a NetPrimer (Premier Biosoft, Palo Alto, CA, U.S.A.) analysis.

Flight Cage Experiments

Mesh-covered flight cages of 4 × 2 × 2 m were installed in the Botanical Garden of the Ruhr-Universität Bochum in the summers of 2011 and 2012 (Fig. 1). The cages contained two longitudinal rows of freely planted *Betonica officinalis* (for pollen and nectar), potted plants of *Stachys byzanthina* (for plant wool, which females use to construct brood cells), potted plants of *Pelargonium* sp. and *Crepis capillaris* (for trichome secretions, which females apply on collected plant wool; Müller, Töpfl, & Amiet, 1996), and clusters of split bamboo internodes (length 9–27 cm, inner diameter 1.2–2.2 cm) fixed to wooden poles (see also Payne, Schildroth, & Starks 2011). Wild female and male *A. manicatum* were captured at their food plants in the Botanical Gardens of Bochum and Düsseldorf, marked individually with dots of acrylic paint on the mesosoma, and introduced into these cages. They usually habituated quickly to the new situation, with males engaging in territorial behaviour around the food plants, and females collecting plant wool, trichome secretions, nectar and pollen to provision brood cells, which were constructed as linear nests in bamboo internodes (Fig. 1). Copulations were frequently observed when females entered male territories. Three experiments were conducted.

Wild versus caged male paternity experiment

This was a preliminary experiment conducted from 24 May to 12 July 2011 to see whether bees would breed in the cages but also to examine whether offspring of caged females were sired by (genotypically known) caged males or by (genotypically unknown) wild males to which females had mated before their captivity (spermathecae of wild-caught dissected females ($N = 4$) were all filled with sperm). A total of four caged males were present during the experiment, two during the first half and two during the second half. Six females were introduced consecutively between 24 May and 28 June, with two or three females actively provisioning brood cells at any given time. Individuals that were exchanged intermittently or removed at the end of the experiment were killed by freezing and fixed in abs. EtOH (Sigma Aldrich). At the end of the experiment bamboo internodes with nests were transferred to the laboratory for later freezing and fixation of larvae (13 nests with a

Table 1
Details of the six polymorphic microsatellite loci developed for *Anthidium manicatum* (Anm) from Germany

Locus	Repeat motif	Primer sequence (5'–3')	Size range (bp)	N_1 (♀/♂)	N_A	H_o	H_e	Gen Bank Accession no.
Anm01	(CA) ₁₄	F: TCAAACCGTTGAGCCGA R: *CGACGGGCAAGAAGGGACCG	250–280	48 (22/26)	11	0.86364	0.81818	KF998175
Anm02	(GT) ₁₈	F: GCCATCGTCATTCCCTGCGGA R: *CGGTTGAACCACCGTAACAGACCG	176–252	41 (23/18)	18	0.91304	0.91014	KF998176
Anm03	(GA) ₃₅	F: TGAACCGTGCCCAAACGCCA R: *CCCCGATCACCTGTTAGGCTCA	218–242	45 (22/23)	11	0.63636	0.78858	KF998177
Anm04	(AAG) ₁₇	F: AGCTACTCCCGGACAGCGGA R: *ACGGTTCACCCCTCGCCTT	188–212	43 (24/19)	11	0.91304	0.8657	KF998178
Anm05	(AG) ₁₇	F: *TGCTCGGTACCAGGGCTTCCT R: CCCCGTACACGTCGTTGATCCC	192–190	50 (26/24)	12	0.84615	0.83861	KF998179
Anm06	(GT) ₂₀	F: ACGTGCCCGTGACAAACGA R: *AGGGTAGACGCACGTGTGGAC	261–309	40 (23/17)	15	0.86957	0.91884	KF998180

For each locus the repeat motif, fragment sizes (bp) and number of alleles (N_A) are given. N_1 (♀/♂) is the total number of individuals and the numbers of females and males that could be genotyped. The size range (bp), N_1 and N_A are based only on adult individuals. H_o and H_e are the expected and observed heterozygosities, based only on adult diploid females. No significant deviations from HWE were detected. The primer sequences that include an M13 tail (5'-CACGACGTTGTAACACGAC-3') are marked with an asterisk.



Figure 1. Breeding *Anthidium manicatum* in the Botanical Garden of the Ruhr-University Bochum. (a) Flight cage with food plants and bamboo trap nests, (b) copulating pair, (c) female drinking nectar and collecting pollen from *Betonica officinalis* (note pollen-filled hairy scopa at metasoma), (d) female scraping off plant-wool from *Stachys byzanthina*, (e) female provisioning brood cell in bamboo internode, and (f) completed nest in bamboo internode with five brood cells and nest plug made from substrate particles.

total of 53 larvae; individual females built one to three discrete nests).

Paternity dynamics experiment

This is the main experiment of which two replicates were conducted in parallel in separate cages (A and B), from 31 May to 3 August 2012. The set-up was similar to the previous experiment, but males were exchanged at intervals of 4 days whenever this was possible. Effectively, bad weather periods led to slightly longer intervals (see Fig. 2). Normally, two of a total of 15 males were active in each cage at any given time. In each cage four females provisioned brood cells, and bamboo internodes were opened once every day to keep track of brood cell construction. This brood cell monitoring allowed us to establish the temporal dynamics of paternity over the entire experiment. At the end of the experiment bamboo internodes with nests were transferred to the laboratory for later freezing and fixation of larvae (32 nests with a total of 148 larvae; individual females built two to seven discrete nests).

Male exclusion experiment

For this experiment, conducted from 6 June to 15 August 2012, four females and two males were first introduced into the cage. On 7 July, we removed the males from the cage and left the females to

breed without access to mating partners for the rest of the experiment. No individual observations were made. By the time the males were removed three nests with two, two and three brood cells had already been provisioned. At the end of the experiment 23 additional brood cells had been provisioned in the absence of males. All were transferred to the laboratory for microsatellite analysis.

DNA Extraction, Microsatellite Genotyping and Paternity Assignment

DNA was extracted from adults (legs of females and males present in the cages) and offspring produced during the experiments (larval tissue) using the Qiagen blood and tissue kit (Qiagen, Hilden, Germany). Extractions were performed according to the manufacturer's instruction with the following adaptations: initial incubation at 56 °C was done overnight and the final elution was done two times with 75 µl TE buffer each. PCRs of the six microsatellite loci were performed in a total volume of 20 µl, containing 1× buffer, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.24 mM of the untailed as well as fluorescently labelled primer, 0.05 mM of the primer with the M13 tail, 1 µl dimethyl sulphoxide (DMSO) and 0.25 U Taq polymerase (EuroTaq – BioCat, Heidelberg, Germany).

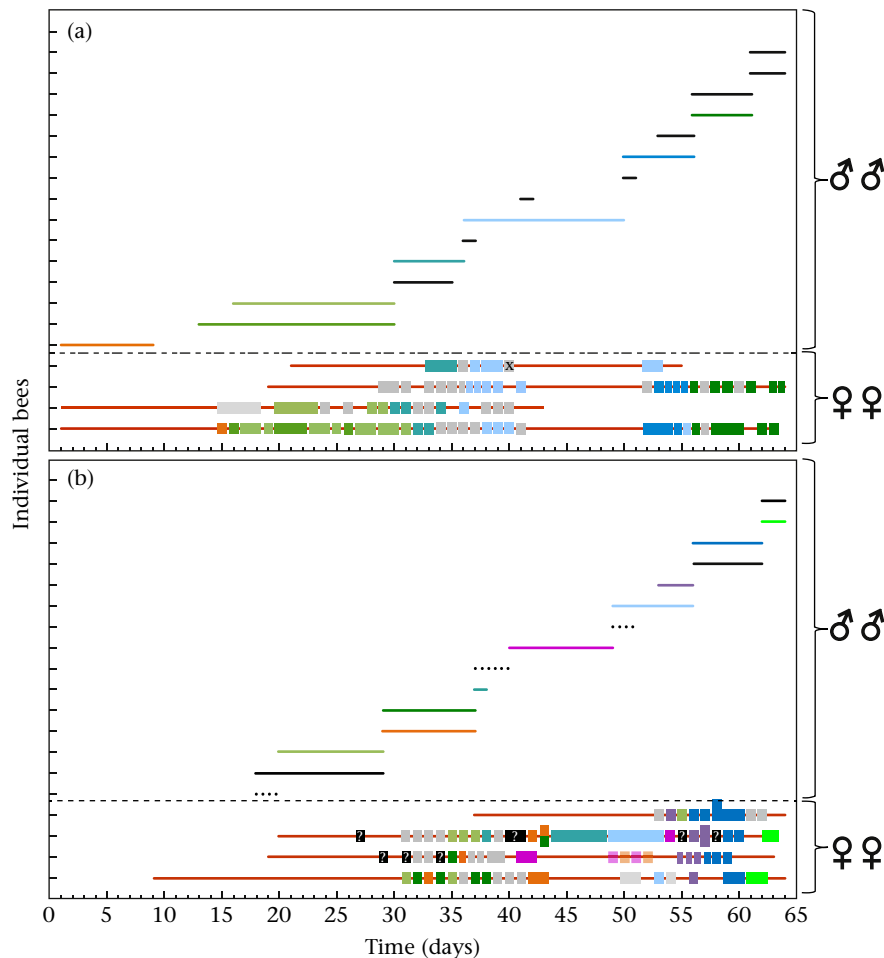


Figure 2. Individual activity spans of adults and paternity assignment of brood of *Anthidium manicatum* during the paternity dynamics experiment carried out from 31 May to 2 August 2012. (a) Cage A; (b) cage B. Note period of cold and rainy weather between days 42 and 51. Breeding females are represented by red lines, males by various colours. Rectangles superimposed on female lines represent active provisioning times of individual brood cells of the respective female. Colours correspond to the father inferred from microsatellite genotypes in the case of diploid daughters. Haploid male offspring are grey; the larva marked with an x was not genotyped. Males represented by solid black lines were genotyped but did not sire any offspring; those represented by dashed black lines were lost during the experiment and not available for genotyping, but may have fathered some or all of the not assignable brood marked with '?' in cage B.

The amplification program started with an initial denaturation step at 95 °C for 5 min followed by 40 cycles including 30 s at 95 °C, 30 s at 55 °C annealing and 30 s at 72 °C. A final elongation step was done at 72 °C for 5 min. Fragment size was determined on a Licor 4300 DNA Analyzer using the implemented software Saga2 (Licor Biosciences, Lincoln, NE, U.S.A.).

Statistical Procedures

As individuals came from two different field sites, the Botanical Gardens of Düsseldorf and Bochum which are about 60 km apart, we first tested for population differentiation using Arlequin version 3.5.1.3 (Excoffier & Lischer, 2010). Only adult females ($N_{\text{total}} = 26$) were used for this analysis and 10 000 permutation steps were used to evaluate the statistical significance of the result. We found no significant genetic differentiation between sites ($F_{\text{ST}} = 0.0015$, $P = 0.40$). Consequently, for further analyses the data were treated as a single population. For each locus we tested for an independent inheritance of all loci (linkage disequilibrium, LD) and calculated levels of heterozygosity as well as deviations from Hardy–Weinberg equilibrium (HWE) also using Arlequin version 3.5.1.3, for females exclusively. Parentage of brood in cage experiments was assessed assuming unlinked Mendelian inheritance at the six microsatellite loci (as confirmed by LD analysis). We used the program COLONY (Jones & Wang, 2010) to calculate the probability that males present in the cage were the fathers of the diploid bee offspring. For the COLONY analysis we used the default analysis settings with a haploid–diploid sex determination and assumed male and female polygamy. To estimate the probability that an unknown male with the identical genotype might have sired the offspring, we calculated the multilocus probability of identity (P_{ID}) for each male's genotype by multiplying the specific allele frequencies over all loci. Only bees (adults and larvae) that could be genotyped at at least five of the six loci were included in the analyses.

To test the hypothesis of last male sperm precedence in the paternity dynamics experiment we calculated for each individual larva the expected probability (P_{exp}) of being fathered by a male present at the time of brood cell construction under the assumption of complete sperm mixing (the null model):

$$P_{\text{exp}} = N_{\text{pres}} / (N_{\text{pres}} + N_{\text{prev}}),$$

where N_{pres} is the number of males present at the time of brood cell construction and N_{prev} is the number of males that were present earlier in the cage life of the mother. To avoid bias we excluded from the analysis all males that had not left any offspring at all, that is, may not have copulated during their time in the cage (see Fig. 2). We then averaged P_{exp} values across the larvae of each individual female and tested it against P_{obs} , the observed proportion of each female's brood fathered by males present at the time of brood cell construction, using a paired t test. P_{obs} was scored in two ways. In the first analysis we simply excluded the seven unassigned female offspring in cage B. For an alternative analysis we assumed that they were derived from sperm storage, that is, not from males present at the time of brood cell construction (to reduce as much as possible the likelihood of a type 1 error).

RESULTS

Molecular Analyses

DNA extraction was successful for all adults and larvae. Six of the nine loci that were selected for screening could be amplified very reliably and showed high levels of polymorphism (11–18 alleles;

Table 1). There was no linkage disequilibrium. Levels of heterozygosity were very high and no significant deviations from HWE were detected.

Mating Experiments

Wild versus caged male paternity experiment

Twenty-eight brood cells were parasitized by chalcidoid wasps, *Melittobia* sp., so that only 25 *Anthidium* larvae were available for genotyping. Among these, 13 larvae were monomorphic at all six loci, that is, haploid (hemizygous) males derived from unfertilized eggs. The remaining 12 larvae were dimorphic at two to six (average 4.4) loci, that is, diploid females derived from fertilized eggs (sex ratio 0.92:1). The 12 daughters were derived from four different mothers and could all be assigned to one of three of the four potential fathers present in the cage (probability always = 1). The likelihood that a daughter was sired by an unknown father with identical alleles prior to the experiment was very low for all cases (P_{ID} between 1.9×10^{-5} and 6.7×10^{-7} ; mean + SD: $5.4 \times 10^{-6} + 9.1 \times 10^{-6}$).

Paternity dynamics experiment

Early sampling of larvae (directly after the experiment) prevented chalcidoid wasp-induced mortality in 2012, so that we had 73 (cage A) and 75 (cage B) larvae available for genotyping. Of these, 24 (cage A) and 19 (cage B) were monomorphic at all loci, that is, haploid (hemizygous) males derived from unfertilized eggs. The remaining 105 larvae were dimorphic at two to six (average 4.8) loci, that is, diploid females derived from fertilized eggs. Thus, the sex ratio of brood was 2.4:1, strongly biased in favour of daughters. Figure 2 shows paternity of daughters as a function of the time of brood cell provisioning for each of the eight breeding females, which had produced between six and 29 offspring. In cage A all daughters could be assigned with high probability ($P = 1.0$) to the experimental males and the probability that an unknown male could have sired the offspring was very low ($P_{\text{ID}} = 1.5 \times 10^{-5} + 2.7 \times 10^{-5}$; mean + SD; range 8.5×10^{-5} – 3.2×10^{-8}). In most cases (41 of 49, or 84%) the father was a male that was present in the cage at the time when the respective brood cell was being provisioned. In eight cases a daughter was sired by a male that had been removed 1–6 days earlier, and in one of these cases the mother had intermittently fertilized an egg with the sperm of another male present at the same time. In cage B three males were lost during the experiment and were not available for genotyping. Thus we could not assign the paternity of seven larvae, and the overall COLONY assignment probability was lower ($P = 0.89$). However, as in cage A, the majority of female brood in cage B (33 of 56, or 59%) were fathered by males that were present at the time of brood cell construction. In 16 cases a daughter was sired by a male that had been removed 1–18 days earlier (see Fig. 2). We tested the hypothesis of last male sperm precedence as outlined in the Methods (Statistical Procedures). The observed probability of an offspring being derived from a male present in the cage at the time of brood cell construction ($P_{\text{obs}} = 0.77 + 0.16$) was statistically different from and more than twice as high as the likelihood expected from random sperm mixing ($P_{\text{exp}} = 0.31 + 0.08$) across all females ($N = 8$, $P < 0.01$). This analysis had excluded the seven unassigned offspring in cage B. However, the result remained essentially unaltered when we included these offspring assuming that they were derived from sperm storage, that is, not from males present at the time of brood cell construction ($P_{\text{obs}} = 0.74 + 0.19$; $P_{\text{exp}} = 0.33 + 0.08$; $P < 0.01$).

Individual females produced daughters derived from 2 to 10 different males (mean of 5.0 ± 2.8). The fertilization success of males was unevenly distributed. In cage A seven of 15 males sired

1–13 offspring, while in cage B 10 of 15 males sired 1–12 offspring. Often, and in both cages, paternity was biased in favour of one of the two males present at a given point in time. The successful males tended to be the territorially dominant ones (data not shown). They chased subordinate males away from flowers, where copulations occurred.

Male exclusion experiment

Of the 23 larvae from brood cells that were provisioned in the absence of males (later than 7 July), 19 were monomorphic at all six loci, that is, haploid (hemizygous) males derived from unfertilized eggs. Four were dimorphic at four to six (average 4.75) loci, that is, diploid females derived from fertilized eggs. Thus, the sex ratio was 1:4.75, strongly in favour of males. Unfortunately, in this experiment we could not assign paternity of the remaining four female brood because the samples of adult males were lost. However, all four daughters were in brood cells that had been provisioned later than 18 July, that is, more than 11 days after the males had been removed. Thus, it is clear that sperm had been stored by the mothers for at least 11 days, possibly much longer.

DISCUSSION

Our experiments clearly demonstrate that males that copulate late in a sequence of mating partners have an above average chance of siring the about-to-be laid egg of a female *A. manicatum*. Evidently, sperm from sequential mates do not mix within the female on a timescale that would promote a 'fair raffle' in the sense of Parker, Simmons, and Kirk (1990). Last male sperm precedence, while being common in other groups of insects (Simmons, 2001), has to our knowledge not been demonstrated in any other aculeate hymenopteran (bees, ants, wasps), or in fact in any Hymenoptera at all (Franck et al., 2002; Khanh, Bressac, & Chevrier, 2005; see Table 2.3 in Simmons 2001). This may largely be explained by the relative scarcity of polyandry and the resulting scarcity of sperm precedence studies in solitary Hymenoptera. Sperm precedence has been studied in detail in the tiny parasitic wasp *Dahlbominus fuscipennis* (Wilkes, 1965, 1966), which may serve as a model for comparisons. As in *A. manicatum* (T. Eltz, personal observation) and other bees (Martins & Serrao, 2002), the spermatheca of female *D. fuscipennis* is a spheroid chitinous structure with a fixed volume. It is connected to the bursa copulatrix via a narrow duct that allows entry and exit of spermatozoa. When *Dahlbominus* females were subjected to three sequential matings, fewer ejaculated sperm were found to be able to enter the spermatheca with each successive copulation (30% and 17% of sperm added by the second and the third copulation in relation to the number added by the first), and paternity of female progeny approximated the numerical representation of sperm in the spermatheca (Wilkes, 1966). The average contribution of the second male (P2) was 0.32 (Wilkes, 1965, 1966). These studies suggest that sperm used for fertilization by female *D. fuscipennis* does indeed derive from the spermatheca, where sperm from males mixes to a substantial degree. Such a scenario is highly unlikely for *A. manicatum*, where previous mating partners leave much less genetic trace among female offspring than expected by chance. Two mechanisms may contribute to the observed 'late' male sperm precedence: (1) sperm from previous copulations is actively removed from the spermatheca either by the female or by the last-copulating male, or (2) sperm is stratified in the female sexual organs so that only 'late' entering sperm has a chance of fertilizing an egg. There is no clear evidence for the first mechanism (active sperm displacement) in any Hymenoptera, but clearly this mechanism cannot be ruled out given its surprising intricacies in other groups of insects (Simmons, 2001; Waage, 1979). However, the second mechanism (stratification) seems more parsimonious

for *A. manicatum* given the rapid succession of copulations observed. In fact, the timescale over which copulations occur suggests that fertilizing sperm may not even have entered the spermathecal duct but was recruited from the bursa copulatrix directly. It can be safely assumed that spermatozoa are able to survive in the bursa copulatrix for at least some minutes, and thus sperm from the last copulation will be viably retained here to fertilize any passing egg on its way to the ovipositor. The bursa copulatrix could also be the arena for active sperm expulsion or replacement (by the female or by a successive male), a possibility that we plan to investigate in future experiments. In any case, we hypothesize that the spermatheca in *A. manicatum* is mostly a backup device that stores sperm for when no males are available as mating partners in the short term. In accordance with this scenario, our experiments also demonstrated the long-term retention and occasional use of stored sperm from males that have copulated more than 11 days prior to egg laying. Applying microsatellite genotyping to spermathecal sperm samples would provide insight into whether sperm mixing occurs in the hypothetical 'backup spermatheca'.

Irrespective of the mechanism that creates it, 'late' male sperm precedence may have promoted the evolution of male resource defence in *A. manicatum*, and possibly other resource-defending Hymenoptera. Male resource defence is clearly beneficial to female *A. manicatum* visiting the nectar- and pollen-enriched flower patches (Wirtz et al., 1988), but it will be more beneficial for males if the temporal dynamics of female receptivity promote a 'sit-and-wait' mating strategy in males. In contrast, the active search (patrolling) for females at nesting substrates or food plants should be more adaptive in first come–first served situations. Thus, a certain degree of LMSP could be a prerequisite for male resource defence to evolve, and LMSP may become more pronounced over evolutionary time in such systems. LMSP was also assumed by Thornhill and Alcock (1983, p. 249), in their discussion of *Anthidium* male mating strategies. Other resource-defending species of Hymenoptera, for example the native American *A. maculosum* (Alcock et al., 1977) as well as other anthidiine bees with similar behaviour, are also predicted to show late male sperm precedence, but no data are available.

LMSP appears to be present in the resource-defending pollen wasp *Ceramius fonscolombeii* (Groddeck, Mauss, & Reinhold, 2004), in which matings can be observed rather frequently at patches of food plants and at sites where females collect water for brood cell construction. Both types of sites are patrolled and defended by males, but males defending water collection sites mate 2.5 times more frequently than males that defend floral resource sites (Groddeck et al., 2004). This bias is related to the timing of oviposition in the species, which takes place immediately after collection of water to excavate and stabilize the brood cell but prior to food provisioning of the cell. Thus, assuming LMSP, copulations at water collection sites are perfectly timed to fertilize an about-to-be-laid egg in *C. fonscolombeii* (Groddeck et al., 2004). These observations indirectly argue for the existence of LMSP in at least one other species of resource-defending aculeate Hymenoptera.

LSMP was also considered likely in the male-dimorphic *Perdita portalis* (Danforth & Desjardins, 1999). Here, one 'small-headed' male morph is winged and defends female forage plants flowering in the desert, where encounters and copulations are frequently observed. The other 'large-headed' morph is flightless, never leaves the communal nests, and exclusively mates with females within the nest. In *Perdita*, eggs are laid at the end of the provisioning phase, that is, when a pollen ball has been formed by the female and briefly before the cell is closed. Matings of the large-headed morph are precisely timed at this moment, that is, they occur immediately before egg laying. At no other time were matings observed although encounters between males and females in the nest

occurred regularly. The large-headed morph is derived within this group of andrenid bees (Danforth & Desjardins, 1999), and its well-timed mating behaviour strongly argues for the existence of LMSP in *P. portalis*.

Other observational/circumstantial evidence could probably be accumulated for LMSP in aculeate Hymenoptera, but it has only been directly demonstrated in *A. manicatum* (this study). It remains to be investigated whether LMSP is a derived character in *Anthidium*, or whether the proximate mechanism(s) that create it are plesiomorphic in Megachilidae or possibly all aculeate Hymenoptera. In this context it may be worth exploring the role of LMSP in the evolution not only of male resource defence, but also of polyandry (multiple mating), which is often but not always coupled with male resource defence (Paxton, 2005). In any case, we conclude that data on sperm precedence are dearly missing in nonsocial aculeate Hymenoptera (bees and wasps), and that the question of LMSP needs to be addressed in studies on their mating systems.

Acknowledgments

We thank Manfred Reimann and the staff of the Botanical Garden of the Ruhr-University Bochum for providing space, plants, expertise and logistic help with setting up the flight cage experiments. Without their support the study would not have been possible! Thanks also to Jennifer Küttner for help with rearing the bees. We are grateful to Volker Mauss, Andreas Müller and Jim Cane for discussions on anthidiine bees and mating systems, and to Volker Mauss and the Evolutionary Ecology Journal club members for comments on the manuscript. Many thanks also to two anonymous referees for helpful comments that improved the manuscript. This study was supported by the Ruhr-University Bochum.

References

- Alcock, J., Eickwort, G. C., & Eickwort, K. R. (1977). Reproductive behavior of *Anthidium maculosum* (Hymenoptera: Megachilidae) and the evolutionary significance of multiple copulations by females. *Behavioral Ecology and Sociobiology*, 2(4), 385–396.
- Brown, M. J. F., & Schmid-Hempel, P. (2003). The evolution of female multiple mating in social Hymenoptera. *Evolution*, 57(9), 2067–2081.
- Crozier, R. H., & Fjerdingstad, E. J. (2001). Polyandry in social Hymenoptera—disunity in diversity? *Annales Zoologici Fennici*, 38(3–4), 267–285.
- Danforth, B. N., & Desjardins, C. A. (1999). Male dimorphism in *Perdita portalis* (Hymenoptera, Andrenidae) has arisen from preexisting allometric patterns. *Insectes Sociaux*, 46(1), 18–28.
- Drummond, A., Ashton, B., Buxton, S., & Cooper, A. (2011). *Geneious v5.XX*. Retrieved from <http://www.geneious.com>.
- Excoffier, L., & Lischer, H. E. L. (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, 10(3), 564–567.
- Franck, P., Solignac, M., Vautrin, D., Cornuet, J. M., Koeniger, G., & Koeniger, N. (2002). Sperm competition and last-male precedence in the honeybee. *Animal Behaviour*, 64(3), 503–509.
- Groddeck, J., Mauss, V., & Reinhold, K. (2004). The resource-based mating system of the Mediterranean pollen wasp *Ceramius fonscolombeii* Latreille 1810 (Hymenoptera, Vespidae, Masarinae). *Journal of Insect Behavior*, 17(3), 397–418.
- Haberl, M., & Tautz, D. (1998). Sperm usage in honey bees. *Behavioral Ecology and Sociobiology*, 42(4), 247–255.
- Hughes, W. O. H., Oldroyd, B. P., Beekman, M., & Ratnieks, F. L. W. (2008). Ancestral monogamy shows kin selection is key to the evolution of eusociality. *Science*, 320(5880), 1213–1216.
- Jones, O. R., & Wang, J. L. (2010). COLONY: a program for parentage and sibship inference from multilocus genotype data. *Molecular Ecology Resources*, 10(3), 551–555.
- Khanh, H. D. T., Bressac, C., & Chevrier, C. (2005). Male sperm donation consequences in single and double matings in *Anisopteromalus calandrae*. *Physiological Entomology*, 30(1), 29–35.
- Leese, F., Mayer, C., & Held, C. (2008). Isolation of micro satellites from unknown genomes using known genomes as enrichment templates. *Limnology and Oceanography: Methods*, 6, 412–426.
- Martins, G. F., & Serrao, J. E. (2002). A comparative study of the spermatheca in bees (Hymenoptera: Apoidea). *Sociobiology*, 40(3), 711–720.
- Mayer, C. (2011). *Phobos Version 3.X. A tandem repeat search program*. Retrieved from http://www.rub.de/spezoo/cm/cm_phobos.htm.
- Müller, A., Töpfl, W., & Amiet, F. (1996). Collection of extrafloral trichome secretions for nest wool impregnation in the solitary bee *Anthidium manicatum*. *Naturwissenschaften*, 83(5), 230–232.
- Nolte, A. W., Stemshorn, K. C., & Tautz, D. (2005). Direct cloning of microsatellite loci from *Cottus gobio* through a simplified enrichment procedure. *Molecular Ecology Notes*, 5, 628–636.
- Oldroyd, B. P., Smolenski, A. J., Cornuet, J. M., Wongsiri, S., Estoup, A., Rinderer, T. E., et al. (1996). Levels of polyandry and intracolony genetic relationships in *Apis dorsata* (Hymenoptera: Apidae). *Annals of the Entomological Society of America*, 89(2), 276–283.
- Parker, G. A., Simmons, L. W., & Kirk, H. (1990). Analyzing sperm competition data: simple models for predicting mechanisms. *Behavioral Ecology and Sociobiology*, 27(1), 55–65.
- Paxton, R. J. (2005). Male mating behaviour and mating systems of bees: an overview. *Apidologie*, 36(2), 145–156.
- Payne, A., Schildroth, D. A., & Starks, P. T. (2011). Nest site selection in the European wool-carder bee, *Anthidium manicatum*, with methods for an emerging model species. *Apidologie*, 42(2), 181–191.
- Rozen, S., & Skaletsky, H. J. (2000). Primer3 on the WWW for general users and for biologist programmers. In S. Krawetz, & S. Misener (Eds.), *Bioinformatics methods and protocols: Methods in molecular biology* (pp. 365–386). Totowa, NJ: Humana Press.
- Schlüns, H., Koeniger, G., Koeniger, N., & Moritz, R. F. A. (2004). Sperm utilization pattern in the honeybee (*Apis mellifera*). *Behavioral Ecology and Sociobiology*, 56(5), 458–463.
- Schlüns, H., Moritz, R. F. A., Neumann, P., Kryger, P., & Koeniger, G. (2005). Multiple nuptial flights, sperm transfer and the evolution of extreme polyandry in honeybee queens. *Animal Behaviour*, 70(1), 125–131.
- Severinghaus, L. L., Kurtak, B. H., & Eickwort, G. C. (1981). The reproductive behavior of *Anthidium manicatum* (Hymenoptera, Megachilidae) and the significance of size for territorial males. *Behavioral Ecology and Sociobiology*, 9(1), 51–58.
- Simmons, L. W. (2001). *Sperm competition and its evolutionary consequences in the insects*. Princeton, NJ: Princeton University Press.
- Strange, J. P., Koch, J. B., Gonzalez, V. H., Nemelka, L., & Griswold, T. (2011). Global invasion by *Anthidium manicatum* (Linnaeus) (Hymenoptera: Megachilidae): Assessing potential distribution in North America and beyond. *Biological Invasions*, 13(9), 2115–2133.
- Strassmann, J. (2001). The rarity of multiple mating by females in the social Hymenoptera. *Insectes Sociaux*, 48(1), 1–13.
- Thornhill, R., & Alcock, J. (1983). *The evolution of insect mating systems*. Cambridge, MA: Harvard University Press.
- Waage, J. K. (1979). Dual function of the damselfly penis: Sperm removal and transfer. *Science*, 203(4383), 916–918.
- Westrich, P. (1989). *Die Wildbienen Baden-Württembergs: Allgemeiner Teil*. Stuttgart, Germany: Eugen Ulmer GmbH & Co.
- Wilkes, A. (1965). Sperm transfer and utilization by arrhenotokous wasp *Dahlbominus fuscipennis* (Zett) (Hymenoptera—Eulophidae). *Canadian Entomologist*, 97(6), 647–657.
- Wilkes, A. (1966). Sperm utilization following multiple insemination in wasp *Dahlbominus fuscipennis*. *Canadian Journal of Genetics and Cytology*, 8(3), 451–461.
- Wirtz, P., Kopka, S., & Schmoll, G. (1992). Phenology of two territorial solitary bees, *Anthidium manicatum* and *A. florentinum* (Hymenoptera, Megachilidae). *Journal of Zoology*, 228(4), 641–651.
- Wirtz, P., Szabados, M., Pethig, H., & Plant, J. (1988). An extreme case of interspecific territoriality: Male *Anthidium manicatum* (Hymenoptera, Megachilidae) wound and kill intruders. *Ethology*, 78(2), 159–167.