Ecological Entomology

Ecological Entomology (2010), **35**, 175–182 DOI: 10.1111/j.1365-2311.2009.01167.x

Diploid male production in a leaf-cutting ant

SOPHIE ARMITAGE,[1*] JACOBUS BOOMSMA[1] and BORIS BAER[1†] [1]Department of Biology, Centre for Social Evolution, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen, Denmark

Abstract. 1. In haplodiploid social insects where males are haploid and females are diploid, inbreeding depression is expressed as the production of diploid males when homozygosity at the sex-determining locus results in the production of diploid individuals with a male phenotype. Diploid males are often assumed to have reduced fitness compared with their haploid brothers.

2. While studying the reproductive biology of a leaf-cutting ant, *Atta sexdens*, in Gamboa, Republic of Panama, we detected the presence of a larger male morph. Using microsatellite markers we were able to confirm that the large male morph was diploid in 87% of cases.

3. We infer that the Gamboa population of *A. sexdens* experiences inbreeding depression because diploid males were found in three out of five mature colonies. However, their frequencies were relatively low because queens were multiply mated and our estimates suggest that many diploid male larvae may not survive to adulthood.

4. We measured two traits potentially linked to male reproductive success: sperm length and sperm number, and showed that diploid males produced fewer but longer sperm. These results provide indirect evidence that diploid male reproductive success would be reduced compared with haploid males if they were able to copulate.

5. We conclude that diploid male production is likely to affect the fitness of *A. sexdens* queens with a matched mating, as these males are produced at the cost of workers and, if the colony survives to reach mature size, also gynes.

Key words. *Atta sexdens*, fitness reduction, haplodiploid, inbreeding depression, matched mating, sperm.

Introduction

Mating between related individuals increases offspring homozygosity. This results in inbreeding, which has well-documented negative effects on offspring such as a reduction in survival (e.g. Keller, 1998) or disease resistance (e.g. Calleri *et al*., 2006; see Keller & Waller, 2002 for a review). Furthermore, inbreeding can have substantial consequences for male reproductive success (Slate *et al*., 2000) because inbred males can produce ejaculates of low quality (Asa *et al*., 2007) or be more likely to be sterile (Gomez & Shaw, 2006).

Haplodiploid insects are an interesting group to study effects and consequences of inbreeding, which we define here as the increased frequency of individuals that are homozygous for alleles identical by descent (Keller & Waller, 2002). In these species, inbreeding often inflicts a genetic load (Heimpel & de Boer, 2008) that is phenotypically expressed as the production of diploid males. The sex of an individual is often, but not always, determined at one locus (single-locus complementary sex determination *sl*-CSD) (Heimpel & de Boer, 2008) where heterozygous individuals develop into females and hemizygous individuals develop into males. Inbreeding can result in the CSD allele of the father being identical to one of the two CSD alleles of the female he mates with (termed a matched mating). All individuals that are homozygous at the CSD locus will develop into diploid males (Beye *et al*., 2003). The appearance of diploid males as a consequence of inbreeding and a low variation at the CSD locus can have dramatic consequences

Correspondence: Boris Baer, ARC Centre of Excellence in Plant Energy Biology, The University of Western Australia, 6009 Crawley, Australia.

*Current address: Institute for Evolution and Biodiversity, Westfälische Wilhelms University, Hüfferstrasse 1, 48149 Münster, Germany.

†Current address: Centre for Evolutionary Biology, School of Animal Biology (MO92), The University of Western Australia, 6009 Crawley, Australia.

at the population or species level, as it theoretically results in a substantially increased risk of extinction (Zayed & Packer, 2005).

Diploid males can impose direct fitness costs in solitary Hymenoptera. For example, in the parasitic wasp *Bracon* sp. near *hebetor* diploid males have low survival to adulthood, and females to which they are mated produce only haploid offspring, indicating that the diploid males are sterile (Holloway *et al*., 1999). Furthermore, in the solitary wasp *Cotesia vestitalis* diploid males sire triploid females (de Boer *et al*., 2007). However, diploid males do not always appear to impose fitness costs because in some species they are capable of siring normal diploid female offspring, for example in the solitary vespid wasp *Euodynerus forminatus* (Cowan & Stahlhut, 2004) and in the parasitoid wasp *Cotesia glomerata* (Elias *et al*., 2009). In social Hymenoptera, fitness costs are also likely, but are less easy to quantify, because diploid males mostly replace workers rather than reproductives. Diploid honeybee (*Apis mellifera*) males have been shown to have low survival to adulthood (Beye *et al*., 2003) and diploid bumblebee (*Bombus terrestris*) males have a lower immune response (Gerloff *et al*., 2003). Diploid bumblebee males also have lower fitness because they produce fewer sperm and sire a reduced number of viable worker offspring compared with haploid males (Duchateau & Marien, 1995). In addition, in the social wasp *Polistes dominulus*, diploid males sire triploid females (Liebert *et al*., 2005) but it remains to be tested whether the triploid sexual offspring are fully fertile.

Eusociality has evolved repeatedly in haplodiploid insects and adds an interesting dimension to potential effects of inbreeding and diploid male production, as reproductive females only copulate during a brief period early in their life and store a lifetime's supply of sperm that cannot be replenished later in life (Baer, 2005; Boomsma *et al*., 2005). In the absence of queen remating after colony founding, inbreeding increases worker relatedness. However, the storage of sperm that will ultimately result in the fertilisation of diploid male eggs inflicts fitness costs for these colonies (Whitehorn *et al*., 2009). The first reason for this is that these sperm cells need to be physiologically maintained within the volume-limited spermatheca (Baer *et al*., 2006; Baer & Schmid-Hempel, 2005), reducing the total number of workers and virgin queens that a colony can produce (Cook & Crozier, 1995; Whitehorn *et al*., 2009). The second reason is that the colony will waste resources rearing diploid males. Consequently diploid males are produced at the expense of a proportion of the worker force and female reproductives. The costs of matched matings are expected to be especially pronounced during colony foundation, because the production of diploid male eggs implies substantial energetic costs and delays early colony development (Cook & Crozier, 1995). For example, in the fire ant, *Solenopsis invicta*, colonies headed by monogyne diploid male-producing queens exhibited 100% mortality early in development (Ross & Fletcher, 1986). Also, in the bumblebee *B. terrestris*, queens mated to a brother had reduced colony foundation success and smaller colonies in the laboratory (Gerloff & Schmid-Hempel, 2005) as well as in the field (Whitehorn *et al*., 2009). The production of diploid males can also have negative population-level implications (Heimpel & de Boer, 2008), for example when sex ratios and population dynamics are affected (Ross & Fletcher, 1986). For these reasons, strong selection against inbreeding is expected in social insects, especially in clades with large, long-lived colonies without queen replacement. Indeed, some social insect queens seem to actively avoid copulating with relatives (Foster, 1992; Keller & Passera, 1993), but diploid males have repeatedly been detected in more than 40 social hymenopteran species (van Wilgenburg *et al*., 2006), indicating that inbreeding might be more common than has been assumed. Also the proportion of sampled males that are diploid varies widely across species, for example from 1.3% in *Leptothorax acervorum* (Hammond *et al*., 2001) up to 100% in some populations of *Euglossa tridentata* (Roubik *et al.,* 1996), although it should be noted that different male sampling methods were used for these studies.

Understanding the evolutionary significance of diploid males and quantifying the costs and benefits of inbreeding at the colony level, depends on a detailed knowledge about the biology of diploid males, especially their potential fitness contributions in comparison to their haploid brothers. However, few studies have investigated diploid male production in the field. During excavations of mature fungus-growing leaf-cutter ants (Formicidae: Attini) *Atta sexdens* and *A. cephalotes* colonies, we detected two distinct male morphs. We collected a large number of male *A. sexdens*, a species that is known to have multiply mated queens (Fjerdingstad & Boomsma, 2000) and exceptionally large and long-lived colonies containing up to 5–8 million workers (Weber, 1972). We used microsatellite markers to determine whether the males were haploid or diploid and to genotype worker offspring to estimate the number of matched matings per queen relative to the total number of queen matings. Using diploid males collected in the field we further investigated variation in body size, sperm number, and sperm length in order to compare them with normal haploid males from the same colony.

Material and methods

In May 2001–2004 and 2006 we surveyed the surroundings of Gamboa (9°07′N, 79°42′W, Republic of Panama) and identified all mature colonies of *A. sexdens*. To check colonies had reached sexual maturity, we excavated several fungus chambers per colony and confirmed the presence of virgin queens and males.

Male size difference

In three out of five large colonies (A, B and C), a larger male morph was present. To quantify the observed size difference, we excavated a large number of available males from one of these colonies both before and after a mating flight in 2001 and 2002. Male size was measured as head width, which is the maximal distance (in mm) between the compound eyes, which has previously been shown to be a reliable indicator of body size in leaf cutting ants (Fjerdingstad & Boomsma, 1997; Baer & Boomsma, 2004; Baer *et al*., 2009).

Determining ploidy of large and small males, and queen mating frequency using microsatellites

As our statistical analysis confirmed the presence of two distinct size classes of males, we determined the ploidy of both male morphs. To do this we initially used seven microsatellite loci: Etta5-6TF (Fjerdingstad *et al*., 1998), Atco12, Atco13, Atco15, Atco37, Atco 47 (Helmkampf *et al*., 2008) and Etta1-2TF (Fjerdingstad & Boomsma, 2000). From these seven microsatellites, only Atco12 and Atco37 (five and three alleles respectively) were polymorphic and unambiguous to score; we therefore used these two markers for the final analysis. We found no evidence that the two microsatellites used amplified more than one locus, as we only detected a maximum of two alleles in our worker samples.

DNA was extracted from a leg of each individual by boiling for 15 min in a 5% Chelex® (Fluka 22477) ddH_20 solution. The extracted DNA was amplified for the two polymorphic microsatellite loci: Atco12 and Atco37. PCR reactions for Atco 12 contained 4 µl GATC mix, 1 µl PCR buffer (Applied Biosystems, Foster City, California), 0.75 µl of each primer, 0.1 µl AmpliTaq Gold polymerase (Applied Biosystems), 1.2 µl $MgCl_2$ solution, 1 µl DNA extract, and 1.2 µl ddH_2O. Where needed, 0.2 µl of bovine serum albumin (BSA) solution was also added, and ddH_2O was reduced to 1 µl. PCR reactions for Atco37 were the same as above, except we used 1 µl of each primer and no BSA. DNA was amplified on a Hybaid PXE PCR Express Thermal Cycler. For Atco12 we used the following conditions: an initial denaturing step of 95°C for 5 min followed by a sequence of 18 cycles (95°C for 30 s, touchdown cycle of 67–62°C for 30 s, 72°C for 90 s), a sequence of 25 cycles (95°C for 30 s, 62°C for 30 s, 72°C for 90 s), and a final elongation step of 72°C for 30 min. For Atco37 the following conditions were used: an initial denaturing step of 94°C for 4 min followed by a sequence of 15 cycles (94°C for 30 s, touchdown cycle of 57–53°C for 30 s, 72°C for 30 s), a sequence of 15 cycles (94°C for 30 s, 53°C for 30 s, 72°C for 30 s), and a final elongation step of 72°C for 60 min. PCR products were analysed on a Hitachi Applied Biosystems 3130XL Genetic Analyser. The microsatellite data were analysed with Genemapper® software (version 4.0; Applied Biosystems).

To determine queen mating frequency and the number of matched matings per queen, we genotyped males as well as 15 small-, 15 medium- and 15 large-workers from each of the three colonies. For colony A we also genotyped three virgin queens. To determine genotypes and deduce the number of matings, we pooled all data available for the two polymorphic microsatellites, and discarded any individuals for whom it was not possible to score alleles for both microsatellite markers. We used haploid male genotypes to reconstruct the queen genotype for each colony. With queen and worker genotypes we then deduced the minimum number of queen matings and the number of matched matings. Diploid males were inferred from heterozygous genotypes at at least one of the two microsatellite loci.

Differences in sperm characteristics between the male morphs

For a subset of the small and large males for which we obtained ploidy assessments by microsatellites, we also compared reproductive traits between the morphs. We investigated two traits that may contribute to male and colony reproductive success: sperm number and sperm length. We used total sperm number from males as a measure of total male fertility because in the vast majority of social hymenopterans, including *A. sexdens*, spermatogenesis is discontinued early in adult life (Boomsma *et al*., 2005). We chose sperm length as a second measurement of potential reproductive success in order to test for a possible trade off between sperm length and sperm number, so that females receiving longer sperm would face a reduction in the total number of sperm they are able to store. Our earlier work on sperm length across 19 species of attine ants, including *A. sexdens* (Baer *et al*., 2009), has indicated that sperm size may evolve under such sperm storage constraints across species.

To estimate sperm number and length, males of each morph were collected from the three colonies (colony A: $n = 10$; colony B: $n = 10$; colony C: $n = 7$) and dissected to obtain all the sperm from the right accessory testis, which was transferred to 2-ml boar semen extender (Merk III diluent, Minitüb, Tiefenbach, Germany). To do this, accessory testes were ruptured with watchmaker forceps and the out-flowing sperm was diluted by vortexing the sample for 20–30 s. We used 50 µl of this stock solution and transferred it to 1.95 ml of distilled water and vortexed the solution for 20–30 s. Single drops of 1 µl of this solution were placed on a microscope slide and air-dried. Sperm counts were performed after staining the sperm cells with DAPI (4,6-diamidino-2-phenylindole; Merck, Darmstadt, Germany). Using an Olympus fluorescence microscope at 400× magnification we counted all fluorescent-stained sperm heads within a single 1-µl drop of diluted sperm. For the same set of males we also measured sperm length. To do this we used a LEICA DMRXA microscope with a Nomarski filter and inspected slides at 400× magnification. Photographs of single non-damaged sperm were taken from dried 1-µl drops using a digital camera and Image Pro 6.1. Afterwards we used the NIH image program (http://rsb.info.nih.gov/nih-image) to measure sperm length in µm of five sperm per male.

Statistics

Data were analysed using SPSS version 11.0 for Macintosh and the R statistical package (R Development Core Team, 2005) version 2.5.1, and all errors given are one standard error. To test for significant deviations from normal distributions for head widths we used calculations as described in Sokal and Rohlf (1981). To test the explanatory power of male size (measured as head width) on allele number (one or two) we performed a binary logistic regression. For the sperm number and length analyses, two ANOVAs were performed, using sperm number and sperm length as dependent variables. In both cases male morph (diploid/haploid male) was a fixed factor and colony of origin was a random factor. We performed backwards elimination of non-significant terms until only significant terms

remained. The paternity pedigree for each of the three colonies was based on microsatellite data and was deduced by hand.

Results

Male size differences

The distribution of the pooled head widths of 571 males from colony A collected in 2001 and 2002 differed significantly from normality (one-sample Kolmogorov–Smirnov test $z = 4.713$, $P < 0.001$). Head width distribution was significantly right-skewed ($t_s = 22.81$, $n = 571$, $P < 0.001$) and leptokurtic ($t_s = 78.17$, $n = 571$, $P < 0.001$) justifying placing males into two (non-overlapping) groups with all males having head widths larger than 2.7 mm belonging to the large male morph (Fig. 1). After doing so, head widths differed significantly between the two groups (ANOVA, $F_{1,569} = 1026.01$, $P < 0.001$ see also Table 1).

In colony A, the larger male morph was rare: out of the 571 males excavated before ($n = 345$ males) and after ($n = 226$ males) a mating flight, only 22 were of the larger morph. We found that the proportion of large males was significantly higher before the mating flight (6.08% of all males sampled) compared with a sample taken after the mating flight (0.4% of all males, $\chi^2 = 4.224$, d.f. $= 1$, $P = 0.04$). During the mating flight, large males appeared on the surface of colonies attempting to take off, suggesting that they may participate in nuptial flights.

Male ploidy and queen mating frequency

Microsatellite analyses allowed us to unambiguously confirm diploidy in 27 out of 31 large males investigated (Table 2).

Table 1. Summary of characteristics (mean with standard error) from small and large males.

	Males of *Atta sexdens*		
	Small	Large	p
Head width (mm)	2.42 ± 0.003 (549)	2.84 ± 0.008 (22)	<0.001
Sperm length (μm)	56.04 ± 1.13 (15)	84.08 ± 2.23 (12)	<0.001
Sperm number × 10^7	8.19 ± 0.74 (15)	2.33 ± 0.51 (12)	<0.001

Data for head width originated from a sample of 571 males collected from colony A. Males from all three colonies studied were used to calculate differences in sperm length and sperm number. Total sample sizes are given in parentheses.

The remaining four large males showed only one band at both of the microsatellite loci examined, so we were not able to distinguish between whether they were homozygous diploid or hemizygous at the loci studied. We were therefore not able to conclude that these four males were also diploid. All 76 small morph males had single bands, which strongly suggest that they were indeed haploid. However, by applying similar reasoning to above, we cannot completely discount the possibility that a hemizygous individual was a homozygous diploid. However, when we tested for an association between band number and male head width we found that head width was a reliable predictor of allele number (binary logistic regression, $z = 4.44$, single band $n = 80$, double band $n = 27$, $P < 0.001$), providing confirmative evidence that our previous inference based on kurtosis of the overall distributions was correct. We therefore conclude that male size can be used as a phenotypic marker to predict ploidy in *A. sexdens*, although we cannot prove that

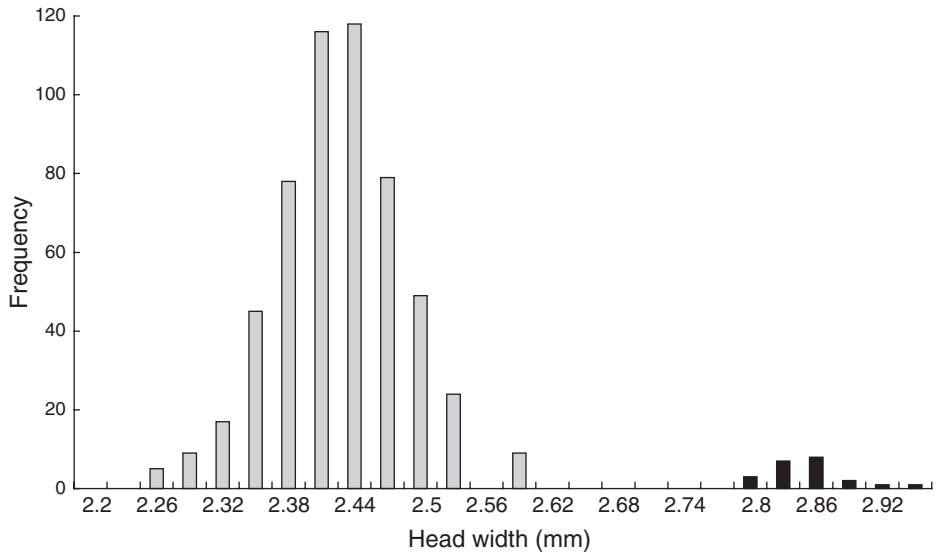

Fig. 1. Head widths of 571 *Atta sexdens* males originating from colony A and excavated in May 2001 and 2002. Head width distributions differed significantly from a normal distribution, because the size distributions of the small (grey bars) and large male morphs (black bars) were completely separated.

Table 2. Minimal estimates of the mating frequency of *Atta sexdens* queens and the number of matched matings observed for each of the three colonies.

Colony	Minimum no. matings	Minimum no. matched matings	Number of genotyped individuals		
			Haploid males	Workers	Diploid males
A	6*	3	41 (5)	47 (1)	18† (2)
B	5	1	18 (1)	43 (1)	6 (0)
C	5	1	17 (1)	43 (1)	3 (0)
Totals			76 (7)	133 (3)	27 (2)

*Assuming monogyny.

†Excluding four large males for which we could not discriminate between whether they were homozygous diploid or hemizygous haploid with our microsatellite markers.

The number of individuals whose genotype did not match that of the queen is shown in parentheses.

the predictor variable explains 100% of the variation in ploidy because of the limited resolution of our genetic markers.

Amongst the diploid males from colonies A, B, and C, we detected 6, 3, and 2 genotypes, respectively, and amongst the workers 15, 15, and 13 genotypes, respectively. Our estimate of the worker genotypes is conservative because only two microsatellites were available, so we may have missed additional patrilines. However, using these data we were able to calculate minimum estimates of queen mating frequencies and the minimal number of matched matings. This allowed us to infer that the number of matched matings was three for colony A and one for colonies B and C (see Table 2 for more details). For colony A, eight genotypes were detected where workers, haploid males or diploid male genotypes could not be explained by the presence of a single queen (Table 2) suggesting that a second reproductive queen might have been present. However, as only two microsatellite loci were available for the analysis, we were not able to perform any meaningful analysis of the pedigree for such a polygynous scenario. Similarly to colony A, we also found two individuals in each of colonies B and C whose genotypes did not have one of the queen alleles, suggesting that worker drifting may occur.

Differences in sperm characteristics between the male morphs

Male morph had a significant effect on sperm number ($F_{1,25} = 38.08$, $P < 0.0001$), but there was no influence of colony of origin ($F_{2,23} = 0.30$, $P = 0.742$) so colony was removed from the final model. Haploid males produced about 3.5 times more sperm compared with diploid males (see Table 1). The same was found for sperm length ($F_{1,25} = 142.35$, $P < 0.0001$), as sperm from haploid males was approximately 33% shorter than sperm from diploid males (see Table 1). Also, there was no significant effect of colony of origin on sperm length ($F_{2,23} = 2.52$, $P = 0.102$). All the males used were confirmed as haploid or diploid except for one large male, but running the analysis without this male did not make a difference to the result (data not shown).

Discussion

We have shown that diploid males are produced in *A. sexdens*, that these diploid males are larger than haploid males, and that they produce fewer and longer sperm. Our results provide indirect evidence that diploid males in a wild population of *A. sexdens* may have reduced reproductive success compared with haploid males, and that they may be costly to the colonies that produce them, because they hatch at the expense of female workers and reproductives. However, despite the inferred inbreeding leading to these matched matings, our results also imply that colonies managed to become reproductively mature in spite of this handicap. It would be interesting to compare colony foundation success between queens with matched versus non-matched matings. This could be feasible in the population studied here and would allow the estimation of a direct cost of inbreeding during the most crucial part of the colony life cycle. Our results also indicate that workers are unable to detect at least part of the diploid male brood, in contrast, for example, to honeybees where the diploid larvae are actively removed by the workers (Woyke, 1963). Our finding that diploid *A. sexdens* males are substantially larger than haploid males is similar to findings reported for two other ant species, *Solenopsis invicta* (Ross & Fletcher, 1985) and *Lasius sakagamii* (Yamauchi *et al*., 2001). However, diploid males in bumblebees are smaller than their haploid brothers (Duchateau & Marien, 1995; Gerloff *et al*., 2003). Whatever causes the observed size difference, the differences between the male phenotypes suggest that diploid males may have reduced reproductive success compared with their haploid brothers.

Our data support the idea of a single-locus complementary sex determination system in *A. sexdens*, as this provided the most parsimonious interpretation of the observed variation for our microsatellite markers in males and workers. However, further work will be required to confirm the single-locus sex determining mechanism in attine ants, because it is noteworthy that CSD in at least one ant species is based upon more than one locus (Schrempf *et al*., 2006). From our data we could estimate the number of diploid male offspring that would be expected to be produced based upon the assumptions that: (1) all of the queen's mates provided equal numbers of sperm, (2) on average half of the fertilised eggs from a matched mating developed as diploid male larvae (Cook & Crozier, 1995), and (3) all eggs fertilised by matched matings developed to adulthood. For colonies B and C, where our pedigree data were clear-cut, this calculation indicated

that *c*. 10% of the diploid brood developed into diploid males. However, our field observations indicated that the ratio between diploid males and workers was orders of magnitude lower than 1/10, suggesting either that many diploid males do not survive to adulthood, or that diploid males are more likely to disperse away from the colony than haploid males. The limited resolution of our markers and the assumption of equal sperm contributions imply that these inferences are approximate. We note, however, that previous studies have shown that paternity contributions in *Atta* are more equal than in the sister genus *Acromyrmex* (Fjerdingstad *et al*., 1998; Fjerdingstad & Boomsma, 2000; Sumner *et al*., 2004; Hughes & Boomsma, 2008). Assuming that our calculations are roughly correct, it would be interesting to know when diploid male brood is removed as this would have important fitness ramifications for the colony. In *Formica* ants and the wasp *Polybioides tabidus*, diploid males are only produced in association with haploid males (Pamilo *et al*., 1994; Henshaw *et al*., 2002), which implies that they are effectively removed at all times when colonies do not reproduce. We have not excavated *A. sexdens* colonies outside the reproductive season, but the large size difference between worker and male larvae would make it likely that diploid male brood is detected in the absence of haploid brothers and virgin queens.

Despite the fact that diploid males are larger than haploid males, they produce 3.5 times fewer sperm than their haploid brothers and about one-third longer sperm. Similar patterns were found in the ant *L. sakagamii* (Yamauchi *et al*., 2001) where the nuclei of diploid male sperm were considerably larger than those of haploid sperm, and also contained twice the amount of DNA compared with haploid sperm. Diploid male sperm was also confirmed to be diploid in *Apis mellifera* (Herrmann *et al*., 2005). It therefore seems reasonable to assume that the longer sperm of *A. sexdens* is diploid as well.

Our field observations showed that, similar to normal haploid male behaviour immediately before the nuptial flight, diploid *A. sexdens* males also appeared on the surface of the colony attempting to take off. However, we do not know whether diploid males can achieve successful copulations. In *A. colombica* and *A. cephalotes*, males possess a specific genital structure that attaches to a counterpart in the female, termed the mussel organ (Baer & Boomsma, 2006). It is unclear whether the larger diploid males are still able to attach to the female's mussel organ and successfully engage in copulations. Further work is needed to investigate whether male genitalia differ in size between the two morphs and whether queens can copulate with the larger, diploid males.

Multiple mating of queens reduces the variance in diploid male production among colonies (Ratnieks, 1990; Cook & Crozier, 1995) and could thus reduce the average costs of a matched mating for an individual queen, provided queen fitness is mostly determined by colony level selection (late in life) rather than individual selection (early in life). This mechanism has been particularly successful in explaining the evolution of extreme multiple queen mating in honeybees (Moritz *et al*., 1995; Palmer & Oldroyn, 2000) and army ants (Kronauer *et al*., 2007). *Atta sexdens* is also a polyandrous species with a comparable queen mating frequency to another *Atta* species, *A. colombica* (Fjerdingstad *et al*., 1998; Baer *et al*., 2006), but *Atta* incipient queens found their nests independently and mortality during the colony founding stage is very high, so that selection as a result of diploid male load should push for lower rather than higher mating frequencies (Ratnieks, 1990). This may explain in part why mating frequencies in *Atta* are lower than in the sister genus *Acromyrmex*, as the latter have much smaller colonies and therefore larger effective population sizes. However, we do not think this form of selection would be very efficient within particular species, because the main reason for the *A. sexdens* population in Gamboa having very low colony densities compared with sites closer to the Pacific ocean (pers. obs.) is that Gamboa is at the edge of the species distribution across the isthmus. It may therefore be that inbreeding is a by-product of low population density, which provided us with an interesting opportunity to study diploid males in the field, but does not necessarily mean that our results are representative for all *A. sexdens* populations.

Acknowledgements

We would like to thank Barbara Baer-Imhoof, Susanne den Boer, Henrik de Fine Licht, Bill Hughes, David Nash, and Klaus Skov Petersen for help in collecting ants in the field, the Smithsonian Tropical Research Institute in Panama for providing logistical help and facilities to work in Gamboa, and the Autoridad Nacional e Ambiente (ANAM) of Panama for issuing collection and export permissions. Sylvia Matthiasen, Charlotte Andersen, and Sandra Breum Andersen gave invaluable help with the microsatellite work and Jens Høeg kindly allowed us to use one of his microscopes. This work was supported by an Individual Marie Curie Fellowship from the European Commission and a Volkswagen Foundation Postdoctoral Fellowship to SAOA; by a Swiss National Science Foundation stipend for advanced scientists and two Discovery Grants offered by the Australian Research Council grant to B.B.; and funding from the Danish Natural Science Research Council to J.J.B. and B.B. We also thank two anonymous referees for helpful comments.

References

Asa, C., Miller, P., Agnew, M. *et al*. (2007) Relationship of inbreeding with sperm quality and reproductive success in Mexican gray wolves. *Animal Conservation*, **10**, 326–331.

Baer, B. (2005) Sexual selection in *Apis* bees. *Apidologie*, **36**, 187–200.

Baer, B. & Boomsma, J.J. (2004) Male reproductive investment and queen mating frequency in fungus growing ants. *Behavioral Ecology*, **15**, 426–432.

Baer, B. & Boomsma, J.J. (2006) Mating biology of the leaf-cutting ants *Atta colombica* and *A. cephalotes*. *Journal of Morphology*, **267**, 1165–1171.

Baer, B. & Schmid-Hempel, P. (2005) Sperm influences female hibernation success, survival and fitness in the bumblebee *Bombus*

terrestris. *Proceedings of the Royal Society of London Series B*, **272**, 319–323.

Baer, B., Armitage, S.A.O. & Boomsma, J.J. (2006) Sperm storage induces an immunity cost in ants. *Nature*, **441**, 872–875.

Baer, B., Dijkstra, M.B., Mueller, U.G., Nash, D.R. & Boomsma, J.J. (2009) Sperm length evolution in the fungus growing ants. *Behavioral Ecology*, **20**, 38–45.

Beye, M., Hasselmann, M., Fondrk, M.K., Page, R.E. & Omholt, S.W. (2003) The gene csd is the primary signal for sexual development in the honeybee and encodes an SR-type protein. *Cell*, **114**, 419–429.

de Boer, J.G., Ode, P.J., Vet, L.E.M., Whitfield, J.B. & Heimpel, G.E. (2007) Diploid males sire triploid daughters and sons in the parasitoid wasp *Cotesia vestalis*. *Heredity*, **99**, 288–294.

Boomsma, J.J., Baer, B. & Heinze, J. (2005) The evolution of male traits in social insects. *Annual Review of Entomology*, **50**, 395–420.

Calleri, D.V., Reid, E.M., Rosengaus, R.B., Vargo, E.L. & Traniello, J.F.A. (2006) Inbreeding and disease resistance in a social insect: effects of heterozygosity on immnnocompetence in the termite *Zootermopsis angusticollis*. *Proceedings of the Royal Society of London Series B*, **273**, 2633–2640.

Cook, J.M. & Crozier, R.H. (1995) Sex determination and population biology in the hymenoptera. *Trends in Ecology and Evolution*, **10**, 281–286.

Cowan, D.P. & Stahlhut, J.K. (2004) Functionally reproductive diploid and haploid males in an inbreeding hymenopteran with complementary sex determination. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 10374–10379.

Duchateau, M.J. & Marien, J. (1995) Sexual biology of haploid and diploid males in the bumble bee *Bombus terrestris*. *Insectes Sociaux*, **42**, 255–266.

Elias, J., Mazzi, D. & Dorn, S. (2009) No need to discriminate? Reproductive diploid males in a parasitoid with complementary sex determination. *PLoS ONE*, **4**, e6024.

Fjerdingstad, E.J. & Boomsma, J.J. (1997) Variation in size and sperm content of sexuals in the leafcutter ant *Atta colombica*. *Insectes Sociaux*, **44**, 209–218.

Fjerdingstad, E.J. & Boomsma, J.J. (2000) Queen mating frequency and relatedness in young *Atta sexdens* colonies. *Insectes Sociaux*, **47**, 354–356.

Fjerdingstad, E.J., Boomsma, J.J. & Thoren, P. (1998) Multiple paternity in the leafcutter ant *Atta colombica* - a microsatellite DNA study. *Heredity*, **80**, 118–126.

Foster, R.L. (1992) Nestmate recognition as an inbreeding avoidance mechanism in bumble bees (Hymenoptera: Apidae). *Journal of the Kansas Entomological Society*, **65**, 238–243.

Gerloff, C.U. & Schmid-Hempel, P. (2005) Inbreeding depression and family variation in a social insect, *Bombus terrestris* (Hymenoptera: Apidae). *Oikos*, **111**, 67–80.

Gerloff, C.U., Ottmer, B.K. & Schmid Hempel, P. (2003) Effects of inbreeding on immune response and body size in a social insect, *Bombus terrestris*. *Functional Ecology*, **17**, 582–589.

Gomez, N.N. & Shaw, R.G. (2006) Inbreeding effect on male and female fertility and inheritance of male sterility in *Nemophila menziesii* (Hydrophyllaceae). *American Journal of Botany*, **93**, 739–746.

Hammond, R.L., Bourke, A.F.G. & Bruford, M.W. (2001) Mating frequency and mating system of the polygynous ant, *Leptothorax acervorum*. *Molecular Ecology*, **10**, 2719–2728.

Heimpel, G.E. & de Boer, J.G. (2008) Sex determination in the Hymenoptera. *Annual Review of Entomology*, **53**, 209–230.

Helmkampf, M., Gadau, J. & Feldhaar, H. (2008) Population- and sociogenetic structure of the leaf-cutter ant *Atta colombica* (Formicidae, Myrmicinae). *Insectes Sociaux*, **55**, 434–442.

Henshaw, M.T., Queller, D.C. & Strassmann, J.E. (2002) Control of male production in the swarm-founding wasp, Polybioides tabidus. *Journal of Evolutionary Biology*, **15**, 262–268.

Herrmann, M., Trenzcek, T., Fahrenhorst, H. & Engels, W. (2005) Characters that differ between diploid and haploid honey bee (*Apis mellifera*) drones. *Genetics and Molecular Research*, **4**, 624–641.

Holloway, A.K., Heimpel, G.E., Strand, M.R. & Antolin, M.F. (1999) Survival of diploid males in *Bracon* sp. near *hebetor* (Hymenoptera: Braconidae). *Annals of the Entomological Society of America*, **92**, 110–116.

Hughes, W.O.H. & Boomsma, J.J. (2008) Genetic royal cheats in leaf-cutting ant societies. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 5150–5153.

Keller, L.F. (1998) Inbreeding and its fitness effects in an insular population of song sparrows (*Melospiza melodia*). *Evolution*, **52**, 240–250.

Keller, L. & Passera, L. (1993) Incest avoidance, fluctuation asymmetry, and the consequences of inbreeding in *Iridomyrmex humilis*, an ant with multiple queen colonies. *Behavioral Ecology and Sociobiology*, **33**, 191–199.

Keller, L.F. & Waller, D.M. (2002) Inbreeding effects in wild populations. *Trends in Ecology and Evolution*, **17**, 230–241.

Kronauer, D.J.C, Johnson, R.A. & Boomsma, J.J. (2007) The evolution of multiple mating in army ants. *Evolution*, **61**, 413–422.

Liebert, A.E., Sumana, A. & Starks, P.T. (2005) Diploid males and their triploid offspring in the paper wasp *Polistes dominulus*. *Biology Letters*, **1**, 200–203.

Moritz, R.F.A., Kryger, P., Koeniger, G., Koeniger, N., Estoup, A. & Tingek, S. (1995) High degree of polyandry in *Apis dorsata* queens detected by DNA microsatellite variability. *Behavioral Ecology and Sociobiology*, **37**, 357–363.

Palmer, K.A. & Oldroyn, B.P. (2000) Evolution of multiple mating in the genus *Apis*. *Apidologie*, **31**, 235–248.

Pamilo, P., Sundstrom, L., Fortelius, W. & Rosengren, R. (1994) Diploid males and colony-level selection in *Formica* ants. *Ethology, Ecology and Evolution*, **6**, 221–235.

R Development Core Team (2005) *R:A Language and Environment for Statistical Computing*. R foundation for statistical computing, Vienna, Austria [WWW document]. URL http://www.R-project.org [accessed in 2009].

Ratnieks, F.L.W. (1990) The evolution of polyandry by queens in social hymenoptera - The significance of the timing of removal of diploid males. *Behavioral Ecology and Sociobiology*, **26**, 343–348.

Ross, K.G. & Fletcher, D.J.C. (1985) Genetic origin of male diploidy in the fire ant *Solenopsis invicta* (Hymenoptera, Formicidae) and its evolutionary significance. *Evolution*, **39**, 888–903.

Ross, K.G. & Fletcher, D.J.C. (1986) Diploid male production—a significant colony mortality factor in the fire ant Solenopsis invicta (Hymenoptera: Formicidae). *Behavioral Ecology and Sociobiology*, **19**, 283–291.

Roubik, D.W., Weight, L.A. & Bonilla, M.A. (1996) Population genetics, diploid males, and limits to social evolution of euglossine bees. *Evolution*, **50**, 931–935.

Schrempf, A., Aron, S. & Heinze, J. (2006) Sex determination and inbreeding depression in an ant with regular sib-mating. *Heredity*, **97**, 75–80.

Slate, J., Kruuk, L.E.B., Marshall, T.C., Pemberton, J.M. & Clutton-Brock, T.H. (2000) Inbreeding depression influences lifetime breeding success in a wild population of red deer (*Cervus elaphus*). *Proceedings of the Royal Society of London Series B*, **267**, 1657–1662.

Sokal, R.R. & Rohlf, F.J. (1981) *Biometry: The Principles and Practice of Statistics in Biological Research*. Freeman, San Francisco, California.

Sumner, S., Hughes, W.O.H., Pedersen, J.S. & Boomsma, J.J. (2004) Ant parasite queens revert to mating singly. *Nature*, **428**, 35–36.

Weber, N.A. (1972) *Gardening Ants: The Attines*. The American Philosophical Society, Philadelphia, Pennsylvania.

Whitehorn, P.R., Tinsley, M.C., Brown, M.J.F., Darvill, B. & Goulson, D. (2009) Impacts of inbreeding on bumblebee colony fitness under field conditions. *BMC Evolutionary Biology*, **9**, 182.

van Wilgenburg, E., Driessen, G. & Beukeboom, L. (2006) Single locus complementary sex determination in Hymenoptera: an "unintelligent" design? *Frontiers in Zoology*, **3**, 1.

Woyke, J. (1963) What happens to diploid drone larvae in a honeybee colony. *Journal of Apicultural Research*, **2**, 73–75.

Yamauchi, K., Yoshida, T., Ogawa, T. *et al*. (2001) Spermatogenesis of diploid males in the formicine ant, *Lasius sakagamii*. *Insectes Sociaux*, **48**, 28–32.

Zayed, A. & Packer, L. (2005) Complementary sex determination substantially increases extinction proneness of haplodiploid populations. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 10742–10746.

Accepted 17 November 2009
First published online 26 January 2010

