



## RESEARCH PAPER

# Similar Performance of Diploid and Haploid Males in an Ant Species without Inbreeding Avoidance

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**Abstract**

Under haplodiploidy, a characteristic trait of all Hymenoptera, females develop from fertilised eggs, and males from unfertilised ones. Males are therefore typically haploid. Yet, inbreeding can lead to the production of diploid males that often fail in development, are sterile or are of lower fertility. In most Hymenoptera, inbreeding is avoided by dispersal flights of one or both sexes, leading to low diploid male loads. We investigated causes for the production of diploid males and their performance in a highly inbred social Hymenopteran species. In the ant *Hypoponera opacior*, inbreeding occurs between wingless sexuals, which mate within the mother nest, whereas winged sexuals outbreed during mating flights earlier in the season. Wingless males mate with queen pupae and guard their mating partners. We found that they mated randomly with respect to relatedness, indicating that males do not avoid mating with close kin. These frequent sib-matings lead to the production of diploid males, which are able to sire sterile triploid offspring. We compared mating activity and lifespan of haploid and diploid wingless males. As sexual selection acts on the time of emergence and body size in this species, we also investigated these traits. Diploid males resembled haploid ones in all investigated traits. Hence, albeit diploid males cannot produce fertile offspring, they keep up with haploid males in their lifetime mating success. Moreover, by fathering viable triploid workers, they contribute to the colonies' work force. In conclusion, the lack of inbreeding avoidance led to frequent sib-matings of wingless sexuals, which in turn resulted in the regular production of diploid males. However, in contrast to many other Hymenopteran species, diploid males exhibit normal sexual behaviour and sire viable, albeit sterile daughters.

Social insects generally reproduce in large outbreeding mating swarms, followed by independent nest foundations by queens and, as in the case of termites, also kings. However, in some species, alternative sexual phenotypes evolved, which can be regarded as dispersal polymorphisms (Molet et al. 2009; Peeters 2012). These wingless sexuals circumvent the risks of dispersal and independent colony foundation (Pusey & Wolf 1996), but their restricted dispersal capabilities may lead to inbreeding with all its potential negative consequences. Outbreeding can be maintained if at least one sex, most often the male, maintains the ability to fly. In several ant species, wingless females can

attract unrelated winged males by releasing sexual pheromones, a behaviour that has been termed 'female calling' (Peeters 1991; Passera & Keller 1994; Boomsma et al. 2005). Inseminated queens then return to their nest to reproduce or they take part of the work force to start a new nest in the vicinity. Wingless males however usually mate with nestmate queens that are in most cases close relatives. This non-dispersive male morph is much less common than winged males, but has been found in nine genera (Heinze & Tsuji 1995).

As wingless males do not disperse but mate within their natal nest, they compete with each other for the

access to queens. Local competition selected for competitive behaviours and morphologies in these males: some engage in deadly fights (Heinze & Hölldobler 1993; Kinomura & Yamauchi 1994; Yamauchi et al. 1996), defend territories within the colony (Frohshammer & Heinze 2009) or show courtship or guarding behaviours towards females (Yamauchi et al. 2001a; Foitzik et al. 2002; Mercier et al. 2007).

A negative consequence of inbreeding in Hymenoptera is the production of diploid males (Cook 1993). In most Hymenopteran species, sex is determined by complementary sex determination: unfertilised, haploid eggs are hemizygous on the sex-determining locus/loci and will develop into males. Diploid larvae develop into females if they are heterozygous and into males if they are homozygous on the sex locus/the sex loci. Under outbreeding, most fertilised eggs will develop into females, because sex loci are highly variable (Yokoyama & Nei 1979). In social insects, diploid males can be very costly for a colony, as they are produced at the expense of workers or queens. Diploid males are often non-viable or sterile and, in contrast to workers, never take over work chores in the nest (Plowright & Pallett 1979; Ross & Fletcher 1986; Cook 1993; Tarpay & Page 2002). In honeybees, diploid male larvae are recognised by workers and killed to lower the cost for the colony (Woyke 1963). In species in which diploid males survive to adulthood, they often exhibit a shorter lifespan (De Camargo 1982; Duchateau & Mariën 1995), but apparently normal sexual behaviour (Naito & Suzuki 1991; Krieger et al. 1999; De Boer et al. 2007). In evolutionary terms, diploid males are a dead end as, due to a lack of meiosis in sperm production of Hymenopteran males, the triploid offspring of diploid males are invariably sterile (Ayabe et al. 2004; Liebert et al. 2004; De Boer et al. 2007; Cournault & Aron 2009; but see Cowan & Stahlhut 2004; Elias et al. 2009).

The ant *Hypoponera opacior* exhibits two alternative reproductive tactics: winged males and queens conduct large-scale mating flights in early summer, while wingless queens and males emerge in late summer and mate within the mother colony, leading to high inbreeding levels (Foitzik et al. 2010, 2011). In response to local mate competition within the nests, wingless males evolved an unusual mating behaviour: they mate with pupal queens shortly before their emergence and guard their mating partners by embracing their cocoons for hours (Foitzik et al. 2002; Kureck et al. 2011). Males can mate multiply, but spermatogenesis does not continue into adult life so that sperm is limited (Foitzik et al. 2002). Although the level of inbreeding is

very high in *H. opacior*, inbred colonies do not exhibit a reduced productivity, but shift their allocation towards the production of sexuals, especially in the outbreeding winged summer generation. Our microsatellite analyses detected a low frequency of diploid males: about 0.5% of the diploid brood develops into males. We found diploid males only among the wingless, nest-mating males, whereas all analysed winged males were haploid. Fertility of diploid males was indicated by the occurrence of triploid workers and queens (Kureck et al. 2012), but it was not investigated how the ploidy level affects behaviour, life history and morphology of wingless males. In contrast to many other studies on Hymenopteran males, the intranidal mating behaviour and short reproductive season of about 3–4 weeks gave us the opportunity to observe and compare lifetime mating success of diploid and haploid males.

Previous studies indicate that *H. opacior* not only does conduct regular sib-matings, but is indeed adapted to inbreeding as indicated by the absence of inbreeding depression and alternative investment strategies (Kureck et al. 2012). The occurrence of diploid males and the fact that they can reproduce indicate that selection does not act on early removal of diploid male larvae, but rather on restoring a high fitness in diploid males. We therefore compared the performance of diploid and haploid males by focussing on the traits lifespan, number of matings and the average duration of matings. The latter trait is important because mating duration is expected to be associated with paternity certainty in this species with extensive post-copulatory mate-guarding behaviour (Kureck et al. 2011). As diploid *H. opacior* males are able to sire worker daughters and thereby to contribute to the colonies' work force, the costs of their production are reduced compared with species in which diploid males are entirely useless (Kureck et al. 2012). The more a diploid male can contribute to worker production, the lower are the costs for the colony. We hypothesised that *H. opacior* is well adapted to inbreeding, so that we expected a similar mating activity (i.e. number and duration of matings) of haploid and diploid males.

Body size often is an important trait in sexual selection. In *H. opacior*, male size was found to be under positive selection, with larger males exhibiting a higher mating success (Kureck et al., own data). These males might outcompete their smaller rivals by being better able to mount and hold on to queen pupae (Villabos & Shelly 1991) or by producing more sperm, enabling them to mate more often (Pech-May

et al. 2012). We measured and compared body size between haploid and diploid males, because earlier studies on several Hymenopteran species reported size differences between the two male types. In most species, diploid males are larger (Petters & Mettus 1980; Ross & Fletcher 1985; Packer & Owen 1990; El Agoze & Periquet 1993; Armitage et al. 2010), but in bumblebees, they are smaller than haploid males (Duchateau & Mariën 1995; Gerloff et al. 2003). Next to body size, an early emergence was found to be under positive sexual selection (Kureck et al., own data), which led us to compare emergence rank between haploid and diploid males. We hypothesised that if haploid and diploid males differ in body size or emergence rank, they should also exhibit a different mating success.

Moreover, we studied whether wingless males actively try to avoid mating with close kin, by choosing less-related nestmate queens as mating partners. As nests usually contain more than one reproductive queen, relatedness values strongly vary between nestmates (Foitzik et al. 2010, 2011). The ability of males to choose more distantly related queens would lower the inbreeding rate. However, as true kin recognition is mostly absent in social insects (Crozier 1988; Ratnieks 1991; DeHeer & Ross 1997; Strassmann et al. 1997) and genetic analyses indicate high inbreeding levels in this species, we did not expect inbreeding avoidance. Moreover, our studies so far (Kureck et al. 2012) revealed no inbreeding depression so that the selection pressure for evolving kin recognition in ant males is rather low.

## Material and Methods

### Ant Collection and Maintenance

Ant nests were collected in the Chiricahua Mountains of south-eastern Arizona in late summer, at a time of the year when only wingless males are produced. In 2010, we collected nests for the mate choice analyses, while nests collected in 2011 were used for the comparison of haploid and diploid males. Ant nests were detected by turning stones, and the ants were collected with an aspirator. We transferred the colonies to the laboratory at the Southwestern Research Station (31°52.0000N, 109° 12.6090W) and kept them at room temperature in three-chamber boxes (10 × 10 cm and 3 cm high). The boxes contained a moistened plaster floor with a cavity of 3 mm depth and 1.5 cm radius that was covered with a microscope slide and served as a nest chamber. Colonies were daily provided with water and dead insects.

### Comparison Between Haploid and Diploid Males

A total of 133 males from 40 nests were observed from their emergence until their death by scanning the nests three to six times per day for newly emerged males and for matings. Nests were checked once to twice in the morning, once at noon, once to twice in the afternoon and once in the late evening. All males were individually marked with enamel paint. For each male, the day of emergence and death as well as the number of matings and their mating durations were recorded.

To analyse whether diploid and haploid males differ in the timing of emergence within a nest, we calculated an emergence rank for each male. We therefore ranked males by their emergence day on a per-colony basis with nestmate males that emerged on the same day obtaining the same ranking number. In nests that already contained males at the time of collection, numbering was started with the number 'two' for the first male(s) emerging in the laboratory. 'Emergence rank' was then calculated by dividing the ranking number of a male by the number of males emerging in the nest, that is, low values reflect an early emergence in the nest. Males that remained the only ones in their nest ( $N = 7$ ) were excluded from the analysis of emergence order.

'Mating' was defined as all situations in which males embraced and guarded a cocoon as this is the typical sexual behaviour of wingless males before, during and after genital contact (Kureck et al. 2011). As our recordings were based on scan samplings and not on continuous observations, the exact onset and termination of matings could not be recorded in most cases. Hence, the first and the last time a male was observed interacting with a queen was defined as onset/termination point, resulting in lower bound estimates of mating durations. Further, our scan sampling method might have missed short matings. However, comparisons should be possible as these errors were made systematically for all males (Kureck et al. 2011). As measurements for mating activity, the total number of matings and the mean mating duration per male were used. Dying or dead males were preserved in 100% ethanol and observations were terminated after all males had died.

We compared lifespan, emergence rank, number of matings, mean mating duration (excluding males that never mated during their life) as well as head width and thorax width of haploid and diploid males using a generalised linear mixed model with a binomial error distribution. Each of the variables was entered as a fixed factor and 'nest' as a random factor, and we

tested for associations between each factor. Our statistical model was based on a cloglog link function (Zuur et al. 2009), which allows the analysis of data sets with a strong asymmetry in sample sizes (many more zeros than ones, i.e. more haploid than diploid males). Analyses were conducted with the statistical software R, version 2.14.0 (R Development Core Team 2011) using the package lme4 (Bates et al. 2011).

### Genetic Analysis

All males from the observation nests and 48 males and queens each from the mate choice experiments were genotyped on 11 highly variable microsatellite loci (protocol and primer description: Kureck et al. 2012). Amplification products were detected in a capillary sequencer (Applied Biosystems GA 3130xl Genetic Analyzer, Darmstadt, Germany) and analysed using the program GENEMARKER (V1.97 demo version, SoftGenetics, State College, PA, USA).

### Morphometric Comparisons

We measured the head width and thorax width of ETOH-preserved males under  $40\times$  magnifications using a stereomicroscope coupled to a computer and the measuring software LAS (Leica Application Suite version 3.8 from Leica Microsoft, Wetzlar, Germany).

To enlarge our sample size for the morphometric comparisons, we added males from 15 colonies (collected in the same season and year) that contained diploid males. By including these colonies into our data set, we were able to add morphometric data of 28 diploid males and 29 haploid males. For some individuals, the measurement of head or thorax width was impossible, because the ETOH-preserved individuals were deformed. In total, we were able to measure head width of 21 diploid and 87 haploid males and the thorax width of 38 diploid and 141 haploid males.

### Mate Choice Experiments

To investigate mate choice, 24 mating pairs were removed from 24 nests collected in late summer 2010 and stored in 100% ethanol for genetic analysis. As a control, an additional, randomly chosen, wingless queen and a wingless male was taken from each of the nests and preserved in 100% ethanol. The caste of the female was determined under the stereomicroscope to ensure that only mating pairs between wingless queens and males were included in the analyses. Wingless males sometimes accidentally try to copulate with sterile workers (Kureck et al. 2011). Individuals

were genotyped on 11 microsatellite loci as described above. Unidirectional relatedness values from male to female in both groups ('mating pairs' and 'nestmate controls') were calculated with the program RELAT-EDNESS 5.0.8 (Goodnight Software, Houston, TX, USA) and compared using a paired *t*-test.

### Results

Microsatellite analyses revealed that 12 of the 133 (9%) genotyped males were diploid, and these males stemmed from 10 nests. In general, the statistical power for the following comparisons of the mating behaviour of haploid and diploid males is limited by the low number of diploid males.

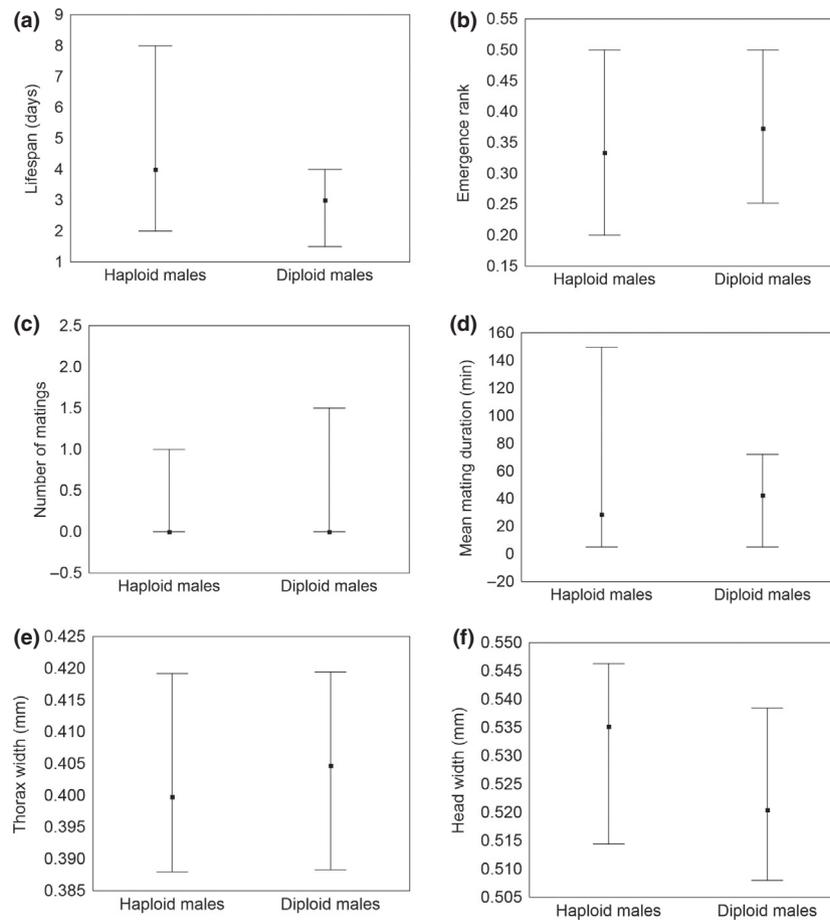
Five of these diploid males (42%) and 52 of the haploid males (43%) showed sexual activity, so that the proportion of males that mated at least once during their lifetime did not vary with ploidy ( $\chi^2$  test:  $p = 0.920$ ,  $N_{\text{haploid}} = 121$ ,  $N_{\text{diploid}} = 12$ ). The sexually active diploid males mated either once ( $N = 2$ ) or twice ( $N = 3$ ), and their matings lasted up to 360 min. Diploid males lived up to 10 d, and the longest-lived haploid male lived for 33 d. Diploid males did not differ from haploid ones in lifespan (Fig 1 a), emergence rank (Fig 1 b) and the number of matings (Fig 1 c). Further, the mean mate-guarding durations of sexually active males did not differ between the two male types (Fig 1 d). Diploid males did not significantly differ from haploid males in thorax width (Fig 1e) or head width (Fig 1f), but diploid males tended to have smaller heads ( $p = 0.066$ , Table 1).

Relatedness values between nestmate males and queens varied between  $r = -0.212$  and  $r = 0.918$ . However, male-to-queen relatedness did not differ between mating pairs and control pairs (male  $\rightarrow$  queen:  $r_{\text{mating pair}}: 0.232 \pm 0.083$ ,  $r_{\text{control pair}}: 0.258 \pm 0.0933$ ; mean  $\pm$  95% CI; paired *t*-test:  $t_{23} = 0.268$ ,  $p = 0.523$ ), demonstrating an absence of inbreeding avoidance in wingless males.

### Discussion

Our study revealed that wingless diploid males of the species *H. opacior* resemble normal haploid males in lifespan, mating success, time of emergence and body size. Furthermore, we found that nest-mating wingless males generally did not show active inbreeding avoidance, as they did not select less-related queens as mating partners.

As in *H. opacior*, sexually active diploid males were found to sire triploid offspring under inbreeding in other Hymenopteran species (Ayabe et al. 2004;



**Fig. 1:** Comparison of the male traits (a) lifespan, (b) emergence rank, (c) number of matings, (d) mean mating duration (only taking sexually active males into account), (e) thorax width and (f) head width. None of the traits differed significantly between haploid and diploid males. Points: median, whiskers: 25th and 75th percentiles.

**Table 1:** Comparison between haploid and diploid males. None of the analysed male traits differed between the two male types, but diploid males tended to have smaller heads

	$\chi^2$	p	Haploid males		Diploid males	
			N	median; min – max	N	median; min – max
Lifespan (days)	2.39	0.122	121	4; 1 – 33	12	3; 1 – 10
Emergence rank	0.40	0.530	114	0.33; 0.07 – 1	12	0.37; 0.13 – 1
N of matings	0.30	0.586	121	0; 0 – 8	12	0; 0 – 2
Mean mating duration (min)	0.10	0.751	52	27.25; 5 – 691	5	42.5; 5 – 360
Head width (mm)	3.37	0.066	87	0.54; 0.48 – 0.59	21	0.52; 0.46 – 0.58
Thorax width (mm)	0.34	0.561	141	0.40; 0.29 – 0.46	38	0.40; 0.35 – 0.46

Liebert et al. 2004; De Boer et al. 2007). In two regularly inbreeding wasp species (Cowan & Stahlhut 2004; Elias et al. 2009), diploid males are even able to sire fertile diploid offspring either by meiosis during sperm production or by the elimination of a chromosome set during fertilisation and resulting in a diploid zygote (Cowan & Stahlhut 2004). The detection of

viable triploid workers and queens in *H. opacior* (Kureck et al. 2012) indicates that at least some, but probably all diploid males, are unable to produce diploid offspring. Triploid queens are likely to be sterile, and therefore, the production of diploid males remains an evolutionary dead end. However, by being able to father workers that have been shown to live at

least for several months, diploid males can contribute to the colonies' work force (Kureck et al. 2012). Workers raise close relatives and thereby contribute to their own and their fathers' inclusive fitness. Whether diploid males show a similar fertility as haploid ones, for example, as measured by the number and viability of sperm, still remains to be investigated. In *Bombus terrestris* as well as in the parasitoid wasp *Cotesia vestalis*, mating success of diploid and haploid males was similar, but diploid males produced fewer viable offspring (Duchateau & Mariën 1995; De Boer et al. 2007). Hence, although the two male types show no difference in mating activity in *H. opacior*, diploid males might have a lower lifetime reproductive success than haploids. Moreover, it is yet unclear whether triploid workers exhibit the same work performance and lifespan as their diploid nestmates (Kureck et al. 2012).

As cell volume usually rises with genomic content (Olmo 1983), ploidy can affect organism size. For example, in nematodes, body size increases with cell size and polyploidy (Flemming et al. 2000). Yet, studies on plants and vertebrates indicate that larger cells of polyploids do not necessarily result in larger bodies as developmental mechanisms regulate organ growth to compensate for larger cell sizes (reviewed in Comai 2005). Diploid males in Hymenoptera were found to be larger than haploid males in many species (Woyke 1978; El Agoze & Periquet 1993; Yamauchi et al. 2001b; Elias et al. 2009). In the case of social Hymenoptera, these differences were explained by differences in food supply and satiation between diploid and haploid larvae rather than by a higher cell volume (Petters & Mettus 1980; Packer & Owen 1990). However, this is not invariably the case as diploid males were found to be smaller in the bumblebee *Bombus terrestris* (Duchateau & Mariën 1995; Ayabe et al. 2004). In this case, it is unclear whether the reduced body size is due to internal mechanisms or whether diploid male larvae obtain less food during larval development. In our study, we only found a tendency of diploid males to develop smaller heads, but in general, the two male types did not differ significantly in body size. Hence, ploidy level did not strongly influence adult male body size, indicating similar provisioning for haploid and diploid male larvae. Possibly, differential larval provisioning might not have evolved in this species as body size of wingless sexuals and workers is very similar (Foitzik et al. 2010). Next to minor differences in body size, diploid males show a similar emergence time as haploid ones, indicating that ploidy does not affect larval development time. As emergence rank and body size were found to be associated with mating success in wingless

*H. opacior* males (Kureck, own data), the absence of differences in these traits are in line with the finding that haploid and diploid males show a similar sexual performance.

The comparison of relatedness between mating pairs and control pairs revealed that males do not actively choose less-related queens as their mating partners. The lack of such an adaptive behaviour indicates that males are unable to distinguish close kin from less-related individuals. In contrast to the ability to discriminate nestmates from non-nestmates, the absence of true kin recognition has been suggested for many social Hymenoptera (Crozier 1988; Ratnieks 1991; DeHeer & Ross 1997; Strassmann et al. 1997). Some social Hymenoptera can actively avoid incest by choosing non-nestmates over nestmates (Gamboia et al. 1986; Foster 1992). In these species, recognition might be based on environmental cues, such as specific colony odours, rather than on genetically determined cues. Inbreeding avoidance among individuals of the same nest would reveal discrimination by means of genetically determined cues only. However, so far, only an absence of inbreeding avoidance among nestmates has been reported for nest-mating ants, and our results are in accordance with these earlier findings (Blatrix & Jaisson 2002; Keller & Fournier 2002). Hence, not only the production of non-dispersive sexuals in late summer, but also their inability to actively avoid copulations with close kin leads to high levels of inbreeding in *H. opacior*. However, the species seems to have adapted to high inbreeding: first, ant colonies were found to invest more in outbreeding sexuals if the level of inbreeding is high. Second, diploid male production is moderate, which might be due to multilocus complementary sex determination instead of single-locus complementary sex determination (Kureck et al. 2012). Third, the here-shown sexual activity and viability of diploid males lower the costs of diploid male production. Hence, as long as enough haploid males are present that can ensure the production of fertile queens, reproduction within the colony is ensured and the few diploid, worker-producing males can be tolerated.

In conclusion, our study revealed a similar performance of haploid and diploid males in *H. opacior*, indicating low costs of diploid male production. Albeit diploid males sire sterile triploid offspring, their inclusive fitness is still greater than zero as they can father viable workers that contribute to colony productivity. The here-reported normal sexual activity of diploid males is in line with previous findings reporting that *H. opacior* has adapted to a regular inbreeding lifestyle without suffering from inbreeding depression (Kureck

et al. 2012). The low costs of inbreeding results in minor selection for inbreeding avoidance. Indeed, our study shows that wingless males mate randomly with respect to relatedness to their mating partners.

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