# Alternative reproductive tactics, sexual selection and the effects of inbreeding in the ant *Hypoponera opacior*

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# Author contributions

The six chapters presented in this dissertation are based on research articles that have been published in peer-reviewed journals (Chapter I, II, III and V) or are currently under review (Chapter IV and VI). The data for the first two papers were collected by Markus Rüger and Susanne Fotizik between 1998 and 2005. I was involved in data analyses and writing and revising of the manuscripts. The other four chapters are based on data that I have collected during my PhD project from 2009 to 2012. My work involved study design, field collections, data collection and analyses as well as the writing of manuscripts under the guidance of my supervisor Prof. Dr. Susanne Foitzik. Additionally, the following co-authors contributed to the here presented studies: Dirk Metzler (statistical analyses, Chapter I and II), Antje Neumann (field work and chemical extractions, Chapter III), Beate Nicolai (field work, behavioural observations and microsatellite analyses, Chapter IV, V and VI) and Evelien Jongepier (statistical analyses, Chapter V).

# Summary

Inbreeding can lead to a fitness reduction due to the unmasking of deleterious recessive alleles and the loss of heterosis. Therefore, most sexually reproducing organisms avoid inbreeding, often by disperal. Besides the avoidance of inbreeding, dispersal lowers intraspecific competition on a local scale and leads to a spreading of genotypes into new habitats. In social insects, winged reproductives disperse and mate during nuptial flights. Therafter, queens independently found a new colony. However, some species also produce wingless sexuals as an alternative reproductive tactic. Wingless sexuals mate within or close to their colony and queens either stay in the nest or they found a new colony by budding. During this dependent colony foundation, wingless queens are accompanied by a fraction of nestmate workers. The production of wingless reproductives therefore circumvents the risks associated with dispersal and independent colony foundation. However, the absence of dispersal can lead to inbreeding and local competition.

In my PhD-project, I investigated the mating biology of *Hypoponera opacior*, an ant that produces winged and wingless reproductives in a population in Arizona. Besides the investigation of the annual reproductive cycle, I particularly focused on the consequences of wingless reproduction. An analysis of sex ratios in wingless sexuals should reveal the relative importance of local resource competition among queens (that mainly compete for the help of workers) and local mate competition among males. Further, sexual selection was expected to act on wingless males that were previously found to mate with and mate-guard pupal queens in response to local mate competition. We studied whether males are able to adapt their mating behaviour to the current competitive situation in the nest and which traits are under selection in this mating situation. Last, we investigated the extent and effects of inbreeding. As the species appeared to produce non-dispersive males and queens quite frequently, we assumed to find no or only weak negative effects of inbreeding and potentially mechanisms that moderate inbreeding levels despite frequent nest-matings.

We found that winged and wingless males and queens are produced during two separate seasons of the year. Winged sexuals emerge in early summer and conduct nuptial flights in July, when climate conditions due to frequent rainfalls lower the risks of dispersal and independent colony foundation. In fall, wingless sexuals are produced that reproduce within the colonies leading to an expansion on the local scale. The absence of dispersal during this second reproductive season resulted in a local genetic population viscosity and high levels of inbreeding within the colonies. Male-biased sex ratios in fall indicated a greater importance of local resource competition among queens than local mate competition among males. Males were observed to adjust mate-guarding durations to the competitive situation (i.e. the number of competing males and pupae) in the nest, an adaptation that helps maximising their reproductive success. Further, sexual selection was found to act on the timing of emergence as well as on body size in these males, i.e. earlier emerging and larger males show a higher mating success. Genetic analyses revealed that wingless males do *not* actively avoid inbreeding by choosing less related queens as mating partners. Further, we detected diploid males, a male type that is produced instead of diploid females if close relatives mate. In contrast to many other Hymenopteran species, diploid males were here viable and able to sire sterile triploid offspring. They did not differ in lifespan, body size and mating success from "normal" haploid males. Hence, diploid male production in *H. opacior* is less costly than in other social Hymenopteran species. No evidence of inbreeding depression was found on the colony level but more inbred colonies invested more resources into the production of sexuals. This effect was more pronounced in the dispersive summer generation. The increased investment in outbreeding sexuals can be regarded as an active strategy to moderate the extent and effects of inbreeding.

In summary, my thesis describes an ant species that has evolved alternative reproductive tactics as an adaptation to seasonal environmental variations. Hereby, the species is able to maintain its adaptive mating system without suffering from negative effects due to the absence of dispersal flights in fall.

# Zusammenfassung

Inzucht verursacht oftmals hohe Fitnesskosten, was mit der Demaskierung schädlicher, rezessiver Allele, sowie mit dem Verlust von Heterosis zu erklären ist. Daher vermeiden viele Arten Inzucht, oftmals durch Abwanderung vom Geburtsort. Neben der Vermeidung von Inzucht führt Abwanderung auch zu einer Verminderung innerartlicher, lokaler Konkurrenz, sowie zu einer Ausbreitung von Genotypen in neue Areale.

Geflügelte Geschlechtstiere sozialer Insektenarten verlassen in der Regel die Mutterkolonie und paaren sich auf ihren Hochzeitsflügen. Nach der Paarung gründen Jungköniginnen dann eigenständig eine neue Kolonie. Als alternative reproduktive Taktik produzieren jedoch einige Arten ungeflügelte Königinnen und/oder Männchen. Diese ungeflügelten Geschlechtstiere paaren sich innerhalb oder nahe ihrer Ursprungskolonie. Jungköniginnen bleiben im Anschluss entweder in ihrer Kolonie, oder sie gründen eine neue Kolonie in unmittelbarer Nähe, wobei sie von einigen Arbeiterinnen aus dem Mutternest begleitet werden (‚Budding'). Durch die Produktion ungeflügelter Geschlechtstiere werden somit die Risiken, die mit einem Hochzeitsflug oder einer eigenständigen Koloniegründung durch eine einzelne Königin verbunden sind, umgangen. Auf der anderen Seite führt diese reproduktive Taktik zu einer stärkeren Konkurrenz auf lokaler Ebene und zu einem erhöhten Inzuchtrisiko.

In meinem Promotionsprojekt habe ich mich mit der Paarungsbiologie der Ameisenart *Hypoponera opacior* beschäftigt. Diese Art produziert, in einer Population in Arizona, sowohl geflügelte als auch ungeflügelte Königinnen und Männchen. Neben der Untersuchung des jährlichen Reproduktionszyklus habe ich mich insbesondere mit den Konsequenzen der Produktion flügelloser Geschlechtstiere beschäftigt. Eine Analyse des Geschlechterverhältnisses in ungeflügelten Königinnen und Männchen sollte zeigen, ob die Konkurrenz zwischen Königinnen (um die Hilfe von Arbeiterinnen) oder die Konkurrenz zwischen Männchen (um Paarungen mit Königinnen) einen stärkeren Einfluss auf die Investition der Kolonien in Geschlechtstiere hat. Des Weiteren war aus früheren Studien bekannt, dass sich ungeflügelte Männchen, in Anpassung an die Paarungskonkurrenz im Nest, bereits mit Jungköniginnen paaren, bevor diese vollständig aus ihrem Kokon geschlüpft sind. Hierbei bewachen sie ihre Partnerinnen oftmals stundenlang. Nun sollte untersucht werden ob die Männchen in der Lage sind ihr Partnerbewachungsverhalten aktiv an die

lokale Konkurrenzsituation im Nest anzupassen und inwiefern sexuelle Selektion auf die Männchen in dieser speziellen Paarungssituation wirkt. Darüber hinaus wurden das Ausmaß und die Effekte der Inzucht untersucht. Aufgrund der beobachteten regelmäßigen Produktion ungeflügelter Geschlechtstiere lag die Vermutung nahe, dass Inzucht keine oder nur schwache negative Auswirkungen auf *H. opacior*-Kolonien hat. Eine weitere Hypothese war, dass die Art möglicherweise Mechanismen entwickelt hat, die es ihr ermöglichen, die Inzuchtrate trotz regelmäßiger Nestverpaarungen moderat zu halten.

Unsere Untersuchungen zeigten, dass die Produktion geflügelter und ungeflügelter Geschlechtstiere an zwei reproduktive Phasen im Jahr geknüpft ist. Geflügelte Männchen und Königinnen schlüpfen im Frühsommer und begeben sich im Juli auf ihren Hochzeitsflug. Regelmäßige Regenfälle begünstigen in dieser Zeit den Hochzeitsflug und die unabhängige Nestgründung durch einzelne Königinnen. In einer zweiten reproduktiven Phase im Herbst werden ausschließlich ungeflügelte Nachkommen produziert und Königinnen und Männchen paaren sich im Ursprungsnest. Diese zweite Reproduktionsphase sorgt für einen Massezuwachs der Kolonien sowie für eine Ausbreitung auf lokaler Ebene durch Budding. Der Verzicht auf einen Paarungsflug und damit auf eine Ausbreitung in weiter entfernte Regionen, führt zu einer lokalen genetischen Viskosität sowie zu hohen Inzuchtraten in den Kolonien. Herbstkolonien wiesen ein männchenlastiges Geschlechterverhältnis auf, was darauf hindeutet, dass die lokale Konkurrenz unter Königinnen einen stärkeren Effekt auf die Investition in neue Geschlechtstiere hat, als die Paarungskonkurrenz unter Männchen. Beobachtungen zeigten, dass ungeflügelte Männchen die Bewachungsdauer ihrer Partnerinnen an die Konkurrenzsituation im Nest (die Zahl der Rivalen und Puppen) anpassen, um dadurch einen optimalen Paarungserfolg zu erzielen. Die sexuelle Selektion wirkt bei diesen Männchen auf den Schlupfzeitpunkt und auf die Körpergröße: Früh geschlüpfte und große Männchen haben einen höheren Paarungserfolg. Genetische Analysen konnten zeigen, dass die Männchen sich *nicht* bevorzugt mit entfernter verwandten Königinnen im Nest paaren um Inzucht aktiv zu vermeiden. Des Weiteren wurden diploide Männchen gefunden. Diploide Männchen werden in sozialen Hymenopteren unter Inzuchtbedingungen gelegentlich anstelle von Arbeiterinnen oder Königinnen produziert. Im Gegensatz zu vielen anderen Hymenopterenarten waren diploide Männchen hier lebensfähig und in der Lage, triploide, sterile Nachkommen zu zeugen. Sie unterschieden sich in Bezug auf

Lebensdauer, Körpergröße und Fortpflanzungserfolg nicht von „normalen“ haploiden Männchen. Somit sind die Kosten ihrer Produktion hier niedriger als in vielen anderen Arten. Es wurden keine Hinweise auf eine inzuchtbedingte Depression gefunden. Vielmehr steigerten stark ingezüchtete Kolonien die Produktion von Geschlechtstieren, ein Effekt der insbesondere die geflügelte Sommergeneration betraf. Die Abwesenheit einer Inzuchtdepression sowie die gesteigerte Investition in geflügelte, Auszucht betreibende Königinnen und Männchen kann als Anpassung an regelmäßige Verwandtenpaarungen betrachtet werden, die die Inzuchtrate und die damit verbundenen negativen Effekte mäßigt.

Meine Dissertation beschreibt eine Ameisenart, welche alternative reproduktive Taktiken in Anpassung an saisonbedingte Umweltbedingungen entwickelt hat. Der Verzicht auf Paarungsflüge im Herbst wirkt sich nicht negativ auf die hier untersuchte Population aus. So kann diese ihr spezielles Paarungssystem aufrechterhalten ohne unter den Kosten eingeschränkter Ausbreitungsmöglichkeiten zu leiden.

# Introduction

Sexual reproduction is widespread in multicellular organisms. Many hypotheses have been proposed to explain the evolution of sexual reproduction (reviewed in Hartfield and Keightley 2012). The most commonly accepted explanation is the advantage resulting from the creation of genetic diversity though recombination. By recombining novel mutations, sexually reproducing populations are more likely to create new genotypes that are able to adapt to environmental variations. Hence, although it is more costly than asexual reproduction, sexual reproduction can be beneficial as it results in high genetic variation for natural selection to work upon. At the same time, it establishes heterozygosity in di- or polyploid organisms, i.e. organisms receive more than one allele for each genetic locus. In more heterozygous organisms, dominant, beneficial alleles can compensate for deleterious recessive ones. Further, a higher number of alleles often results in an enhanced viability, larger size, better disease resistance and higher fertility (Shull 1952; Berger 1976; Calleri et al. 2006; Fitzpatrick and Evans 2009). These positive effects, however, disappear under inbreeding. As close relatives share a high number of alleles, their joint offspring carry a high proportion of homozygous loci. Hence, if not many new mutations occur in an inbreeding community, offspring will become more and more homozygous in every new generation. Consequently, inbreeding has negative effects on many sexually reproducing species (e.g. Ralls et al. 1979; DeRose and Ross 1999; Packer and Pusey 1993; Keller and Waller 2002), which are explained by the loss of heterosis and the unmasking of deleterious recessive alleles (Wright 1977; Charlesworth and Charlesworth 1999). Therefore most species evolved mechanisms to avoid inbreeding, such as the active recognition and avoidance of close kin as mates or a delayed maturation if parents or related individuals of the other sex are present (reviewed in Pusey and Wolf 1996). One important and widespread mechanism that helps avoiding inbreeding is dispersal, often conducted by only one of the two sexes (Pusey 1987). Through dispersal, alleles are not only mixed within individuals of the same local habitat, but also transferred to other populations. The combination of the two, dispersal and recombination of genotypes through sexual reproduction, ensures fast evolution and spreading of improved genotypes. Further, dispersal reduces local competition for mating partners and resources such as food or nesting sites.

### *Sexual dimorphism and competition for mating partners*

In most sexually reproducing species we find differences between female and male gametes (anisogamy). Female gametes, which are equipped with resources and nutrition, are mostly larger and more costly to produce than male gametes. Thus, males that contribute relatively few resources to a newly formed zygote usually follow the strategy of trying to inseminate as many females as possible, while females are resource limited in their reproduction (Parker et al. 1972). Additionally, in species with long gestation periods or maternal care, reproduction is more time-consuming for the female sex. Therefore, males usually compete with each other for copulations with females.

But females and males do not only differ on the level of their gametes. Many sexually reproducing animals exhibit a sexual dimorphism that goes beyond the general differences in the reproductive organs. This dimorphism evolved through sexual selection, which mostly works on the competitive male sex (Darwin 1859). Driven by the two main forces of sexual selection, female choice and male-male competition, males developed traits that either make them more attractive to females or help during competitive interactions with their rivals. Sexual selection therefore explains male characteristics like ornamentations (e.g. colourful feathers), weapons (e.g. antlers or horns) or a large body size (Darwin 1871). But also sensory, physiological or behavioural traits have evolved under sexual selection. A good olfactory sense, for example, can help a male to find a female by following her pheromones (insects: Linn and Roelofs 1989). Likewise, fast development to sexual maturity can reduce male-male competition in early adulthood and therewith help finding a mating partner before rivals are present (insects: Mynihan and Shuker 2011; birds: Saino et al. 2012). Behavioural traits that evolved under sexual selection are for example courtship behaviours (insects: Mercier et al. 2007; birds: Fusani et al. 2007), fighting behaviours (mammals: Mattiangeli et al. 1998; insects: Anderson et al. 2003), the defence of territories (mammals: Carey 1991; insects: Frohschammer and Heinze 2009), or mate-guarding (insects: Connor and Itagaki 1984; fish: Morbey 2002; birds: Estep et al. 2005; mammals: Schubert et al. 2009).

By guarding females, males try to prevent other males from mating with their partner. This behaviour is beneficial if a male encounters a female before she becomes receptive or if, under multiple mating of females (polyandry), male-male competition continues after copulation on the level of sperm (sperm competition; reviewed in

Birkhead and Møller 1998). Sperm competition is especially common in species in which sperm is long-lived and can be stored over long periods of time. This is for example the case in many insects and birds. In the majority of cases, the last male that mates with a female fertilizes most of her eggs (last male precedence). But also a fair raffle of sperm within the genital tract of the female and a first male precedence have been described (reviewed in Birkhead and Hunter 1990). Hence, depending on the mechanism of sperm competition either a pre- or a postcopulatory mate-guarding behaviour is more advantageous to ensure paternity. The highest reproductive success is ensured if a male achieves to remain the only mating partner of a female. On the other hand mate-guarding is time consuming and lowers the quantitative mating success of males. This means that there is trade-off between guarding a female and trying to copulate with as many females as possible. Consequently, in order to enlarge their reproductive success, males should adapt their mate-guarding behaviour to the local competitive environment (Parker 1974): If females are difficult to find and intrasexual competition is high, they should guard their partners longer. If several receptive females are present and intrasexual competition is low, they should rather try to mate multiply. Indeed, an adaptation of mate-guarding durations to the local competitive environment has been found both in invertebrates (insects: Ward 1983, García-González and Gomendio 2006; crustaceans: Takeshita and Hemnmi 2010) and vertebrates (reptiles: Cuadrado 2000; birds: Komdeur 2001). As an alternative mechanism to circumvent sperm competition, males in some animal species seal the genital opening of females after copulation with a ‘mating plug’ that hinders other males from mating with their partner (reptiles: Devine 1975; mammals: Marten and Shepherd 1976; insects: Matsumoto and Suzuki 1992; spiders: Masumoto 1993). In the order Odonata, males even have evolved utensils with that they remove their rivals’ sperm from the genital tract of their partner (Córdoba-Aguilar et al. 2003).

### *Alternative reproductive tactics*

Sexual dimorphism is not the only variation within a species that evolved in response to the competition over reproduction. While in some species selection favours a single optimal phenotype per sex, alternative phenotypes within a sex (mostly within the male sex) are common in others. These alternative phenotypes often use different behavioural tactics to achieve copulations. In the majority of cases different sexual phenotypes are genetically monomorphic and the tactic used by a male depends on its status or the social and environmental conditions (Gross 1996). For instance, larger males often fight with competitors to gain access to females, whereas small males use a ‘sneaking’ strategy and avoid confrontations with competitors. An example for such a two-fold mating strategy is the horned beetle *Onthophagus acuminatus:* Larger males develop prominent horns that they use as weapons to guard the entrances of tunnels containing females. Smaller males develop only small horns or no horns at all. Instead of fighting, they sneak past their competitors, often by digging side-tunnels, in order to gain access to females. The absence of horns makes it easier for this male phenotype to manoeuvre inside the tunnels (Emlen 1997). Sneaking strategies are also common in vertebrates (e.g. fish: Mazzoldi and Rasozzo 2002). In mammals, the strategy used often not only depends on morphological traits, but also on an individual’s age. Older males more often defend territories or harems while younger individuals try to sneak mating opportunities (Le Boeuf 1974; Apio et al. 2007).

Often alternative reproductive tactics are associated with differences in dispersal abilities or dispersal behaviour. Males with a lower competitive ability are more likely to disperse, because their chances to successfully mate within their natal habitat are low (insects: Lawrence 1987; mammals: Solmsen et al. 2011). In insects, the production of either winged or wingless phenotypes is common. These different morphs develop in response to variations in environmental conditions (Simpson et al. 2011; Zera and Denno 1997). For example, high temperatures and long days, which represent good habitat conditions for the aphid *Aphis craccivora,* lead to the development of wingless individuals, while lower temperatures, a high population density and poor host plant conditions result in the development of the winged, disperser phenotype (Johnson 1966).

In social Hymenoptera alternative reproductive tactics are more common in females than in males. In ants, the ancestral sexual morphs are winged females and males that mate on large-scale mating flights. After these nuptial flights, queens shed their wings (now termed ‘dealate queens’) and start a new colony by laying their first eggs and raising a first generation of workers by themselves (independent colony foundation). Alternatively, many species produce wingless queens that resemble the sterile worker caste (‘intermorphic’ or ‘ergatoid’ queens). Wingless queens do not disperse, but mate within or close to their natal colony. Afterwards they either stay in their natal nest, or they leave together with a cohort of workers from their colony on foot and start a new colony close-by (dependent colony foundation). If winged or wingless queens are favoured by selection depends on habitat characteristics such as climate, resource availability, predator pressure and local competition. If, for instance, weather conditions make a nuptial flight risky, selection favours the production of wingless queens. If, on the other hand, local competition for food and nest sites is high, dispersal to another habitat is favoured (reviewed in Heinze and Tsuji 1995).

The loss of wings and the absence of dispersal also occurs in ant males. However wingless males are much rarer than wingless queens and have so far only been described in nine ant genera (Heinze und Tsuji 1995). The reason for the rare evolution of non-dispersive ant males is the high risk of inbreeding with its generally negative consequences. Winglessness in queens does not necessarily lead to sib-matings, because wingless queens often mate with winged males from other colonies, which they can attract by pheromone release (‘female calling’). These males, attracted by sexual pheromones or by the odour of a conspecific colony, either enter colonies or mate with their partners close to the entrance of their nest (reviewed in Peeters 1991; Passera and Keller 1994; Berghoff et al. 2008). Wingless males however usually mate with nestmate queens, because they cannot disperse far on foot and because winged queens of other colonies are usually attacked when they try to approach or enter a colony that is not their own (Heinze and Tsuji 1995). Hence, wingless males mostly engage in sib-matings as nestmates are close relatives.

### *Inbreeding in social Hymenoptera*

As mentioned above, inbreeding results in a loss of heterozygosity and therewith frequently causes a decrease in fitness. In Hymenoptera, males usually develop from unfertilized eggs so that they are haploid whereas females are produced by sexual reproduction and are diploid. In these haplodiploid organisms, negative effects of inbreeding are expected to be lower than in diplodiploid species, because recessive deleterious alleles are purged from the population by haploid males that cannot compensate for these alleles (Werren 1993; Antolin 1999; Henter 2003). But still, inbreeding can have negative effects on Hymenoptera which, in the case of social species, can affect performance and fitness of the whole colony. The most severe cost of inbreeding in social Hymenoptera is the production of diploid males at the expense of workers or queens. The reason why it comes to the production of this rather unusual type of males lies in the sex determination system: In most Hymenopteran species sex is determined by complementary sex determination (Cook 1993). In this system, one or a few genetic loci are responsible for the determination of the sex: Haploid larvae that are hemizygous on the responsible locus/loci develop into males whereas diploid larvae that are heterozygous on the sex locus/loci will become females. Diploid males are produced if individuals are homozygous on the sex loci/all responsible sex loci. This happens rarely under outbreeding, because sex loci are generally highly variable (Yokoyama and Nei 1979; Hasselmann and Beye 2004). In an inbreeding situation on the other hand, chances are high that the two related mating partners share the same alleles on their sex loci resulting in the production of diploid male offspring. Diploid males are often non-viable or sterile and, in contrast to workers, they do not take over any work chores in the nest, which reduces overall colony performance (Plowright and Pallet 1979; Ross and Fletcher 1986; Cook 1993; Tarpy and Page 2002). In honey bees workers detect and kill diploid males in order to lower these costs (Woyke 1963). In some species diploid males are not entirely sterile but can father triploid offspring. This is for example the case in the bumblebee *Bombus terrestris* (Ayabe et al. 2004), in the parasitoid wasp *Cotesia vestalis* (de Boer et al. 2007) and in the ant *Tapinoma erraticum* (Cournault and Aron 2009). The first meiotic division in the spermatogenesis in Hymenopteran males is usually aborted during metaphase I (Hoage and Kessel 1986; Hogge and King 1975). In diploid males this results in the production of two diploid instead if four haploid spermatids that form a triploid zygote when fusing with an egg cell. Triploid individuals are expected to be sterile (de Boer et al. 2007; de Boer et al. 2012). Therefore diploid males, even if they are able to sire triploid offspring, are a reproductive dead end. So far, only two species are known, in which diploid males are able to sire diploid offspring: the solitary hunting wasp *Euodynerus foraminatus* (Cowan and Stahlhut 2004) and the parasitoid wasp *Cotesia glomerata* (Elias et al. 2009). It is yet unclear whether in these exceptional cases a reductional cell division occurs during spermatogenesis or if one chromosome set is eliminated at some point during the fertilization process (Cowan and Stahlhut 2004). Since both species exhibit regular inbreeding, the ability of diploid males to produce fertile offspring can be seen as an adaptation to their inbreeding life style.

Besides the consequences of diploid male production, few studies have reported other effects of inbreeding in social Hymenopteran colonies. In the ant *Formica exsecta*, the relative investment in young queens as well as the body size of newly produced males decreased with increasing homozygosity in adult workers. Since female caste determination as well as body size in ants are generally controlled by larval nutrition, these findings suggest a reduced feeding performance of adult workers in more inbred colonies (Vitikainen et al. 2011). Further, inbreeding was found to reduce queen lifespan and therefore colony survival in this species (Haag-Liautard et al. 2009). Similarly queen lifespan was reduced in the ant species *Cardiocondyla obscurior*, in which also brood mortality increased with inbreeding (Schrempf et al. 2006). In inbred bumble bee colonies queens exhibit a lower winter survival and colony foundation success (Gerloff and Schmid-Hempel 2005).

However, inbreeding can also have positive effects on social Hymenoptera as it can compensate for a reduction of relatedness and thereby restore fitness benefits to altruistic workers. A reduction of relatedness within a colony occurs under multiple matings of queens (polyandry) and/or if multiple queens reproduce within a colony (polygyny). Indeed, in *Plagiolepis pygmaea,* an ant species with polygyny and polyandry, active inbreeding has been described: Workers of this species repel unrelated males that try to enter the colony and queens preferentially mate with close relatives (Thurin and Aron 2009; Thurin et al. 2011).

### *Local mate competition among wingless ant males*

In addition to the risks and negative aspects of inbreeding, the production of wingless males results in a local mate competition (LMC, Hamilton 1967) situation within colonies: Through their inability of dispersal, wingless males compete with their nestmate rivals over matings with queens. Wingless males are therefore under selection to develop tactics to successfully mate in this local mate competition situation. Competitive behaviours like fighting (Hamilton 1979; Heinze and Hölldobler 1993; Yamauchi et al. 1996), territoriality (Frohschammer and Heinze 2009) and mate-guarding (Yamauchi et al. 2001; Foitzik et al. 2002) have been described. In contrast, the much more common winged Hymenopteran males undergo scramble competition during their mating flights. They developed general adaptations such as strong flight muscles, large eyes and good olfactory senses that help them to find queens. However, as they face scramble competition, these males cannot monopolize several females, resulting in low variation in mating success between winged males. Hence, sexual selection is weak on winged males, a reason why the mating biology and sexual selection in social insect males has received so far relatively low attention (Baer 2003; Boomsma et al. 2005). Rather, social insect males have been regarded as “simple small mating machines” (Tsuji 1996).

### *Study system and goals*

In my PhD project, I studied alternative reproductive tactics, sexual selection and inbreeding effects in the ponerine ant species *Hypoponera opacior*. The genus *Hypoponera* is mostly distributed in tropical rain forests but few species also occur in forested habitats in more temperate regions. Colonies are small and nest in the soil, preferentially under rocks. Workers are predators of small soil invertebrates (Fisher and Cover 2007). Many species of the genus exhibit alternative reproductive tactics with winged and wingless queens and/or males (Foitzik et al. 2002, Yamauchi 2001, reviewed in Rüger 2007). As a consequence of LMC wingless males either fight for the access to females within their colonies (*H. punctatissima*: Hamilton 1979; *H. bondroiti*: Yamauchi et al. 1996) or show a mate-guarding behaviour (*H. eduardi*: Le Masne 1956; *H. nubatama*: Yamauchi et al. 1996; *H. opacior*: Foitzik et al. 2002).

*H. opacior* exhibits a winged and a wingless morph in both sexes (Foitzik et al. 2002, Fig. 1). Winged sexuals conduct mating flights, followed by independent nest foundations of queens. Wingless sexuals mate within the nest and males mate-guard their partners. Thereby they show an extraordinary mating behaviour that has so far not been found in other ant genera: They embrace the cocoons of pupal queens and insert their genitalia through a hole at the rear end of the cocoon in order to inseminate the queen inside (Fig. 1 b). Even though sperm is transferred within the first five minutes of copulation, males embrace cocoons for many hours, a behaviour that has been interpreted as a form of mate-guarding (Foitzik et al. 2002). Hence, by mating with very young queens and guarding their partners, males enhance their chance of remaining the only mating partner of the queen. After emergence, the already inseminated wingless queens either stay in their natal nest, resulting in a polygynous nest structure, or they found a new colony by dependent colony foundation. Since successful colony foundation here depends on the help of workers from the mother nest and the availability of suitable nesting sites, wingless queens are expected to compete for these resources (local resource competition, LRC, Clark 1978)

All ant colonies investigated in this thesis were collected in Southeastern Arizona, most of them in the Chiricahua Mountains. The Chiricahua Mountains are part of the so called “Sky Islands”, a group of mountain ranges between the southern Rocky Mountains and the northern Sierra Madre that are separated by dry flatlands and have therefore been compared to islands in the sea (Heald 1951). Suitable habitats for *H. opacior* only occur in forests with an intermediate elevation and therefore the habitat of the species is highly fragmented in this region.

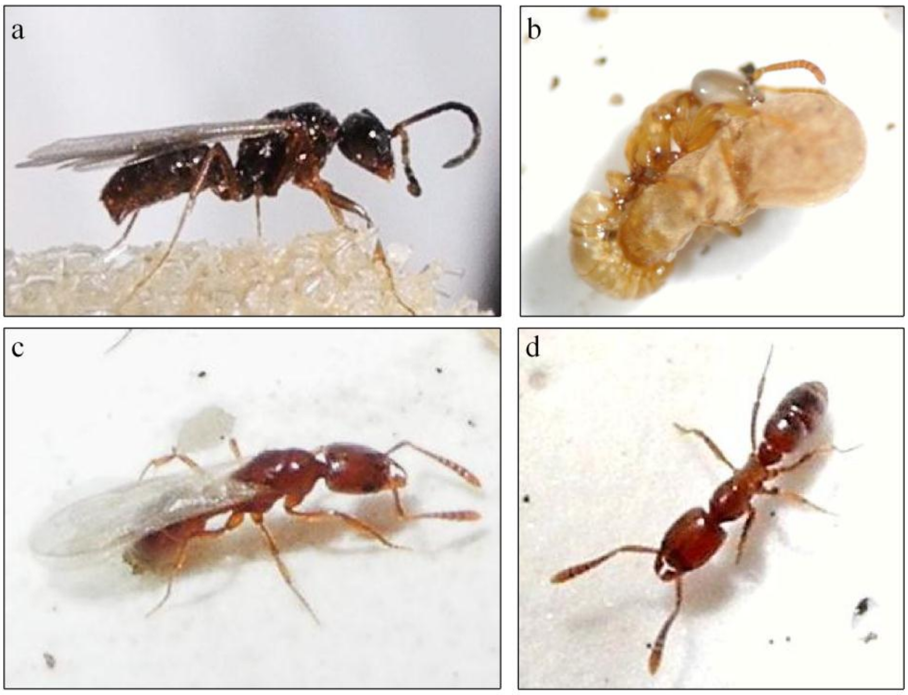

**Fig. 1**: The four sexual morphs of the ant *Hypoponera opacior*:
a) winged male b) wingless male (embracing a cocoon with a queen inside)
c) winged queen d) wingless queen

The production of two morphs in each sex and the extraordinary mating biology of wingless reproductives make *H. opacior* an optimal system to investigate factors influencing the occurrence of alternative reproductive tactics as well as mating behaviour and sexual selection under local mate competition. Since frequent sib-matings among wingless sexuals are expected to result in high levels of inbreeding, the species also offers an insight in the effects of inbreeding in a social insect. In addition, the “Sky Islands” are an interesting geographic region to study large-scale population structure. The analysis of nest structure is of interest to study local population viscosity, which could be the result of dependant colony foundation by wingless queens.

In Chapter I, I will focus on the production of wingless and winged sexuals as well as on investment strategies of the colonies. The aim of this study was to find out if colonies focus on both or either of the two reproductive tactics and which traits are responsible for the production of winged or wingless sexuals. We hypothesized that the production of either of the sexual morphs depends on the status of the colony or on environmental conditions. For example, winged sexuals might only be produced under weather conditions that lower the risks of dispersal and independent colony foundation (i.e. high humidity and moderate temperatures). Alternatively, the development of wings might depend on the genetic background of the mother, that is, intermophic queens might only produce wingless sexuals while dealate queens produce winged sexuals. Further, we studied if and how investment strategies are shaped by local factors such as LRC among wingless queens and LMC among wingless males. We expected to find different sex ratios in wingless and in winged sexuals because only wingless sexuals face local competition. Depending on the strength of these local factors, we hypothesized to find either male-biased (if LRC is stronger: Clark 1978) or female-biased (if LMC is more important: Hamilton 1977; Alexander and Shermann 1977) sex ratios. Last, we analysed if investment strategies are influenced by the relatedness structure within colonies. For example, a high relatedness among competing males could lead to a higher investment in young queens. Likewise relatedness asymmetry could be responsible for a stronger investment in males or queens. In ants, workers are usually more closely related to newly produced females than to males. The stronger this relatedness asymmetry is, the stronger workers should invest in queens in order to increase their inclusive fitness (Boomsma and Grafen 1990, 1991).

As the genetic structure of a population depends on its mating biology, dispersal abilities and habitat structure, the study presented in Chapter II was designed to investigate the population structure of *H. opacior* on three levels: within nests, within the population in the Chiricahua Mountains and between the Chiricahuas and three other mountain ranges of the “Sky Islands”. We expected to find high levels of inbreeding within colonies, because of frequent sib-matings among wingless sexuals as well as a high relatedness among neighbouring nests resulting from dependent colony foundation of intermorphic queens. Due to the highly fragmented habitat in the region of the “Sky Islands”, we expected to find a more pronounced genetic structure in-between than within the mountain ranges, because long stretches of deserts make dispersal from one mountain range to another unlikely.

The mating behaviour of wingless reproductives, in particular of wingless males, will be reported in Chapter III and Chapter IV. As mentioned above, wingless males mate with wingless queens that are still enclosed in their cocoon and mate-guard their partners for hours (Foitzik et al 2002). In the study presented in Chapter III we analysed whether males adapt their mate-guarding durations to the competitive situation in their local environment (i.e. the nest) as predicted by (Parker 1974). Further, as it had previously been observed that sometimes males also embrace cocoons of other workers and males (*H. nubatama*: Yamauchi et al. 2001; *H. opacior*: Foitzik et al. 2002), we investigated how common this behaviour is and if males behave differentially towards queens than towards male or worker cocoons in terms of genital insertion or embracing durations. Since cocoons of all three castes are very similar in outer appearance, we hypothesized that males might use chemical cues to identify the caste of a pupa. Generally cuticular hydrocarbon profiles differ between castes (e.g. Tentschert et al. 2002, Oettler et al. 2008). A high number of “false embraces” could be due to recognition errors as cuticular hydrocarbon profiles might not have fully developed yet in young individuals. We also investigated the survival rate of embraced pupae. It had been observed that pupal males sometimes die after being embraced by another male (Yamauchi et al. 2001; Foitzik et al. 2002). Hence, we were interested in finding out whether the embrace of male pupae might be a strategy to eliminate future competitors rather than just a mistake due to a recognition error. In Chapter IV I will report further investigations on the adaptive abilities of ergatoid males concerning their mate-guarding behaviours. In this study we conducted an experiment in which we manipulated the number of males within a nest in order to find out if and how fast males react to a changed competitive environment. Further, we analysed on which traits sexual selection works in wingless males under the local mate competition situation they face in their nests. Since pupal females cannot actively choose their mating partners and males do not openly fight, female choice and male-male aggression are absent in this mating situation. Therefore we hypothesized that other traits that are not related to attractiveness or fighting abilities might be more important. Hence, we analysed if the timing of emergence and body size influence a male’s mating success. While an early emergence lowers LMC during early adulthood (because the first males to emerge have fewer competitors), a large body size might lead to better abilities of embracing cocoons. Further, lager males might produce more sperm enabling them to mate more often. Sperm amount is a limiting factor in Hymenopteran males because spermatogenesis does not continue during adult life (ants in general: Hölldobler and Wilson 1990; *H. opacior*: Foitzik et al. 2002).

The effects of inbreeding were analysed in the studies I will present in Chapter V and Chapter VI. We analysed if more inbred individuals differ in body size from less homozygous individuals. On the colony level we studied if inbreeding has an impact on colony productivity and allocation ratios. We assumed that *H. opacior* might have adapted to regular sib-matings in the fall generation and therefore be less negatively affected by inbreeding than other species. Further, we here report the production, viability and fertility of diploid males. In the study presented in Chapter VI we then compared mating behaviour, lifespan, body size and sexual performance of diploid and haploid males. Moreover we investigated if ergatoid males are able to actively avoid inbreeding by choosing less related queens as mating partners. This way they might be able to keep inbreeding levels on a moderate level despite the fact that they can only choose among nestmates as mating partners.

In summary, my thesis describes the mating biology of an ant species with alternative reproductive tactics. I will describe reasons for and consequences of the production of non-dispersive sexuals and thereby especially focus on sexual selection on wingless males and consequences of inbreeding.

# Chapter I

## Alternative reproductive tactics and the influence of local competition on sex ratios in the ant *Hypoponera opacior*

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# Abstract

The ant *Hypoponera opacior* exhibits alternative reproductive morphs of males and females associated with distinct sexual behaviours. Our long-term study reports strong seasonality in sexual production with a mating season in early and one in late summer. Winged (alate) reproductives emerge in June, swarm during the monsoon season and establish new colonies independently. In contrast, wingless worker-like (ergatoid) reproductives that appear in late August mate within their natal or adjacent nests and either do not disperse or establish new nests close by. These divergent dispersal patterns allowed us to analyse the impact of local factors on investment strategies by comparing sex allocation between and within the two reproductive events. The optimal sex ratio for ergatoid reproductives should be influenced both by competition for matings between brothers (local mate competition) and rivalry among young locally dispersing queens for workers, nest sites or food (local resource competition). The greater importance of local resource competition was demonstrated both by a male-biased sex ratio for wingless sexuals and a stronger increase in the number of males with total sexual production than for the number of queens. Microsatellite analysis revealed that inter-nest variation in relatedness asymmetry cannot explain split sex ratios in the August generation. Instead, nests with related ergatoid males raised a male-biased sex ratio contrary to the expectations under local mate competition. In conclusion, male bias in wingless *H. opacior* indicates that local mate competition is less strong than local resource competition among ergatoid queens over the help of workers during nest foundation.

# Introduction

In many animal and plant taxa alternative reproductive strategies within a single sex were shown to allow flexible reactions to environmental and social conditions (Gross 1996; Lovett-Doust and Lovett-Doust 1990). The ability to vary morphological, behavioural, physiological and life history traits can enable an individual or - in the case of social insects – a colony to maximise its reproductive success under a wide range of conditions. The majority of alternative reproductive strategies and tactics that have been described are conditional strategies with alternative tactics of the male sex (Gross 1996). Males are under stronger intrasexual selection, because of severe competition for access to females and higher variation in mating success (Gadgil 1972; Trivers 1972). In contrast to the general greater diversity in male reproductive strategies, alternative reproductive phenotypes in social Hymenoptera are more common among females. The ancestral states in ants are winged males and females that conduct mating flights. Males in those large swarms undergo scramble competition and are unable to monopolise mating partners. Consequently, intrasexual selection is low in males leading to general adaptations such as strong flight muscles, excellent olfactory and visual capabilities (Boomsma et al. 2005). Yet, divergent selection frequently occurs in ant queens leading to primarily wingless, worker-like (ergatoid) queens in addition to the typical winged morph. Multiple queen morphs were found in species of all ant subfamilies. In contrast, wingless worker-like (ergatoid) males are only present in nine ant genera (Heinze and Tsuji 1995) with the highest variation in male and female reproductive phenotypes in the ant genus *Hypoponera*.

This study has two core topics. First, we study the occurrence of alternative reproductive phenotypes in the neotropical ant *Hypoponera opacior*, of which winged and ergatoid sexuals of both sexes have been described (Foitzik et al. 2002). We investigate whether ant colonies focus on the production of a single sexual phenotype, a specialisation that could be caused by their genetic background or status-dependant selection. Alternatively, reproductive morphs could be restricted to certain times of the year and thus be adapted to annual variations in the ecological or social environment. Our second main focus lays on the optimal sex allocation for the two types of reproductives. Wingless sexuals have low dispersal capabilities and these ergatoid males and females should therefore compete with members of the same sex for access to mates (local mate competition; Hamilton 1967; Alexander and Sherman 1977) or resources (local resource competition; Clark 1978). An extension of these theories, the constant male or female hypothesis (Frank 1985; 1987a; 1987b) predicts that colonies with any competition among related males should raise males up to a certain threshold and if additional resources are available invest those entirely in females. The reverse should be true under local resource competition. Polygyny, the occurrence of several reproducing queens per colony, mostly arises from adoption of daughter queens. Competition among sister queens reduces their value for these colonies and male-biased sex ratios are consequently expected (Pamilo 1990). These models therefore predict that local competition among males or females should influence optimal sex allocation in the wingless phenotypes, whereas the sex ratio of the outbreeding winged sexuals should be independent of these factors.

Variation in sex allocation between colonies can be due to factors independent of local competition. For example, variation in relatedness asymmetry was shown to co-vary with sex ratios (Sundström 1994; 1995). Split-sex ratio theory (Boomsma and Grafen 1990, 1991) explains this with workers maximizing their inclusive fitness by specialising in colonies with relatedness asymmetries above the population average on queen production and those from colonies with a below-average asymmetry, polygynous or polyandrous colonies, focusing on male production. Finally, resource availability can be a proximate predictor of sex allocation patterns, with well provisioned colonies focussing on costly queen production and colonies with fewer resources raising mainly males (Nonacs 1986a; 1986b). Recent studies indicated that sex allocation under local mate competition and in social Hymenoptera in general are less influenced by relatedness than previously thought (Burton-Chellew et al. 2008; Meunier et al. 2008; West 2009). Individuals that influence sex ratios are often constrained by imperfect information processing or fertility insurance (Shuker and West 2004). In fig wasps, male-male competition is less affected by relatedness. Instead it has been shown that the intensity of competition influences male dispersal behaviour (Nelsona and Greeff 2009). The occurrence of winged outbreeding sexuals and wingless locally competing reproductives in the ant *H. opacior* allows us to test the relative importance of local competition and relatedness on optimal sex allocation. This study addresses both issues – alternative reproductive tactics and sex allocation strategies – together and capitalises on a study system that allows this to be done.

Wingless ergatoid sexuals emerge in *H. opacior* colonies from the Chiricahua Mountains, Arizona in late August. The worker-like males were observed to copulate with ergatoid queens from the same nest for hours before the latter completely emerge from the cocoon (Foitzik et al. 2002). This mating behaviour has been described as mate-guarding, as sperm is transferred within minutes after the onset of the copulation. Inseminated ergatoid queens are either recruited into their mother nest leading to polygyny or they found new colonies with the help of workers by dispersing locally on foot (budding). Due to regular brother – sister matings, the inbreeding coefficient is very high in the study population of *H. opacior* ($F_{IS}$ = 0.447, 95 % CI: 0.33 – 0.60; Foitzik et al. 2011), the second highest value ever found in ants. Colony structure was found to be rather diffuse with dense populations of small interconnected nests in upper soil layers (Foitzik et al. 2002). Dealate, formerly winged queens were mostly collected in founding nests in less dense areas. The few discovered established nests headed by dealate queens were invariably monogynous. The timing of the presumed mating flights of winged sexuals and which colonies contributed to the production of wingless and winged sexuals was unresolved at the start of this study.

One main motivation was therefore to analyse the annual reproductive cycle by collecting colonies headed by winged and wingless queens in different seasons. We analysed whether the observed alternative sexual morphologies have a genetic basis. If true, we expected an association between the phenotype of the mother and offspring phenotypes. The alternative hypothesis states that ecological conditions could influence the production of winged and wingless reproductives. Seasonal variation in environmental conditions for example in precipitation or temperature could lead to distinct reproductive times of the year for the two sexual morphs. Such a seasonal pattern has been shown in the congeneric species *H. nubatama* with reproduction by wingless sexuals in summer and nuptial flights by alates in fall (Yamauchi et al. 2001). Ant nests could also react to unpredictable or nest-specific variation in ecological conditions. For example, environmentally stressed colonies of the ant *Cardiocondyla obscurior* switched to the production of alate males (Cremer and Heinze 2003). If small-scale ecological variation affected *H. opacior* nests, we would expect that colony size or productivity as measure of resource availability influences the production of sexuals of different phenotypes.

For our second main focus of this study – the analysis of sex allocation patterns – we developed the hypothesis that sex ratios in disperser sexuals should differ from sex ratios in ergatoid sexuals. If local mate competition is more important than local resource competition in the study population, we would expect more female-biased sex ratios in ergatoids compared to alate reproductives. If, however, resource competition among sister queens is more severe than male competition, sex ratios should be male-biased among the wingless sexuals compared to the sex ratios of disperser sexuals and vice versa.

Next, we focussed our analysis on sex ratio variation among colonies producing wingless sexuals. The constant male or female hypothesis (Frank 1985; 1987a; 1987b) states that the sex that locally competes more intensely is produced up to a certain threshold and additional resources are invested in the other sex. We therefore predict that for the sex under more intense local competition, the number of individuals of this sex should not increase as strongly with total sexual production as numbers of the respective other sex.

The intensity of competition among relatives can be inferred from the relatedness among interacting males and females. We hypothesise that colonies, in which highly related males compete for access to females, should raise fewer males compared to those with a lower relatedness between males, due to more queens contributing to male production. Under local resource competition, we similarly expect a high relatedness among new queens to lead to a male-biased sex allocation. Relatedness is not only expected to be associated with colony-level sex ratios under local competition, the split sex ratio hypothesis (Boomsma and Grafen 1990; 1991) also predicts that variation in relatedness asymmetry should be correlated with sex ratios. Colonies, in which workers are more closely related to queens than to males compared to the population average, are expected to raise a female-biased sex ratio. A meta-analysis demonstrates that about a fifth of the variation in sex allocation variation among colonies is explained by relatedness asymmetry in social hymenoptera (Meunier et al. 2008). An even less important role of relatedness for sex allocation was suggested for situations under local competition (West 2009). We will therefore analyse how relatedness asymmetry and relatedness among competing males and females influence sex allocation patterns in *H. opacior*.

# Material and methods

*Ant collection, caste determination and nest census*

To study the annual reproductive cycle of *H. opacior* and sex allocation patterns we conducted several field trips over the years 2003 to 2005 and during different seasons (spring, summer, fall) to the site in the Chiricahua Mountains AZ, USA. We did not collect ants in winter, because due to the high elevation (between 1500 – 2500 m above sea level), the winter months are cold and ants are expected to be inactive during this time of the year. We planned at least one field trip per year in August – September, because previous results showed that wingless sexuals are produced during these months (Foitzik et al. 2002). *H. opacior* nests were collected in May 2003 (N = 177), August – September 2003 (N = 80), June – July 2004 (N = 83), August – September 2004 (N =168), June – July 2005 (N = 59) and August –September 2005 (N = 157) in oak-juniper forests close to the Southwester Research station (N 31° 52.000', W 109° 12.609'). As these ants dwell in the upper soil layers preferentially directly under stones, we turned stones and small rocks to collects ant nests. Ants were transported to the laboratory either at the Southwestern Research Station or at the LMU Munich, Germany and nests were censused.

Analysing the demography and reproduction of *H. opacior* nests with their three female castes (winged queens, ergatoid queens and workers) can be challenging. The large winged queens are morphologically distinct with a structured thorax and large eyes, whereas the external morphology of ergatoid queens closely resembles that of workers. These wingless queens can be recognised only by their slightly larger eyes and by dissection. Ergatoid queens have fully functional ovaries with a spermatheca, whereas workers lack reproductive organs. Nests with 10 or fewer individuals (Σ over queens, workers and pupae; N = 254 nests) and all ant nests collected in June 2005 (N = 59) were stored in 100 % ethanol after collection. Cocoons were opened to determine their sex and caste. Larger nests were first kept alive to allow eclosion of developing individuals (N = 470). These ant nests were kept for a few weeks in three-chamber-boxes (10 cm x 10 cm x 3 cm) with a moistened plaster floor and fed with insects three times a week. In order to verify our caste determinations based on external morphology we completely dissected all female ants from ten nests in June 2005. All dissected individuals (N = 89) had been correctly assigned to caste. The sex ratio of all emerging sexuals and the number of produced pupae were determined for a subset of nests (N =

233) collected in 2004 and 2005. For all other nests, we only determined nest demography (N of queens, N of workers, N of pupae and larvae).

We analysed the demography and sex ratio data on the nest level, albeit *H. opacior* colonies occasionally consist of multiple nests (polydomy). Polydomy appears to be relatively rare and continuous exchange of brood between units is more or less absent. Behavioural observations revealed much lower aggression between nestmates than between workers from different, even geographically close nests. In addition, microsatellite analysis showed relatively high relatedness values between diploid nestmates (r = 0.20 – 0.49) and a strong reduction with distance (Foitzik et al. 2011). The diffuse colony structure (Foitzik et al. 2002) made a definition of a nest necessary. We considered a *H. opacior* nest, when we found at least one reproductive female, and/or two or more worker-like individuals, and/or brood. The term “established nests” was used for queen-right nests that contained at least one worker. Nest size was defined as the sum of workers and ergatoid queens. The productivity of nests was calculated as (N of pupae + N of new males + N of new queens) / nest size.

To assess the differential investment in different castes we killed 10 alate queens, 14 ergatoid queens, 21 workers, 5 alate males and 10 ergatoid males by freezing. These ants were dried at 60°C for 48 h and subsequently weighed on a “Satorius micro scale”. We calculated the cost ratio as the mean dry weight of queens through the mean male dry weight. The investment ratio was calculated as (N of queens per nest * cost ratio) / (N of queens * cost ratio + N of males). For the calculation of the investment ratios, we computed the cost ratio for alate and ergatoid reproductives separately. Note that alate queens were collected before the mating flight and were presumably virgins. The 14 ergatoid queens, which were used to determine dry weights, were not dissected, but dissection of a random sample of 64 freshly eclosed ergatoid queens revealed that 63 of them were mated and had developed ovaries.

The population-wide numerical sex ratio was calculated separately for all years and seasons by adding the number of all queens, which were produced and dividing this by the number of all new sexuals, summing over all colonies. The population-wide investment ratio is the product of the number of queens with and the cost ratio.

*Statistical analyses*

We compared the population-wide sex ratio between seasons using $\chi^2$ tests. For the statistical analysis of population-wide sex ratios, social structure, nest size and dry weights we used the program STATISTICA 6.0 from StatSoft.

Data on the frequency of winged and ergatoid sexuals and sex ratio data were analysed using logistic regressions. These analyses allowed us to simultaneously investigate the impact of various parameters on the variance in the occurrence of sexual morphs and the numerical sex ratio. For fitting logistic regression models and log-linear Poisson models (McCullagh and Nelder, 1989, see also Zuur et al., 2009) we used the glm command of the R statistical computing software (R Development Core Team, 2009, see supplementary material for details). We chose the glm option “family = quasibinomial” or “family = quasipoisson”, respectively, to fit overdispersed models, because we had to assume that there were unknown factors that may influence sex ratios and frequencies of winged sexuals. For model selection we used a backward elimination procedure, i.e. we started with a parameter-rich model and iteratively removed the least significant covariable/cofactor. We repeated this until only covariables and cofactors with p-values below 0.15 were left. Note that the common model selection strategy of optimizing AIC values is not applicable here since AIC values are not defined for overdispersed GLMs. As independent variables for the analysis of sex morph (frequency of winged sexuals / all sexuals) we used the cofactor year and the covariates season, nest size and productivity. For analysis of nest-level variation in the numerical sex ratio we started with a model with year, season and the presence of a dealate queen as categorical cofactors and nest size and productivity as covariates.

To analyse whether the number of males or females increases differently with total sexual production we applied overdispersed generalised linear models (GLM) of type Poisson with the canonical log link-funktion (McCullach and Nelder, 1989). We fitted these models to the June and August data separately. The expected number of new females is modelled to be $N \cdot e^{a} \cdot N^{b}$ and the expected number of males $N \cdot e^{a+c} \cdot N^{b+d}$, whereas N is the total number of new sexuals (new winged in June or new ergatoids in August), and $a$, $b$, $c$, and $d$ are parameters to be estimated. Thus, $c$ measures sex-bias and $d$ measures how the sex-bias depends on $N$, (see supplementary material for further explanations).

Using the R command summary.glm we computed p-values for covariables and cofactors, which are based on Student-t-approximations for the null-model distribution of the estimated model coefficients of GLMs. An R script containing the commands that were used in this analysis is available from D.M. upon request.

### *Genetic analysis*

For genetic analysis 572 individuals from 92 *H. opacior* nests were used, of which 510 (89 %) of the individuals and 82 nests were derived from August sampling. The mean number of analysed individuals per nest was 6.22 (range 2 - 29; SD ± 4.33). Individual specimens were preserved in 100% ethanol or frozen at –20°C until extraction. DNA was isolated from individual ants using the Puregene DNA extraction kit (Gentra Systems). The ants were genotyped at the following five microsatellite loci HoP 26, HoP 54, HoP 58, HoP 60 and HoP 64 (Rüger et al. 2005). The applied protocol and program is given in (Rüger et al. 2005) and for amplification we used a PXE 0.2 Thermal Cycler (Thermo Electron Corporation). Amplified fragments were detected on a MegaBACE (Amersham Biosciences) and analysed using the program Fragment Profiler 1.2 (Amersham Biosciences). The regression relatedness values were calculated by weighting nests equally and the standard errors (SEs) were estimated by jackknifing over loci with the program RELATEDNESS 5.0.8 (Goodnight and Queller 1994; Queller and Goodnight 1989). Life-for-life relatedness was used throughout.

## Results

### *Nest demography*

To gain insights into the annual reproductive cycle and founding mode of the different queen morphs we started with a demographic analysis of the 724 *H. opacior* nests, which were collected between 2003 and 2005. 585 of these nests (81 %) were queenless, 100 (14 %) were headed by one or more ergatoid queen(s) and 39 (5 %) were headed by dealate queen(s). Over all years only 1 (2.7 %) of the 37 dealate queen nests was polygynous, while 31 (31 %) of the 100 nests with ergatoid queens were polygynous. The frequency of polygynous nests was significantly higher for ergatoid queen nests than for dealate queen nests over all years (Fisher's exact test: $p < 0.001$).

In nests, in which we discriminated between workers and queens by dissection, the mean number of ergatoid queens was lower in May / June (1.56, range 1 – 7; SD ± 1.54) than in August (4.15; range 1 – 21; $U = 777.0$, $N_{1,\,2} = 62, 53$, $p < 0.001$). A high fraction of dealate queen nests were founding nests without workers (20 nests = 51 %), while only 3 % of ergatoid queen nests were collected during the founding stage (Table 1). Yet, established nests with dealate queens contained more workers than established nests with ergatoid queens. The number of pupae and the productivity per worker-like individual did not differ between the two types of nests (Table 1).

Castes differed in dry weight (ANOVA: $F_{4,55} = 73.36$, $p < 0.001$; Table 2). Alate reproductive morphs were heavier than ergatoid ones: wingless queens weighed 82.5 % of winged queens and ergatoid males weighed about 58.9 % of winged males. Ergatoid queens were only 11.8 % heavier than workers. The cost ratio (mean dry weight of queens / mean dry weight of males) for the production of winged and ergatoid sexuals was 1.63 and 2.28, respectively.

**Table 1** Demographic comparisons between nests with dealate and ergatoid queens. Results are depicted as N or mean ± SE (range). Significant p-values are in bold letters. Nest productivity calculated as (N ((pupae + N (new males) + N (new queens)) / nest size).

| | | Nests with dealate queen | Nests with ergatoid queen | Test |
|---|---|---|---|---|
| N of nests | | 39 | 100 | |
| N of founding nests | | 20 | 3 | $\chi^2 = 47.37$; |
| N of established | | 19 | 97 | **p < 0.00001** |
| Established nests | Workers | 30.94 ± 6.29 (2-90) | 15.14 ± 1.71(1-99) | U = 504.0; **p < 0.005** |
| | Pupae | 13.94 ± 6.93 (0- | 29.38 ± 8.21(0-657) | U = 863.5; p = 0.997 |
| | Nest productivity | 1.60 ± 0.75 (0-10.6) | 1.92 ± 0.38 (0-26.3) | U = 816.0; p = 0.981 |

**Table 2** Dry weight of the different *H. opacior* morphs in µg after drying at 60°C for 48 h were significant different from each other (ANOVA: F4,55 = 73.36, p < 0.001). Asterisks indicate the p-value of the Fisher LSD post hoc test, ** = p < 0.01, *** = p < 0.001.

| | N | Mean ± SE (µg) | Range | Fisher LSD (p) | | |
|---|---|---|---|---|---|---|
| Alate queens | 10 | 246 ± 9 | 190 - 291 | *** | | |
| Ergatoid queens | 14 | 203 ± 6 | 165 - 252 | | ** | *** |
| Workers | 21 | 179 ± 5 | 117 - 217 | | | |
| Alate males | 5 | 151 ± 9 | 151 - 157 | *** | | |
| Ergatoid males | 10 | 89 ± 2 | 79 - 97 | | | |

*Annual reproductive cycle and population-wide sex allocation*

*H. opacior* colonies contained sexuals at two different times of the year. The first reproductive season, in which 97 % of all reproductives were alates, was found to be from mid-June to the beginning of July (18th of June – 2nd of July). Only ergatoid sexuals were produced in *H. opacior* nests in August to mid-September (5th of August to 14th of September) in both years (Table 3). We found no impact of sexual morph of the mother queen on offspring morph: All colonies producing winged sexuals in June were headed by ergatoid queens and all but two colonies raising ergatoid sexuals in August were similarly headed by ergatoid queens. Two August nests with dealate queens raised wingless males.

Then, we tested the hypothesis that the relative strength of local competition among males and among queens should influence sex ratios in the wingless generation. Female-bias relative to the outbreeding winged sexuals would indicate a preponderance of mate competition; male-bias would point to the importance of local resource competition among queens. The population-wide numerical sex ratio (including both types of sexuals) was slightly female-biased in June 2004 (0.600; test for even sex ratio $\chi^2 = 2.6$; $p = 0.11$) and more strongly so in 2005 (0.676; $\chi^2 = 26.2$; $p < 0.0001$). In contrast, the sex ratio was male-biased in August of both years, albeit not significantly in 2004 (tests for even sex ratio: 2004 $\chi^2= 0.9$; $p = 0.36$; 2005 $\chi^2= 9.9$; $p = 0.002$). Population-wide numerical sex ratios differed between seasons in both years (2004 $\chi^2 = 6.12$; $p < 0.015$; 2005 $\chi^2 = 68.9$; $p < 0.0001$). Due to the higher dry weight of queens, the investment ratio in June is even more female-biased than the numerical sex ratio (June 2004: 0.715; June 2005: 0.776). Besides, the investment ratio is also female-biased in August of both years (Table 3). Analysis of nest level sex ratios showed a bimodal distribution with some nests investing exclusively in males, while others produce predominantly female reproductives (Fig. 1). In August, nearly 49 % of *H. opacior* nests with an investment ratio of zero raised only a single (ergatoid) male.

**Table 3** Sex allocation in a *H. opacior* population from Southeastern Arizona from 2004 and 2005. The numerical sex ratio is given as (N of queens / (N of males + N of queens)). The investment ratio was calculated as (N of queens per nest * cost ratio) / (N of queens * cost ratio + N of males).

| Year | Nest (N) | Nests producing pupae (N in %) | Sexual morph | Nests producing sexuals (N) | sexuals (N) | | Population-wide numerical sex ratio | Population-wide investment ratio |
|---|---|---|---|---|---|---|---|---|
| | | | | | Male | Female | | |
| June 2004 | 23 | 15 (65 %) | ergatoid | 4 | 4 | 0 | 0 | 0 |
| | | | alate | 18 | 49 | 79 | 0.617 | 0.724 |
| August 2004 | 31 | 29 (94 %) | ergatoid | 31 | 56 | 43 | 0.434 | 0.637 |
| | | | alate | 0 | 0 | 0 | | |
| June 2005 | 22 | 21 (95 %) | ergatoid | 6 | 7 | 5 | 0.417 | 0.620 |
| | | | alate | 11 | 125 | 271 | 0.684 | 0.779 |
| August 2005 | 157 | 131 (83.4 %) | ergatoid | 85 | 328 | 224 | 0.406 | 0.609 |
| | | | alate | 0 | 0 | 0 | | |

### *Factors influencing the occurrence of winged or ergatoid sexuals*

Besides the apparent effect of season on sexual morph, we investigated which other parameters influence whether winged or ergatoid sexuals were produced. Since there was no variance in August - all emerging sexuals at this time of the year were ergatoids - we focussed on the June data. In June, none of the colonies with dealate queens produced sexuals, so that winged and wingless sexuals were produced solely by nests headed by ergatoid queens. An overdispersed logistic regression model was fitted to the data with year as cofactor, nest size and productivity as covariates and the relative frequency of winged sexuals as response variable (for details see supplementary material). None of the independent variables was significant, so we applied a stepwise variable-elimination procedure. First we removed productivity ($p = 0.46$; Table S1), then year ($p = 0.56$; Table S2) and the final model contained only nest size, which also did not show a significant impact on the frequency of winged reproductives among sexuals ($p = 0.22$; Table S3).

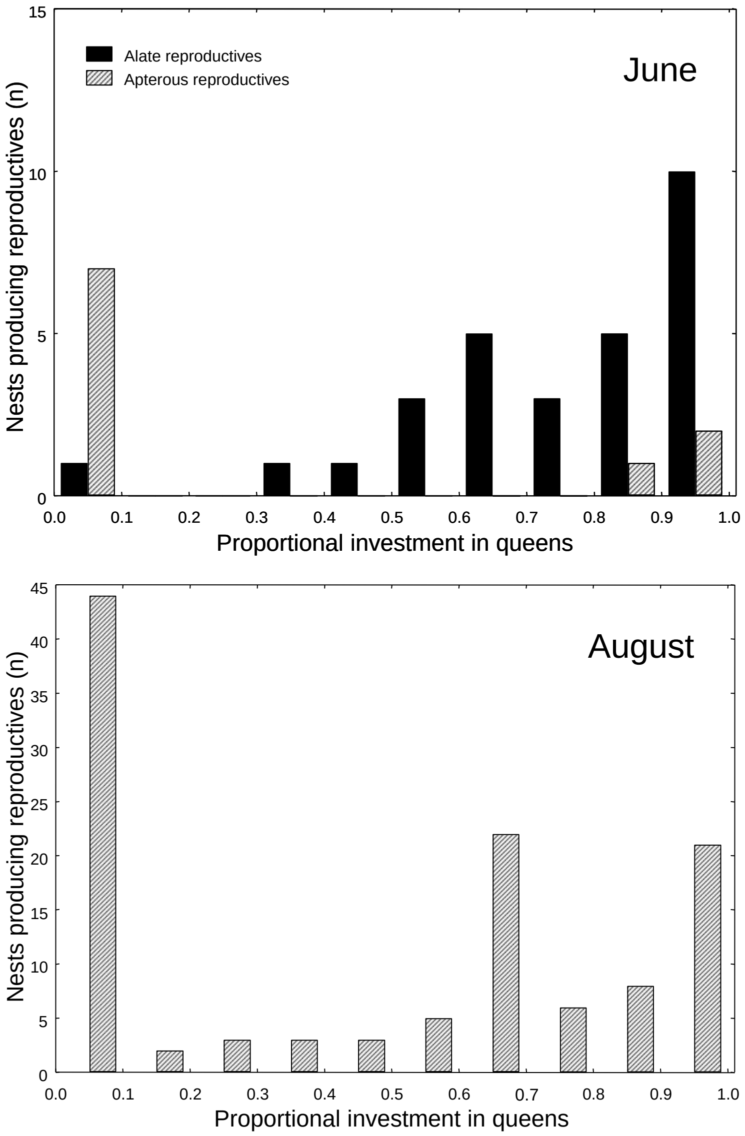

**Fig. 1** Proportional investment for both queen morphs in *H. opacior*, given as proportion of investment in young queens from total sexual investment, including the cost ratio values for alate and apterous morphs. Proportional investments within each reproductive morph did not differ between 2004 and 2005. The bars represent pooled data from June and August of both years, respectively.

### *Factors influencing sex ratios*

Sex ratios are not only influenced by local competition, but also by nest-specific factors such as annual and micro-geographic variation in resource availability, nest size and possibly queen morph. For example, well-provisioned colonies – more productive or larger nests - are expected to raise a female-biased ratio. We fitted an overdispersed logistic regression model to the nest-level data to explore which factors influence the numerical sex ratio in addition to variation due to production of outbreeding winged or locally competing wingless sexuals (see supplementary material for details). We started with a model with year, season and the presence of a dealate queen as categorical cofactors and nest size and productivity as numerical cofactors. First, we removed the presence of a dealate queen (p = 0.88), then productivity (p = 0.84) and finally year (p = 0.67). Only season and nest size remained in the final model, and both were significant (Table 4; Fig. 2). As shown in the population-wide analysis, nest-level data also demonstrate that the numerical sex ratio is more male-biased in August nests compared to June nests. In addition, larger nests showed a more female-biased numerical sex ratio (Fig. 2). Adding interactions between these two co-variables did not significantly improve the model fit (Analysis of deviance p = 0.48; see supplement for details).

**Table 4** Result of overdispersed binomial logistic regression on the influence of season and nest size on the numerical sex ratio. Dispersion parameter was estimated as 2.18.

| | Estimated coefficient | Standard deviation | T | p |
|---|---|---|---|---|
| Intercept | -0.097 | 0.202 | -0.480 | 0.63 |
| Season (effect of August) | -0.844 | 0.185 | -4.557 | 0.00001 |
| Nest size | 0.043 | 0.009 | 4.829 | 0.00001 |

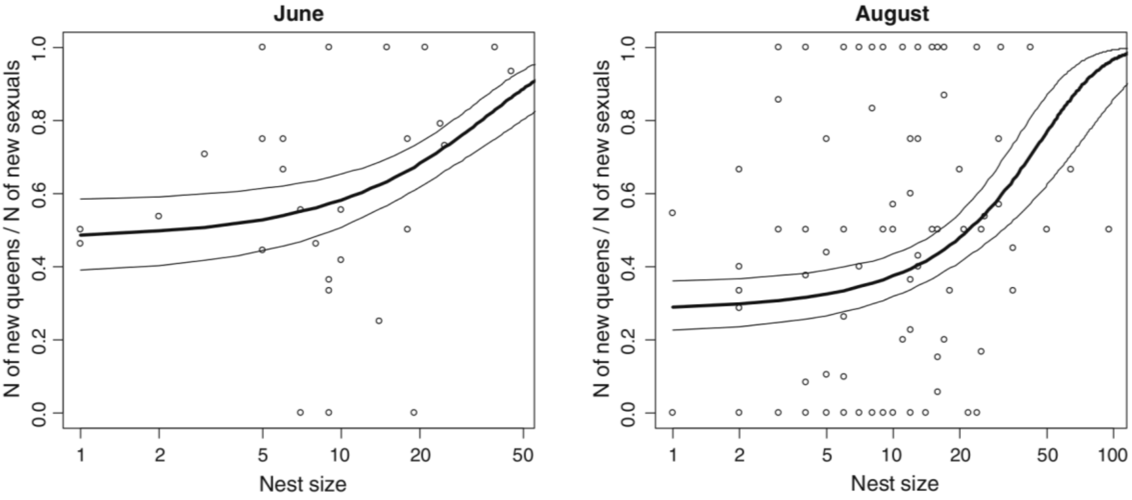

**Fig. 2** Influence of season and nest size on the numerical sex ratio. The numerical sex ratio is given as N of queens / N of sexuals. Nest size is given on the x-axis in logarithmic scale. Thick lines show predictions of an overdispersed logistic regression model (see supplementary material) and thin lines give 95 % confidence bands.

### *Factors influencing the total number of males and females raised per nest*

For locally competing wingless sexuals, we expect a slower increase in the number of sexuals with total sexual production for the sex, which competes more intensely locally. Albeit this prediction can only be made for the wingless sexual generation, we analysed the June data set in an analogous manner to find out whether the patterns are similar across seasons, potentially indicating that local factors are of minor importance.

We fitted overdispersed generalised linear models of type Poisson with the canonical log link-function to the data to assess how the numbers of new queens and new males depended on the total number of new sexuals (see supplementary material for details). Let $N$ be the number of new sexuals produced by a nest. For the June data the expected numbers of new queens and new males are predicted to be $0.54 \cdot N^{1.0513}$ and $0.49 \cdot N^{0.9022}$ (Fig. 3). If we consider these terms as functions of $N$, the coefficients 0.54 and 0.49 approximate the slope of these functions as long as the exponents are close to 1. The slopes correspond to the expected relative frequencies of new queens and males. The difference between the coefficients 0.54 and 0.49 corresponds to $c = -0.102$ and is not significant ($p = 0.70$). However, the two exponents 1.0513 and 0.9022 differ ($d = -0.149$; $p = 0.02$). The number of winged queens and is a slightly convex function of $N$, the function for the males is slightly concave.

The same model applied to the number of ergatoid queens and males in August revealed that the slopes of the functions for queens and males are significantly different (Fig 3; $c = -0.533$; $p = 0.03$) with a stronger increase in the number of males with total sexual production. Differences in the curvatures of the functions are not significant ($d = -0.080$; $p = 0.45$). This analysis shows that the sex-ratio in August is significantly more male-biased, while the sex bias in June depends on the total number of new sexuals. The latter significance vanishes if we exclude a June nest which has an exceptionally high number of more than 200 new sexuals. Details on this analysis and a logistic regression analysis, which leads to the same conclusions, are shown in the supplementary material.

### *Impact of relatedness and relatedness asymmetries on sex ratios*

In the above analysis we uncovered a male-biased sex ratio in the locally competing August generation. This could not only be due to local resource competition among queens, but also due the variation in relatedness asymmetries (split sex ratio theory) between June and August nests. We therefore compared relatedness values and relatedness asymmetries between nests from different seasons. The life-for-life relatedness of workers as calculated from the entire data set was slightly lower in June (mean 0.194 ± SE 0.061) than in August (mean 0.427 ± SE 0.100; -test: $t = 1.73$, $n_{1, 2} = 4, 58$, $p = 0.062$). Life-for-life relatedness values of workers to queens did not significantly vary between the two reproductive periods (June $r_{wq} = 0.379$ ± SE 0.031; August $r_{wq} = 0.389$ ± SE 0.040; t-test: $t = 0.328$, $n_{1, 2} = 5, 39$, $p = 0.745$). Neither differed the life-for-life relatedness of workers to males between June and August (June $r_{wm} = 0.141$ ± SE 0.044; August $r_{wm} = 0.134$ ± SE 0.021; $t = 0.184$, $n_{1, 2} = 5, 19$, $p = 0.856$). The relatedness coefficient of workers to queens was significantly greater than that of workers to males (repeated-measures ANOVA: $F_{1,20} = 33.62$, $p = 0.00001$). Relatedness asymmetry of workers to queens and males was not different between June (ratio = 2.68 : 1) and August (ratio = 2.90 : 1; $F_{1,20} = 0.04$, $p = 0.84$).

Next, we explored the association between nest-level variation in relatedness values and sex ratios for the August data set, where sex allocation could be influenced by local competition. The intensity of local mate competition could be revealed by relatedness among competing males with the expectation of female-biased sex ratios in nests with highly related males. However, albeit we uncovered that the numerical sex ratio of ergatoids in August depended on the relatedness of nestmate males, we found male-biased sex ratios in nests with high relatedness among males (Spearman Rank $r_s$=-0.48; $p < 0.035$ $N = 20$; Fig. 4a). Intra-nest relatedness of workers or queens was not associated with the numerical sex ratio (Spearman Rank worker r: $r_s$=-0.11; $p = 0.67$ $N = 20$; Fig 4b; ergatoid queen r: $r_s$=0.02; $p = 0.91$; $N = 27$; Fig. 4c). To analyse whether inter-nest variation in the numerical sex ratio within a season could be caused by variation in relatedness asymmetry (split sex ratio theory), we tested for an association between these two factors. Only few colonies produced both young queens and males – sex ratios were generally split - so that our statistical power for this analysis was rather low. Relatedness asymmetry varied greatly between nests (min - max: 0.39 – 5.73; SD 1.46). We did not find a significant association between the numerical sex ratio and relatedness asymmetry that is the relatedness of workers to queens divided by the relatedness of workers to males (Spearman Rank $r_s$=-0.44; $p = 0.13$; $N = 13$; Fig. 4d). The non-significant correlation went into the opposite direction as expected: colonies with a higher relatedness of workers to queens than to males raise a male-biased sex ratio.

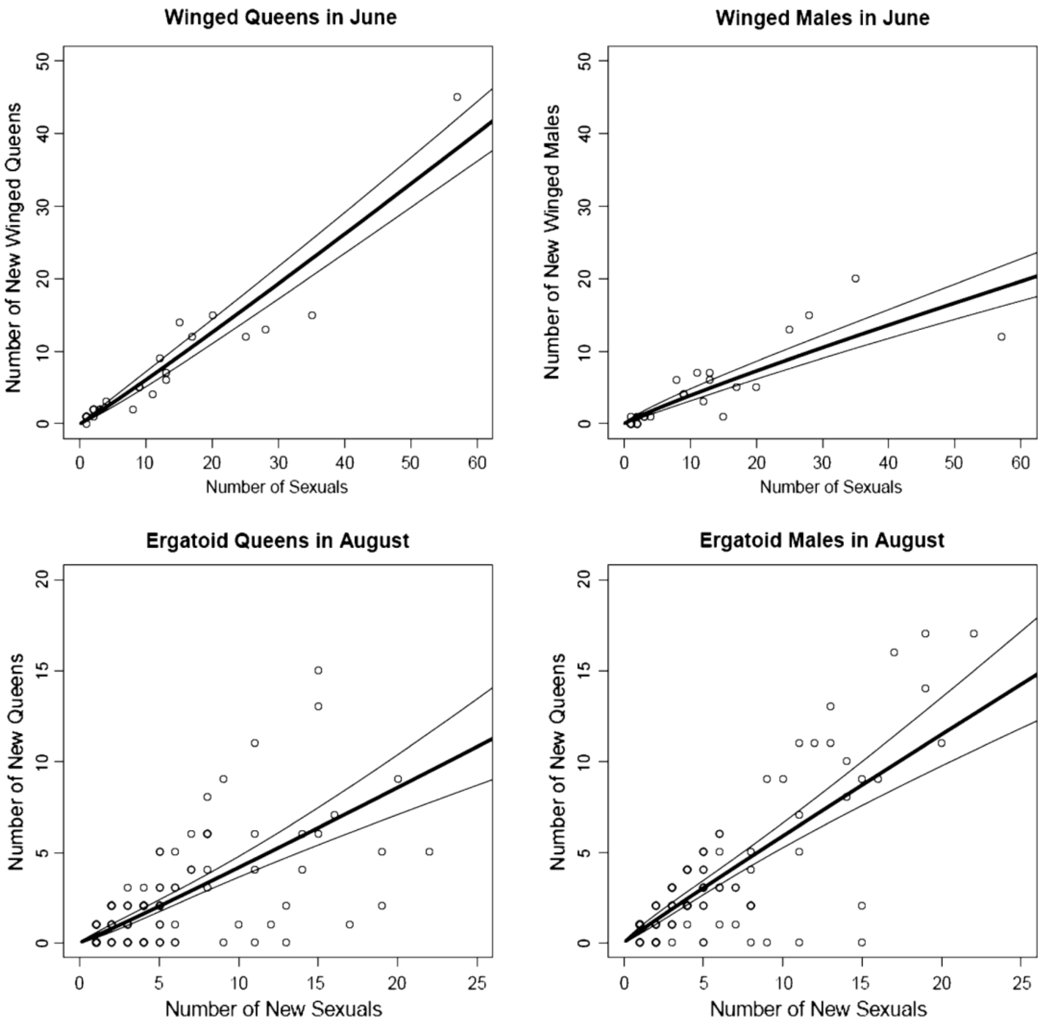

**Fig. 3** The association between sexual production and the number of queens and males in August and June. The thick lines show the predictions of an overdispersed log-linear Poisson regression model (supplementary material) and the thin lines give confidence bands. The plot range of the June data excludes a nest with an exceptionally high number of more than 200 new sexuals. Plots of the full range are shown in the supplementary material.

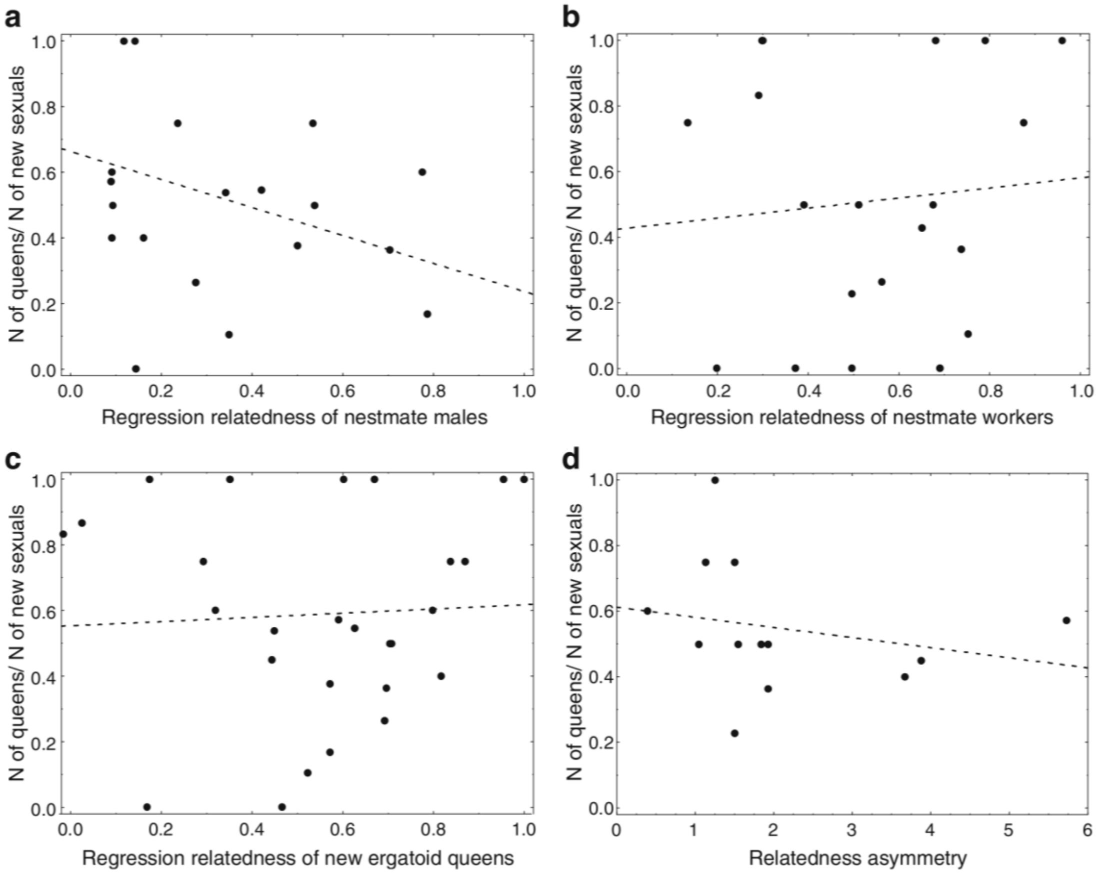

**Fig. 4** The influence of relatedness on the numerical sex ratio of ergatoid sexuals in August. a) Association between regression relatedness of nestmate males with numerical sex ratio. Nests with high male-male relatedness values raise mainly males. b) Association between regression relatedness of nestmate workers with numerical sex ratio. c) Association between regression relatedness of young nestmate ergatoid queens with numerical sex ratio. d) Association of relatedness asymmetry (relatedness of workers to young queens / relatedness of workers to males) with numerical sex ratio.

# Discussion

The ant *Hypoponera opacior* pursues a reproductive strategy with two alternative tactics for queens and males. The heritability of sexual phenotypes appeared to be low or absent, as the morph of the resident queen did not affect offspring morph. Winged sexuals were invariably produced by nests headed by wingless queens and we also found nests with dealate, formerly winged queens, which raised wingless sexuals. Instead we found support for the alternative hypothesis as reproductive tactics varied with season. In early summer, at the onset of the summer monsoon, more than 95 % of all sexuals were outbreeding alates, whereas in August reproductives were invariably ergatoids. We did not detect an impact of nest-specific variation in ecological conditions. Neither nest size nor productivity significantly influenced the frequency of winged or wingless sexuals produced by a nest. Concerning our second main focus – optimal sex allocation patterns – both between and within season comparisons demonstrated that local resource competition is more important than local mate competition among related males. Sex ratios were male-biased for the locally competing wingless sexual generation and male bias further increased with sexual production. Male-male relatedness was associated with sex ratios in the opposite direction as expected under local mate competition, while relatedness asymmetry was not significantly correlated to colony-level sex ratios in contrast to the expectations of the split sex ratio hypothesis (Boomsma and Grafen 1990; 1991). Finally, in both reproductive seasons larger nests showed a more female-biased sex ratio, indicating that resource availability is important for these ants (Nonacs 1986a; 1986b).

In *H. opacior* sex ratios of the wingless sexuals raised in late summer should be influenced by local competition among males for mates (Alexander and Sherman 1977; Hamilton 1967) and among queens for resources such as workers or nest sites (Clark 1978). In contrast to the classical model developed by Hamilton (1967) for species with ergatoid males, in *H. opacior* wingless queens also show strongly reduced dispersal abilities. Their winglessness promotes local resource competition, as the reduced dispersal capability of ergatoid males facilitates local mate competition. We found a more male-biased numerical sex ratio in August compared to June in contrast to the expectations under local mate competition and despite similar relatedness asymmetries in June and August. Furthermore, male-male relatedness co-varied with sex ratios for August nests, but in the opposite direction as expected under local mate competition: nests with a high relatedness among males contained the highest number of males, whereas nests with low male-male relatedness showed more female-biased sex ratios. This counter-intuitive result is difficult to interpret, but we thought of two possible explanations, which need to be tested in future. Nests with female-biased sex ratios might attract non-nestmate males from neighbouring nests, which disperse on foot through subterranean tunnels. If true, a low relatedness among males in nests with female-biased sex ratios could be the consequence of female bias rather than its cause. Alternatively the observed pattern can be the result of male-male competition. Males might accept the presence of highly related males in the nest, while unrelated males displace each other more often.

The constant male or female hypothesis predicts (Frank 1985; 1987a; 1987b) that for the more intensely locally competing sex, the number of individuals of this sex should increase less strongly with total sexual production as numbers of the respective other sex. As we found that August nests with high sexual production raised relatively fewer wingless females we conclude that in the Arizonan population of *H. opacior* the value of ergatoid queens is more strongly reduced through local competition than the value of ergatoid males. However, social and ecological conditions can influence the intensity of competition among males and among queens. A study on *Technomyrmex albipes*, a Japanese ant species with a similar biology (Tsuji and Yamauchi 1996) found a highly female-biased sex ratio among wingless sexuals indicating that under the prevailing conditions local mate competition is more pronounced than local resource competition.

Why do related queens compete so strongly in *H. opacior*? Ergatoid queens, who emerge from the cocoon already mated, have two reproductive options: They can either stay in the mother nest or they can disperse on foot. Only 3 % of the founding nests contained ergatoid queens, so that most ergatoid queens bud with part of the work force from mother nest. Both reproductive strategies of these queens will lead to intense competition with the mother and sister queens. In ant species that reproduce by budding, accompanying workers are part of the resources invested by a colony in female production, if they remain permanently with the new queen in the daughter nest (Hamilton 1975; Pamilo 1991). Here we did not investigate worker production in detail. Including workers into calculation of the investment ratio in August would shift it to a female-bias, approaching the value observed for winged sexuals in June. This indicates a somewhat balanced tug-of-war between the local forces acting on the reproductive values for queens and males.

We found a strong bimodal distribution of sex ratios at the nest level (split sex ratios), especially in the August data set. However, the split sex ratio theory (Boomsma and Grafen 1990; 1991) was not supported as relatedness asymmetry did not co-vary with sex ratios, albeit we observed strong variation in relatedness asymmetries between nests. This analysis was based on a low sample size of only 13 nests, as most nests either raised queens or males so that the relatedness asymmetry could not be determined. Moreover, neither worker nor ergatoid queen relatedness correlated with sex ratio patterns. These data do not support the local resource competition hypothesis as we would have expected that nests where highly related queens compete for resources should raise a male-biased ratio. Only male-male relatedness was associated with the numerical sex ratio, but in the opposite direction as predicted under local mate competition. A recent review (Meunier et al. 2008) concluded that relatedness asymmetry explains about a fifth of the reported variation in colony-level sex ratios in social Hymenoptera. This fraction was similar to that explained by local mate competition or local resource competition, in species in which relatives compete. In *H. opacior* we did not find an effect of relatedness asymmetry, but clearly local resource competition influences optimal sex allocation. Variation in nest-level sex allocation was further associated with nest size with larger nests allocating relatively more resources into queens. These findings can be explained by resource limitation (Nonacs 1986a; 1986b), because in smaller nests with a lower and less stable food supply a larger fraction of the diploid brood develops into workers and not into queens leading to a male-biased sex ratio.

In *H. opacior* only few nests reared ergatoid males in June, which may have the opportunity to copulate with freshly emerged alate queens. Copulation of winged males within the mother nest was never observed and it is therefore likely that, as in many other ant species, winged males need a mating flight to trigger sexual behaviour (Heinze and Tsuji 1995; Boomsma et al. 2005). Intrasexual conflict over reproduction between two male morphs should therefore be rare, but has been observed in *C. obscurior*, where alate males mimic the odour of young queens to be not attacked and killed by apterous fighter males (Cremer et al. 2002).

Why does *H. opacior* pursue a dual strategy with regular production of two reproductive morphs of both sexes? In ants, reproduction by winged queens and males is the ancestral state (Heinze and Tsuji 1995; Heinze and Keller 2000; Boomsma et al. 2005) and this might be also true for the ant genus *Hypoponera*, in which at least two species, *H. nippona* and *H. sauteri*, reproduce only through alates (Yamauchi et al. 2001). While ensuring dispersal, mating flights followed by independent nest foundation are exceedingly risky endeavours. The production of ergatoid reproductives allows mating in the safe haven of the nest and dependant nest foundation with a start-up help of workers. Yet it also leads to inbreeding and competition among sexuals with low dispersal capabilities. Thus, reproduction by both reproductive morphs allows settlement of new habitats through alates, followed by rapid expansion by ergatoid reproductives in suitable patches (Yamauchi et al. 1991).

The success of independent nest foundation by single alate queens is affected by habitat characteristics such as micro-climate, nest site and food availability and the presence of predators, etc. An increase in dispersal costs due to declining ecological conditions may lead to a larger fraction of non-dispersing queens (Heinze and Tsuji 1995). Emergence of *H. opacior* alates in July coincides with the onset of the North American Monsoon in Arizona, which brings the highest annual precipitation rates (Fig 5: precipitation data from 1965-2005 by courtesy of the "Western Regional Climate Centre", http://www.wrcc.dri-edu; Higgins et al. 1999). Nuptial flights are often linked to recent rain fall (Kaspari et al. 2001a; 2001b) and especially Arizonian ant species regularly conduct nuptial flights at the beginning of the summer raining season (Bartz and Hölldobler 1982; Hölldobler 1976; Nagel and Rettenmeyer 1973). The second reproductive period in late summer could be due to the favourable conditions in July and August. Indeed, we found a generally higher productivity of *H. opacior* nests in August than in the comparatively dry June. After *H. opacior* alate queens have successfully started a colony, they have to raise their first workers. Dealate founding queens were relatively common in August, but we found only few successful nest foundations with dealate queens in spring. Indeed, survival during the first winter is low and probably depends on the work force. This might be another important reason for an early in season mating flight as it extends the time to set up a colony and rear the first workers.

Each of the two queen morphs in *H. opacior* exhibits an alternative reproductive tactic that is reflected in the social structure of their nests (Foitzik et al. 2002). Our larger sample corroborates earlier data: all but one established dealate queen nest contained a single queen, whereas nests with ergatoid queens were headed significantly more often by several queens. Furthermore, similar to the situation in *H. nubatama* (Yamauchi et al. 2001) established dealate queen nests were infrequent and represented less than 3 % of all nests. *H. opacior* colonies raise high numbers of alates in early summer so that the minute fraction of dealate nests must be due to low founding success and/or the possible short life span of dealate queens. The number of mated ergatoid queens was highest shortly after their emergence in late summer, while we found fewer queens per nest in June, reflecting nest budding either before or directly after hibernation.

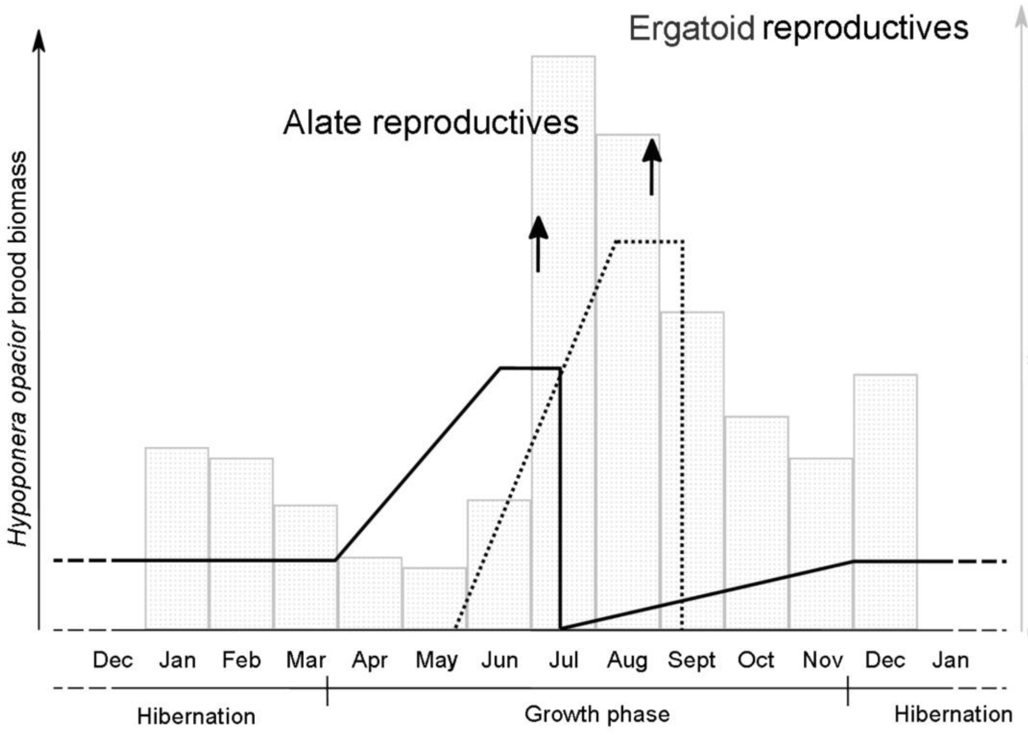

**Fig. 5** The annual cyclic production of sexuals in *H. opacior* as observed from 2003 to 2005. The solid line indicates the brood-biomass of predominantly alate reproductives; the dashed line indicates the brood-biomass of ergatoid reproductives. The grey dotted bars represent the mean precipitation rate at the SWRS near Portal, Arizona, USA, of the years 1965 to 2005. The emerging of predominately alate reproductives and workers in late June coincides with the onset of the North American Monsoon. Apterous reproductives and workers begin to emerge in late August. The left picture shows an alate queen and the right an ergatoid male mating with a still in the cocoon enclosed ergatoid queen.

The ant species *Cardiocondyla obscurior* raises long-distance dispersing alate males only under adverse environmental conditions, e.g. extreme temperature changes or food shortage (Cremer and Heinze 2003). In *H. opacior* the production of alate and ergatoid reproductives predominantly coincides with seasonal changes. Only in June some nests produce both sexual morphs, but factors reflecting microgeographic ecological variation such as nest size or productivity were not associated with the relative production of winged or wingless sexuals. Possible periodic environmental cues for the production of winged sexuals could be low winter temperatures and for wingless sexuals high precipitation rates during the monsoon season. Cold temperatures can influence the developmental avenue of hibernating larvae and the feeding activity of workers, which was shown for the ant *Myrmica rubra*, where hibernated (vernalised), large, third instar diploid larvae developed mainly into queens, while smaller ones developed into workers (Brian 1955; Brian 1975).

he occurrence of alate and ergatoid reproductives of both sexes was also described in the congeneric species *H. eduardi* and *H. nubatama* and in the ant *Technomyrmex albipes* (Le Masne 1956; Yamauchi et al. 1991; Yamauchi et al. 2001). In *Technomyrmex* alate reproductives were produced alongside ergatoid sexuals, while in *H. nubatama* reproductive cycles were reversed to those observed in *H. opacior*. Ergatoid reproductives appeared in this Japanese *Hypoponera* species mainly in summer, whereas alate reproductives conducted nuptial flights in fall (Yamauchi et al. 2001). The climate of the Western part of the Japanese main island Honshu, where this *Hypoponera* species occurs, is characterized by high precipitation rates in September – October. Possibly, alate production in ant species with two reproductive events is generally adapted to local rainfall patterns.

The documentation of two annual reproductive phases in an Arizonan population of the ponerine ant *H. opacior* elucidates the importance of ecological factors on alternative reproductive tactics and sex allocation patterns. The unusual and clear temporal separation of the production and the mating season of the two sets of reproductive phenotypes provides the unique possibility to investigate how seasonal changes influence nest foundation success and how dispersal and competition affect optimal investment strategies. Our study documents a low impact of relatedness on sex ratios and that local resource competition among wingless queens can be more pronounced than local mate competition among wingless males.

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# Supplementary Material

*Software used*

To fit generalised linear models to our data we used the glm command of the R statistical programming environment. Dispersion parameters were estimated by the glm routine. During model selection prodedures we checked significance of regression coefficients based on approximation with Student's t-distribution as implemented in the R command summary.glm. We used the command anova.glm to perform analyses of deviance.

*Factors influencing sex ratios*

Outcome of overdispersed binomial logistic regression for the frequency of new queens among new sexuals:

| | Estimated coefficient | Standard deviation | *t* value | *p*-value Pr (>│t│) |
|---|---|---|---|---|
| (Intercept) | -0.0971 | 0.202 | -0.480 | 0.632 |
| as.factor(month)8 | -0.8441 | 0.185 | -4.557 | 0.00001 |
| Nest.size | 0.0430 | 0.00891 | 4.829 | <0.00001 |

(Dispersion parameter estimated as 2.18.)

This means that the relative frequency of males in a nest of size n is predicted to be

$$\ell(-0.0971 - 0.043 \cdot n) \qquad \text{in June} \qquad (1)$$

$$\ell(-0.0971 - 0.8441 - 0.043 \cdot n) = \ell(-0.9412 - 0.043 \cdot n) \qquad \text{in August} \qquad (2)$$

whereas $\ell$ is the logistic function defined by: $\ell(x) = 1 / (1 + e^{-x})$

Analysis of deviance with *F*-distribution approximation of model with cofactors month and nest against model cofactor month only:

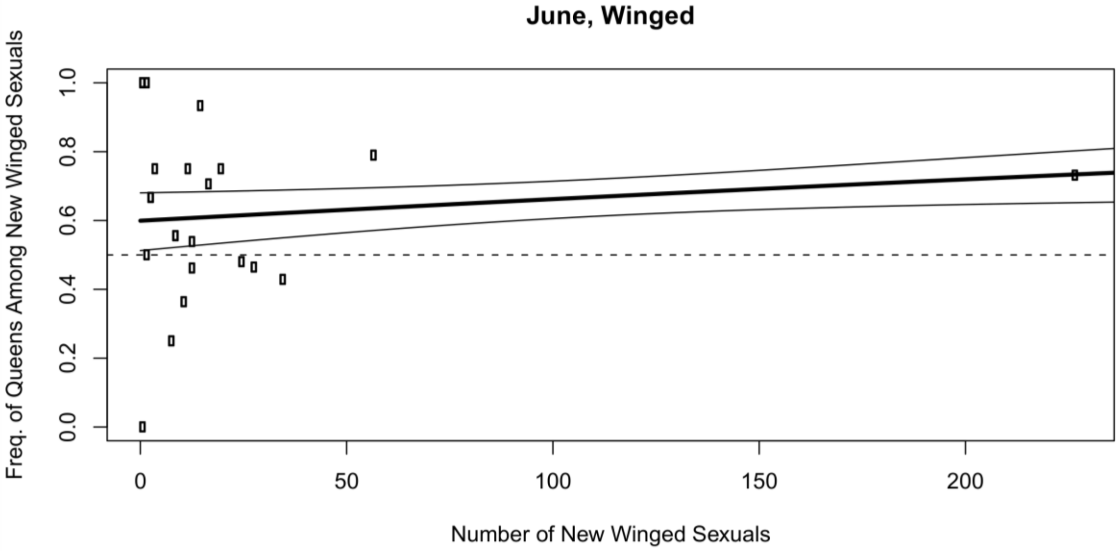
| | Df | Resid. Deviance | Df | Deviance | *F*-value | p-value (Pr (>F)) |
|---|---|---|---|---|---|---|
| Model with month | 144 | 414.54 | | | | |
| Model with month and nest size | 143 | 368.76 | 1 | 45.787 | 20.997 | < 0.00001 |

*Influence of total sexual production on sex ratio*

*New winged in June*

We fit an overdispersed binomoial logistic regression model to the data with the frequency of

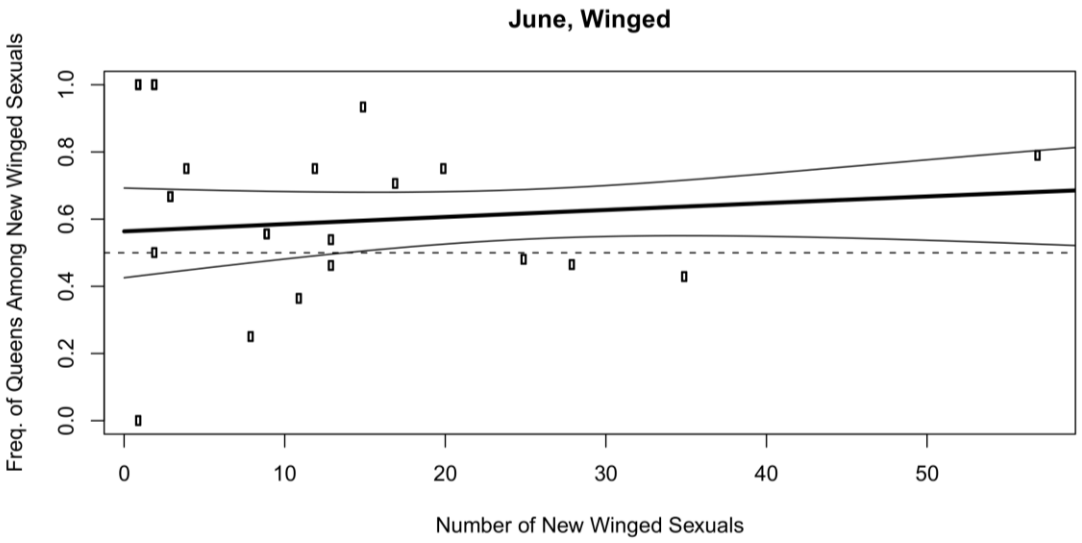
queens among the new winged as response and the total number of new winged as explanatory variable. The results are as follows:



| | Estimate | Standard error | *t* value | Pr (>│t│) |
|---|---|---|---|---|
| (Intercept) | 0.40308 | 0.17614 | 2.288 | 0.0305 |
| Sexuals.winged | 0.00270 | 0.00123 | 2.198 | 0.0371 |

(Dispersion parameter for quasibinomial family taken to be 1.689509)

Thus, the sex ratio (frequency of queens among new winged) is slightly but significantly increased for nests of higher productivity. This slope is not significant anymore when we exclude the exceptionally productive nest with more than 200 new sexuals from the analysis:



| | Estimate | Standard error | *t* value | Pr (>│t│) |
|---|---|---|---|---|
| (Intercept) | 0.256096 | 0.277988 | 0.921 | 0.366 |
| Sexuals.winged | 0.008825 | 0.009075 | 0.973 | 0.3401 |

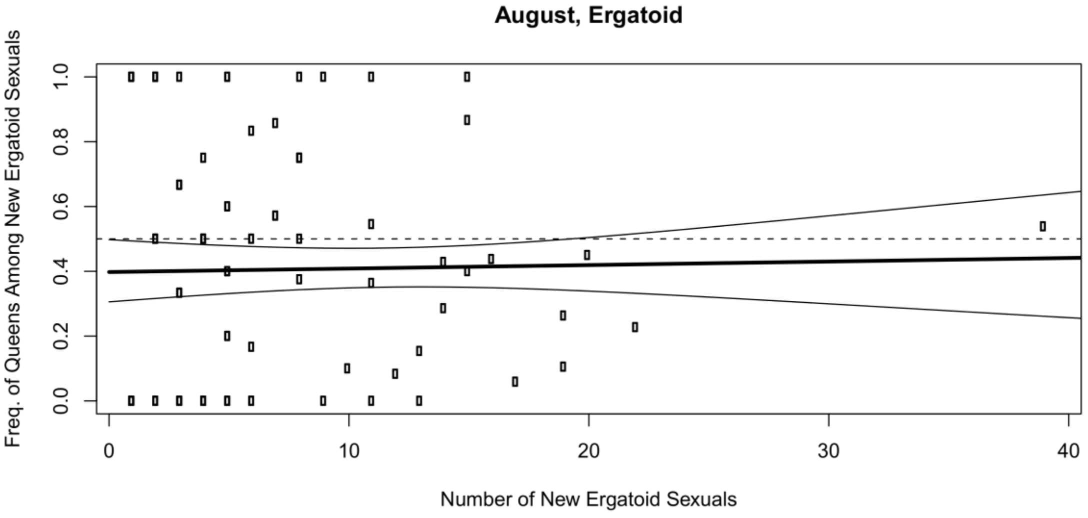
(Dispersion parameter for quasibinomial family taken to be 1.722239)

*New ergatoids in August*

The analogous analysis as above with ergatoids in August gives the following results:

| | Estimate | Standard error | *t* value | Pr (>│t│) |
|---|---|---|---|---|
| (Intercept) | -0.414655 | 0.202845 | -2.044 | 0.0432 |
| Sexuals.ergatoid | 0.004451 | 0.013816 | 0.322 | 0.7479 |

(Dispersion parameter for quasibinomial family taken to be 2.471819)



*Comparing sex ratios in June and August*

| | Estimate | Standard error | *t* value | Pr (>│t│) |
|---|---|---|---|---|
| (Intercept) | 0.318261 | 0.200677 | 1.586 | 0.1150 |
| New.Sexuals | 0.003042 | 0.001392 | 2.185 | 0.0305 |
| month.August | -0.732916 | 0.281079 | -2.608 | 0.0101 |
| New.Sexuals:month.August | 0.001409 | 0.013478 | 0.105 | 0.9169 |

(Dispersion parameter for quasibinomial family taken to be 2.326935)

This means that the relative frequency of queens among the new sexuals in June and August is predicted to be $\ell(0.318 + 0.00304 \cdot \mathrm{N})$ and $\ell(0.318 - 0.733 + (0.00304 + 0.00141) \cdot \mathrm{N}) = \ell\,(-0.415 + 0.00445 \cdot \mathrm{N})$, respectively, whereas N is the total number of sexuals in the same month.

The dependence of the sex ratio on N is significant. The frequency of queens is significantly lower in August than in June. The difference between the slopes of the sex ratios with respect to N in the two seasons is not significant.

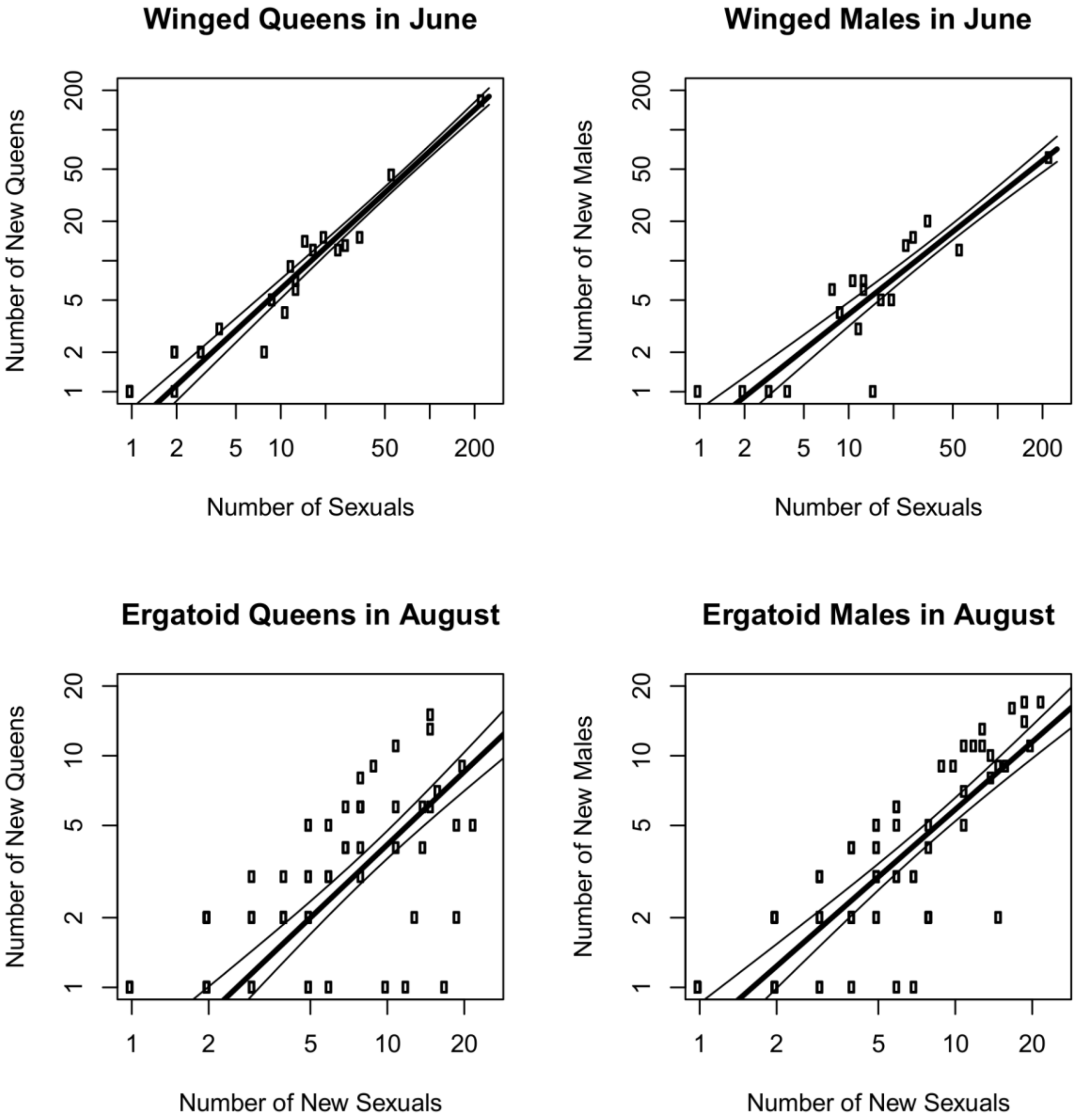
When we fit the same model to the data excluding the exceptionally productive nest, all p-values are above 10 % (details not shown). A simpler model without the interaction term, however, shows that the sex ratios are still significantly different between the seasons:

| | Estimate | Standard error | *t* value | Pr (>│t│) |
|---|---|---|---|---|
| (Intercept) | 0.4082 | 0.1771 | 2.305 | 0.022624 |
| Month.August | -0.7716 | 0.2147 | -3.594 | 0.000447 |

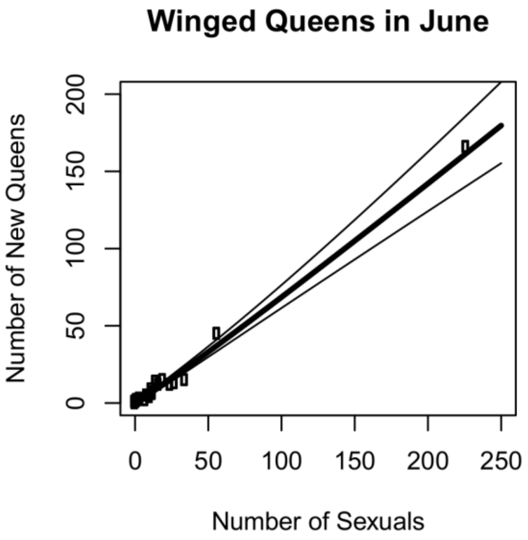
(Dispersion parameter for quasibinomial family taken to be 2.325449)

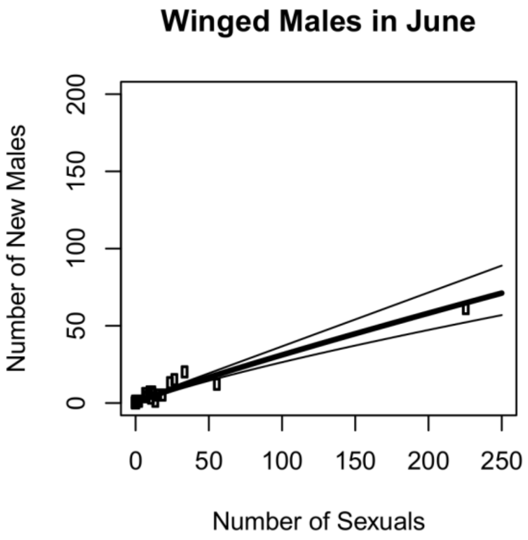
*Numbers of new males and queens depending on total number of new sexuals*

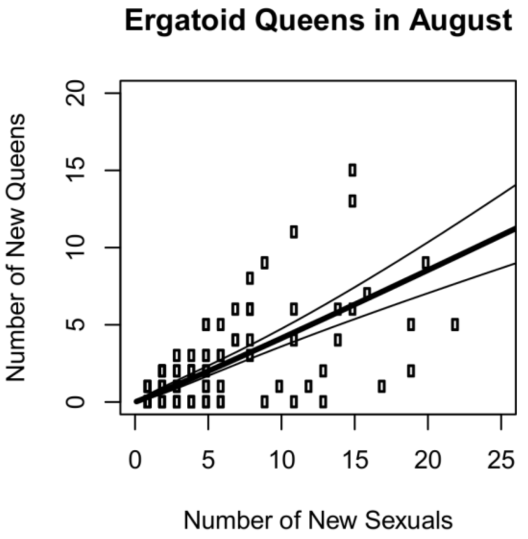
We fit overdispersed log-linear general linear models of type Poisson to the data to assess how the numbers of new queens and new males depend on the total number of new sexuals. Before we explain the details of the models we visualize their outcome. Thick lines show predicted values and thin lines give confidence bands for the expected values.

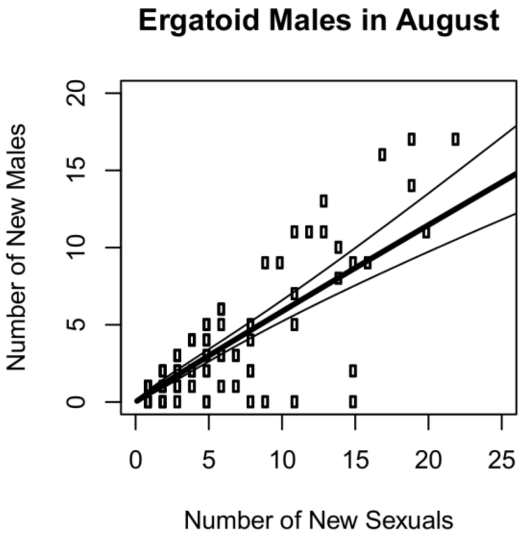


The same data non-log scaled:


Winged Queens in June


Winged Males in June


Ergatoid Queens in August


Ergatoid Males in August

*Numbers of new winged females and males per colony in June*

Overdispersed log-linear GLM of type Poisson: Let N be the numbers of new sexuals produced by a colony. The expected number of new females is modeled to be $N \cdot e^{a} \cdot N^{b}$ and the expected number of males $N \cdot e^{a+c} \cdot N^{b+d}$. The model parameters were estimated as follows:

|   | Estimate | Standard error | *t* value | Pr (>\|t\|) |
|---|---|---|---|---|
| a | -0.6138 | 0.1621 | -3.787 | 0.000397 |
| b | 0.0513 | 0.0373 | 1.378 | 0.174257 |
| c | -0.1024 | 0.26158 | -0.392 | 0.696791 |
| d | -0.1491 | 0.0625 | -2.386 | 0.020719 |

(Dispersion parameter for quasipoisson family estimated as 0.912)

This means that the expected numbers of new queens and new males are predicted to be $0.54 \cdot N^{1.0513}$ and $0.49 \cdot N^{0.9022}$. The significance of d means that the exponents of N are significantly different for queens and males. This significance gets lost when we exclude the exceptionally productive nest with more than 200 new sexuals from the analysis. Then, the results are as follows:

|   | Estimate | Standard error | *t* value | Pr (>\|t\|) |
|---|---|---|---|---|
| a | -0.4504 | 0.2401 | -1.876 | 0.0665 |
| b | 0.0103 | 0.0780 | -1.132 | 0.8954 |
| c | -0.5626 | 0.3932 | -1.431 | 0.1588 |
| d | -0.0272 | 0.1272 | 0.214 | 0.8315 |

(Dispersion parameter for quasipoisson estimated to be 0.9012832)

*Numbers of new ergatoid queens and males per colony in August*

The same model as explained above applied to the new ergatoid queens and males in August gives the following results:

|   | Estimate | Standard error | *t* value | Pr (>\|t\|) |
|---|---|---|---|---|
| a | -0.9927 | 0.1879 | -5.282 | $2.98 \cdot 10^{-7}$ |
| b | 0.0473 | 0.0811 | 0.583 | 0.560 |
| c | 0.5332 | 0.2411 | 2.212 | 0.028 |
| d | -0.0796 | 0.1049 | -0.759 | 0.448 |

(Dispersion parameter for quasipoisson family taken to be 1.237115)

Note that c is significantly positive, which means that significantly more males than queens are produced.

# Chapter II

## Macro- and microgeographic genetic structure in an ant species with alternative reproductive tactics in sexuals

This chapter is based on a research article published in the *Journal of Evolutionary Biology:*

Foitzik S, Rüger MH, **Kureck IM**, Metzler D (2010) Macro- and microgeographic genetic structure in an ant species with alternative reproductive tactics in sexuals. J Evol Biol 24:2721-2730

# Abstract

The genetic structure of social insect populations is influenced by their social organisation and dispersal modes. The ant *Hypoponera opacior* shows diverse reproductive behaviours with regular cycles of outbreeding via winged sexuals and inbreeding via within-nest mating wingless sexuals that reproduce by budding. This unusual life cycle should be reflected in the genetic population structure and we studied this on different scales using microsatellites. On a macrogeographic scale, populations were considerably structured and migration rates within the Chiricahuas were higher than those in-between mountain ranges. On a local scale, our analyses revealed population viscosity through dependant colony foundation and a high genetic diversity with a multi-colonial structure. The latter was also evident from recognition trials revealing consistent aggression between non-nestmates. Within-nest matings led to high inbreeding coefficients. Finally, the observed seasonal changes in relatedness can be explained by variation in queen number and differential dispersal of the two reproductive morphs.

# Introduction

The genetic structure of animal populations is influenced both by internal (species-specific) and external factors that can interact. Species-specific parameters include dispersal capabilities, inbreeding and genetic drift due to variation in population sizes (Hamilton 1964; Clemencet et al. 2005; Ruda et al. 2010). Landscape and climate influence population structure mainly over the distribution, fragmentation and carrying capacity of suitable habitats. For example, populations can be structured, because gene flow is reduced due to geographic barriers like islands, deserts or because of fragmentation in human-dominated landscapes (e.g. Christiansen and Reyer 2011; Zachos and Hartl 2011). Further, population structure can be formed by historic events such as the range expansion from relict populations after the ice ages (Schmitt and Seitz 2001; Grant et al. 2011).

A typical pattern of population structure in continuous habitats is isolation-by-distance (IBD), which occurs when the chances of mating decrease between individuals with geographic distance. The scale of this pattern depends on the dispersal capabilities of the focal species. In ants, winged sexuals disperse in mating flights over distances of fifty meters to a few kilometres (Hölldobler and Wilson 1990). In harvester ants for example, genetic analyses revealed average dispersal distances between 65 and 86 m and IBD on this scale (Suni and Gordon 2010). Mating and dispersal during nuptial flights followed by independent colony foundation of winged queens is the ancestral reproductive mode in ants. However, many ant species developed different mating and dispersal behaviours including within-nest mating and dependant colony foundation during which wingless queens accompanied by workers start new nests in walking distance of their natal nest (Peeters and Ito 2001). Especially this alternative reproductive mode of colony budding or fission, that is frequently shown by polygynous (multi-queen) colonies, can lead to strong structuring of ant populations on a local scale. It results in a high relatedness among neighbouring nests and decreasing relatedness values over the distance of several meters and such population viscosity has been described in a number of ant species with dependant colony foundation (Seppa and Pamilo 1998; Giraud et al. 2000).

Restricted dispersal can also result in a high relatedness among mating partners and inbreeding in turn causes a high degree of homozygosity among offspring. In social

Hymenoptera this can bear fitness costs such as the production of sterile diploid males (e.g. Cook 1993; Agoze et al. 1994; Santomauro et al. 2004), reduced colony growth and survival (Plowright and Pallett, 1979; Ross and Fletcher 1986; Tarpy and Page 2002; Gerloff and Schmid-Hempel 2005), changed sexual investment and smaller body size (Fortelius et al. 1987; Haag-Liautard et al. 2009). Nevertheless, inbreeding and diploid males have been detected in several ant species with local dispersal (Cole and Wiernasz 1997; Foitzik and Heinze 2001; Sundström et al. 2003; Haag-Liautard et al. 2009; Thurin and Aron, 2009). Moreover, in ants of the genera *Cardiocondyla* and *Hypoponera* (Yamauchi et al. 1996; Yamauchi et al. 2001; Foitzik et al. 2002; Schrempf et al. 2005; Schrempf et al. 2006) and in many invasive ant species (Cremer et al. 2008; Drescher et al. 2010) regular intranidal (within-nest) matings occur.

In the ant species *Hypoponera opacior* both winged and wingless sexuals are produced during two discrete mating seasons (Foitzik et al. 2002; Foitzik et al. 2010): Winged males and queens emerge in June, conduct mating flights at the onset of the monsoon season in July and thereafter dealate queens found new colonies independently (Foitzik et al. 2010). The large-scale mating flights of this winged morph should lead to outbreeding. In contrast, later in the year, in August to September, wingless males emerge and mate with young wingless queens within the natal nest, so that sib-matings are expected to occur regularly during this mating season (Foitzik et al. 2002; Kureck et al. 2011). These regular cycles of inbreeding should lead to high rates of homozygosity and potentially the production of diploid males in *H. opacior* colonies. Sterile diploid males can be produced under inbreeding in Hymenoteran species with single locus complementary sex determination (Whiting 1939; Whiting 1943). Inseminated wingless queens either stay in their colony leading to polygynous nests or found new colonies with the help of workers in walking distance of the natal nest. This reproductive mode should consequently lead to population viscosity on the scale of a few meters.

The heritability of sexual phenotypes in *H. opacior* is low or absent as the morph of the resident queen does not affect offspring morph: winged sexuals are produced both by nests headed by wingless and dealate, formerly winged queens and nests headed by either type of queens raise wingless sexuals during the late summer reproductive season. Workers are produced by both sexual morphs, but wingless queen nests produce on average more workers than dealate queen nests (Foitzik et al. 2010).

The low frequency of founding nests headed by dealate queens (5 % of all nests) indicated that long distance dispersal and successful independent colony foundation is risky and rarely successful (Foitzik et al. 2010). The genetic structure of *H. opacior* populations might therefore be strongly influenced by these rare dispersal events. If dense *H. opacior* nest aggregations are the result of a single, long distance colonisation event, low genetic variation is expected on a microgeographic scale. The few alleles introduced by founder queens in a local patch might get lost in time through frequent inbreeding of wingless sexuals. In addition, the local structure should also be affected by the behavioural strategies of wingless sexuals and workers, which could disperse on foot over short distances.

The here conducted investigation of the population structure in *H. opacior* is of interest not only because of the diverse reproductive biology with diverging dispersal capabilities of the two different sexual morphs, but also because these ants occur in a fragmented landscape. Our main study area is situated in the Chiricahua Mountains in Southeastern Arizona, a region that belongs to the so-called “Sky Islands” at the contact zone between the Rocky Mountains and the Sierra Madre. Suitable forest habitats in these mountains are isolated by desert habitat (Heald 1951). Climate changes since the Pleistocene led to the retreat of oak-juniper and pine forests to the more humid sites along the slopes and on-top of these peaks, reaching more than 3000 m in elevation (Marshall 1957). *Hypoponera opacior* mainly occurs in oak-juniper forests at intermediate elevations, and hence its habitat is highly fragmented. Our macrogeographic population genetic analyses may thus provide novel insights into migration rates of the tiny winged reproductives (about 4 mm in length) among habitats of the same and different mountain ranges, which are separated by more than 50 km.

With this population genetic study we aim at a better understanding of the impact of alternative reproductive tactics and dispersal modes onto genetic patterns on different geographic scales. Specifically, we test the following hypotheses:

1. We start on the widest scale of different subpopulations, which inhabit habitat islands on the different mountain ranges in Southeastern Arizona. We hypothesize that due to the fragmented habitat on this scale, isolation-by-distance is not to be expected. However, genetic structure should be more pronounced between than within mountain ranges, because these are separated by long stretches of desert habitat, which are difficult to cross for dispersing winged sexuals.
2. Next, we investigate local structure within meters. We expect to find population viscosity and potentially IBD with geographical close colonies being genetically similar on a fine scale. This genetic pattern should also be reflected in the behaviour

of ant colonies towards each other. Ant nests are expected to react less aggressively to workers of related neighbouring nests than to those from geographically more distant colonies.

3. We study the nest level by investigating colony relatedness, which is expected to vary with social structure and season. Finally, from our observation of regular intra-nidal matings, we predict high inbreeding values in this species.

# Material and methods

*Samples and microsatellite analysis*

*Hypoponera opacior* colonies were collected at Southeastern Arizona (USA) in 1998 and between 2003 and 2005. We collected ant colonies between May and September, with collection times somewhat variable between study years. We genotyped a total of 855 individuals from 376 nests. Samples were preserved in 100 % ethanol and stored at –20 °C until DNA extraction. Ants were washed in ddH$_2$O and thoroughly homogenised after treatment with liquid nitrogen. Subsequently, DNA was isolated using the Puregene DNA extraction kit (Gentra Systems). The five microsatellite loci HoP 26, HoP 54, HoP 58, HoP 60 and HoP 64 (Rüger et al. 2005) were amplified in a PXE 0.2 Thermal Cycler (Thermo Electron Corporation) following the protocol and program given in Rüger et al. (2005). Fragment length was detected on a MegaBACE (Amersham Biosciences) and analysed using the program Fragment Profiler 1.2 (Amersham Biosciences).

To study general patterns of genetic diversity, we used only a single diploid individual per nest. Deviations from Hardy-Weinberg equilibrium observed and expected heterozygosities and the inbreeding coefficient $F_{IS}$ at each locus were calculated with the program GDA (Lewis and Zaykin 2002). Diploid individuals exhibiting only a single allele at one locus can either be homozygous or they can be heterozygous for a non-amplifying null allele. We estimated the potential frequency of these null alleles using the equation: $f = (H_E - H_O) / (1 + H_E)$ (Brookfield 1996). Individuals that are homozygous for null alleles should give no amplification product at this locus, and we therefore contrasted the frequency of potential null alleles with the occurrence of individuals giving no amplification product at certain loci to analyse whether null alleles could explain a preponderance of homozygotes in our data set. A general absence of linkage disequilibrium between these five microsatellites was formerly shown in *H. opacior* for the same study population within the Chiricahua Mountains with the single exception of the loci HoP 26 – HoP 58, which behave not entirely independent (Rüger et al. 2005).

For calculation of relatedness within ant colonies, a sample from the Chiricahua Mountains with multiple individuals and castes per nest was used. Unidirectional and bi-directional relatedness values between castes were calculated with the program RELATEDNESS 5.0.8 (Queller and Goodnight 1989; Goodnight and Queller 1994). For these calculations we weighed colonies equally and 95 % confidence intervals were estimated by jackknifing over nests.

### *Macrogeographic structure*

We sampled four subpopulations in Southeastern Arizona located on the slopes of four different mountain ranges separated by wide stretches of unsuitable desert habitat: the Chiricahua, the Santa Catalina-, the Pinaleno- and the Huachuca Mountains (Fig. 1). We collected only at a single locale (deme) in three mountain ranges, whereas we sampled seven geographically separated demes within the Chiricahua Mountains. For population genetic analysis, we genotyped a single diploid individual each from 344 nests: 327 workers, 3 dealate queens and 14 wingless queens. We sampled on average 34 nests per deme with a range from 27 to 58. To avoid multiple sampling of individuals from the same colony due to polydomy (multiple nest-sites per colony) of *H. opacior*, a minimum distance between sampled nests of at least five meters was maintained. On the other hand, to avoid substructure within demes (e.g. the Wahlund effect; Wahlund 1928) we collected ant colonies in patches of < 100 x 100 m. We conducted a hierarchical F-analysis with the program R (R Development Core Team 2009) using the hierfstat package of Goudet (2005), which allowed us to quantify genetic differentiation between the hierarchical levels ‘demes’, ‘subpopulations’, and total. For the ‘subpopulation’ level we pooled the demes (1–6 and 10) from the Chiricahua Mountains and thus obtained four subpopulations corresponding to the different mountain ranges. We computed bootstrap confidence intervals for the hierarchical F values and performed permutation tests to assess the significances of the deme and subpopulation level for genetic structure.

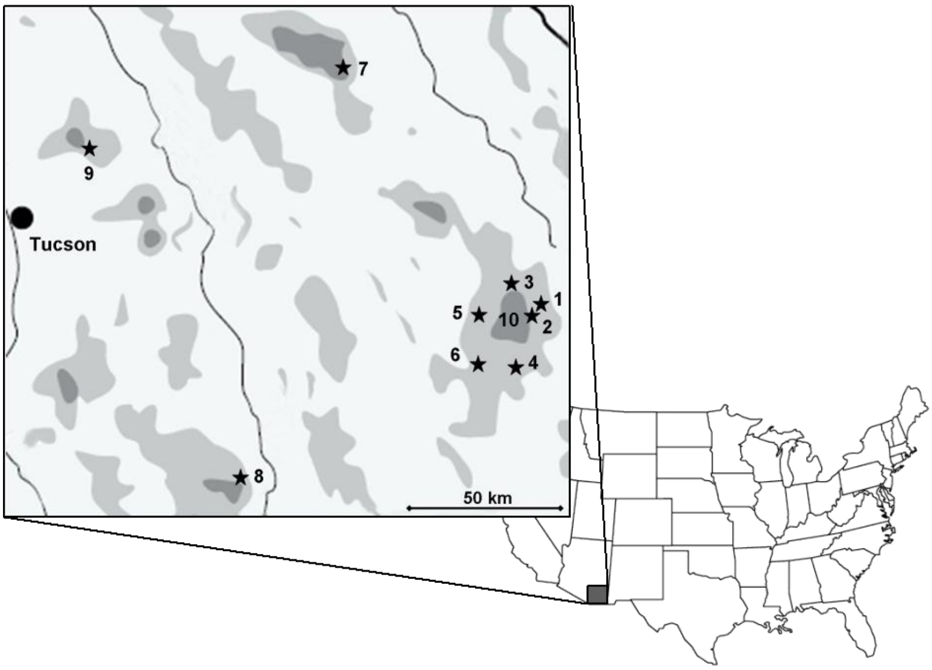


**Fig. 1** Collecting sites of *Hypoponera opacior* in Southeastern Arizona
1 = Cave Creek (Chiricahua Mts, N 31° 52.867’, W 109° 11.682’)
2 = F-Plot close to SWRS (Chiricahua Mts, N 31° 52.000', W 109° 12.609')
3 = Pinery Canyon (Chiricahua Mts, N 31° 57.089', W 109°18.595')
4 = Price Canyon (Chiricahua Mts, N 31° 45.131', W 109° 15.184')
5 = West Turkey Creek (Chiricahua Mts, N 31° 51.883', W 109° 21.489')
6 = Rucker Canyon (Chiricahua Mts, N 31° 45.427', W 109° 22.201')
7 = Mt. Graham (Pinaleno Mts, N 32° 38.740', W 109° 48.707')
8 = close to Nickville (Huachuca Mts, N 31° 26.214', W 110° 16.535')
9 = Mt. Lemmon (Santa Catalina Mts, N 32° 21.863', W 110° 42.659')
10 = Basin Trail (Chiricahua Mts, N31° 53.968’, W109° 14.321’)

### *Estimation of migration rates*

We estimated migration rates between subpopulations and demes with the Bayesian MCMC sampling method implemented in the LAMARC software (Version 2.1.3, cf. Kuhner and Smith 2007). We allowed for two different migration rates, both scaled according to the microsatellite mutation rate. One of the two migration rates was assumed for pairs of demes within the Chiricahua Mountains and the other rate was used for all subpopulation pairs located on different mountain ranges. To convert migration rates into numbers of immigrating gametes per generation we multiplied the estimated migration rates by the LAMARC estimations for $\theta/2=1.5N_r\mu$ and ($k$-1), where $N_r$ is the effective population size of the recipient deme, $k$ is the number of demes and $\mu$ is the mutation rate per generation and per microsatellite. We cannot infer the sex ratio of the migrants from our data. If we estimate that $g$ gametes immigrate into a deme, it could be that $g$/3 queens and $g$/3 males immigrate or that $g$/2 queens and no males or $g$

males and no queens immigrate, or anything in between. After a first visual inspection of the data we decided to run LAMARC with the “MixedKS” option, which means that the microsatellite mutation model in the MCMC procedure prefers step-wise mutations but allows also for other mutations of the microsatellite lengths. We performed eight independent LAMARC runs on the data. Each of these runs consisted of 100 initial chains with 11 000 MCMC steps each and a final run with a burn-in of 10 000 steps and 13 500 000 steps of which we discarded the first 2 700 000 and then took a sample every 27 000 steps. After each of the short chains the ratio of multi-step vs. step-wise mutation rates was optimised. Each LAMARC run took 10 −12 weeks on a core of a Quad-Core AMD Opteron$^{TM}$ 8384 processor (Advanced Micro Devices, Inc. Sunnyvale, CA, USA). Pooling the samples from the eight independent LAMARC runs we obtained 1600 sampled parameter combinations for each locus. From these samples, we estimated the joint posterior probability distribution of the two migration rates. For each locus we applied a two-dimensional kernel density procedure as implemented in the R command kde2d in the MASS package (Venables and Ripley 2002) with a grid size of 200 x 200 to estimate the posterior probability density of the migration parameters given the data for that locus. By multiplying these densities for all loci we got an estimate for the full posterior density.

### *Microgeographic structure and behavioural observations*

The microgeographic genetic structure of *H. opacior* was investigated on a 6.0 x 16.5 m plot with a high nest density, which was located on the eastern slope of the Chiricahua Mountains (Fig. 1; close to deme 2, elevation: 1659 m above sea level). In August 2004, we inspected every potential nest site closely by turning every stone/small rock in this study plot and mapped the exact position of each nest. Ant nests were maintained in three-chamber-boxes (10 x 10 x 3 cm) with a moistened plaster floor in the laboratory of the Southwestern Research Station and fed *ad libitum* with dead insects every other day. We conducted aggression tests in mid-August 2004 with a subsample of 13 nests, which contained more than 15 workers. Ant workers of each nest were marked with enamel paint and aggression behaviour was observed in pair-wise encounters in a neutral arena (diameter: 10 mm) under a stereo microscope. We tested all possible combinations between study nests including nestmate controls. We deliberately only chose the larger nests for the behavioural tests, so that enough workers were available to use each worker only in a single experiment. Behavioural interactions in these encounters were recorded by scan sampling every twenty seconds during a period of five minutes. In addition, we recorded biting *ad libitum* during the duration of the trials. We genotyped five workers per nest (if available) to elucidate the genetic colony structure. To test for an association between geographic distance, relatedness and behaviour, we constructed half-matrices of geographic distance, symmetrical relatedness values between each pair of nests and the seven different behaviours observed during the experiments. Matrix comparisons were conducted with the Mantel test (program: XLSTAT) (Mantel 1967; Mantel and Valand 1970).

## Results

### *General patterns of genetic diversity*

Our five microsatellite markers were highly variable for *H. opacior* with between 15 and 33 alleles per locus (Table 1). Observed heterozygosities per locus were generally high, yet lower than expected under Hardy-Weinberg and the high number of alleles. Consequently, inbreeding coefficients ($F_{IS}$) calculated over all loci, but for each deme separately, varied between 0.332 and 0.514 (Table 1). The mean inbreeding coefficient (averaging over demes; $F_{is}= 0.430$) was very high and hence consistent with the observed regular intra-nest matings of the wingless sexuals. Furthermore, deviations from Hardy-Weinberg equilibrium were significant ($p < 0.00001$, for each of the five loci).

An alternative explanation for the observed high rate of homozgyosity could be the occurrence of null alleles. We calculated the frequency of null alleles for the entire data set under the assumption of the Hardy-Weinberg equilibrium for each locus separately (Brookfield 1996). Consequently, we estimated the potential frequency of homozygotes for the null allele and compared this with the frequency of individuals that gave no amplification product at this locus. For each locus, we found only few non-amplifying individuals, so that null alleles cannot explain the observed deviation from Hardy-Weinberg equilibrium ($\chi^2$ goodness-of-fit test, df = 1: $p < 0.00001$, respectively).

**Table 1** Summary statistics for the population genetic analyses of a data set based on 344 *H. opacior* nests from Southeastern Arizona. We report the number of alleles ($N_A$), mean number of alleles in a deme (A), observed and expected heterozygosity ($H_O$, $H_E$) and inbreeding coefficient ($F_{is}$) per microsatellite locus and population. Calculations were obtained using the program GDA. One diploid individual per nest was analysed. $F_{IT}$ over all loci and demes was 0.471 (95% CI: 0.37-0.62) and $F_{ST}$ over all loci and demes 0.073 (95% CI: 0.05 - 0.1).

| | | N | $N_A$ | $H_O$ | $H_E$ | $F_{is}$ |
|---|---|---|---|---|---|---|
| Locus | | | | | | |
| | HoP 26 | 343 | 26 | 0.420 | 0.863 | 0.514 |
| | HoP 54 | 324 | 29 | 0.586 | 0.932 | 0.371 |
| | HoP 58 | 339 | 15 | 0.183 | 0.767 | 0.762 |
| | HoP 60 | 339 | 33 | 0.569 | 0.940 | 0.395 |
| | HoP 64 | 339 | 27 | 0.563 | 0.851 | 0.339 |
| | Over all loci | | | | | 0.467<br>95% CI:<br>0.32 – 0.59 |
| | | N | A | $H_O$ | $H_E$ | $F_{is}$ |
| Demes | 1 - Cave Creek | 59 | 18.2 | 0.497 | 0.894 | 0.446 |
| | 2 - F Plot SWRS | 29 | 12.6 | 0.344 | 0.872 | 0.506 |
| | 3 - Pinery Canyon | 34 | 11.4 | 0.403 | 0.756 | 0.470 |
| | 4 - Price Canyon | 34 | 14.8 | 0.587 | 0.875 | 0.332 |
| | 5 - West Turkey Creek | 31 | 11.6 | 0.413 | 0.789 | 0.481 |
| | 6 - Rucker Canyon | 36 | 12.8 | 0.585 | 0.851 | 0.324 |
| | 7 - Mt. Graham | 28 | 7.8 | 0.367 | 0.647 | 0.437 |
| | 8 - Huachuca Mts | 34 | 11.4 | 0.466 | 0.747 | 0.380 |
| | 9 - Mt. Lemmon | 31 | 10.4 | 0.433 | 0.736 | 0.415 |
| | 10 - Basin Trail | 28 | 10 | 0.401 | 0.818 | 0.514 |
| | Over all demes | 344 | 12.1 | 0.459 | 0.800 | 0.430 |

Relatedness analyses showed relatively low values for Hymenopteran colonies of r = 0.20, which can be explained by regular polygyny, multiple mating and polydomy (Table 2). Detailed information on the frequency of polygyny and nests headed by dealate or wingless queens are given in (Foitzik *et al.*, 2010). Relatedness coefficients were higher in nests collected in August than in June and these differences were significant for queens (Table 2).

**Table 2** Relatedness coefficients (r) between *Hypoponera opacior* castes in June and August, respectively. Unidirectional relatedness is indicated by an arrow (→) and bidirectional relatedness by a dash (–). The number of nests investigated (N), the standard error (SE), the 95 % confidence interval (CI) and p-values for the Mann-Whitney U-test comparing r-values between June and August are given (significant p-value are in bold).

| June | | | | August | | | | |
|---|---|---|---|---|---|---|---|---|
| $r_{xy}$ | N | r ± SE | CI 95 % | $r_{xy}$ | N | r ± SE | CI 95 % | p |
| worker – alate queen | 9 | 0.300±0.082 | ± 0.189 | worker – wingless queen | 39 | 0.378± 0.046 | ± 0.093 | 0.398 |
| worker → alate male | 9 | 0.196 ± 0.082 | ± 0.189 | worker → wingless male | 18 | 0.247 ± 0.047 | ± 0.100 | 0.396 |
| alate male → worker | 9 | 0.278 ± 0.086 | ± 0.198 | wingless male → worker | 18 | 0.416 ± 0.082 | ± 0.174 | 0.554 |
| alate queen → alate male | 10 | 0.194 ± 0.059 | ± 0.147 | wingless queen → wingless male | 30 | 0.332 ± 0.029 | ± 0.059 | 0.146 |
| alate male → alate queen | 10 | 0.238 ± 0.093 | ± 0.211 | wingless male → wingless queen | 30 | 0.452 ± 0.038 | ± 0.078 | 0.174 |
| worker – worker | 8 | 0.205 ± 0.080 | ± 0.188 | worker – worker | 57 | 0.416 ± 0.035 | ± 0.070 | 0.084 |
| alate queen - alate queen | 9 | 0.244 ± 0.101 | ± 0.233 | wingless queen – wingless queen | 40 | 0.489 ± 0.047 | ± 0.095 | **0.024** |

### *Macrogeographic scale*

The hierarchical F-analysis detected structure on two levels: Differentiation was found at the subpopulation level with $F_{SubpopTot} = 0.050$ (95 % CI: 0.024 – 0.081, permutation test: df = 3; $p < 0.02$), which quantifies the genetic structure between subpopulations located on different mountain ranges. In addition, we found significant genetic differentiation between the seven demes within the Chiricahua Mountains with $F_{DemePop} = 0.049$ (95% CI: 0.026 - 0.073, df = 6; permutation test $p < 0.001$). Note that the two values obtained by the hierarchical F-analysis can be not directly compared as the reference population is different in each calculation. $F_{DemePop}$ quantifies variation within demes in relation to variation within the Chiricahua subpopulation, whereas $F_{Subpop}$ quantifies variation within subpopulations compared to the total.

The results from the LAMARC analysis indicate that there is more gene flow between the demes in the Chiricahuas than between the four subpopulations (cf. Fig. 2). Per deme and generation we obtained the 95 % credibility interval [10.3 - 18.1] for the number of immigrating gametes from other Chiricahua demes. For the four subpopulations, which include one subpopulation consisting of all Chiricahua demes, we obtained the 95 % credibility interval [3.0 - 6.8] for the per-generation number of immigrating gametes per subpopulation from other subpopulations.

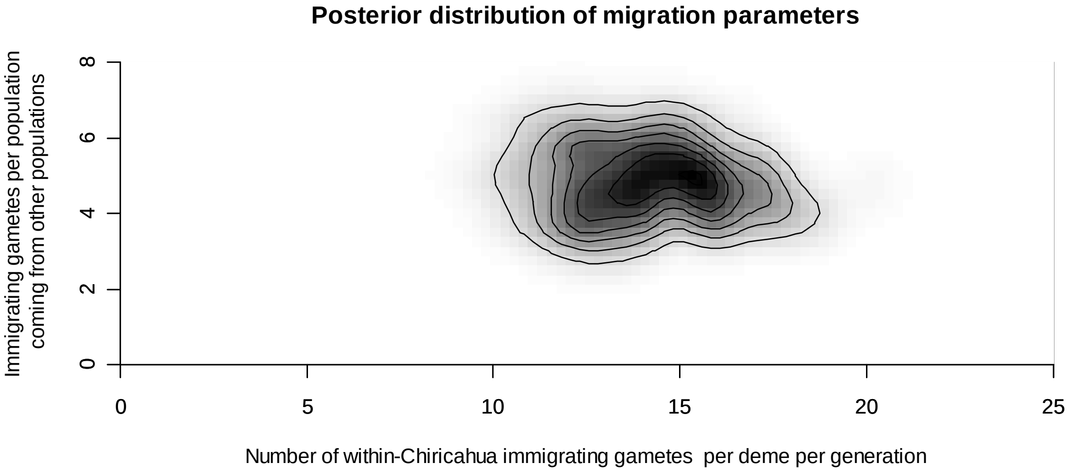


**Fig. 2** Posterior probability density of mutation parameters estimated from five microsatellite loci by eight independent runs of LAMARC. The mutation parameters are scaled such that they can be interpreted as the expected number of immigrants per generation in a Chiricahua deme coming from other Chiricahua demes (horizontal axis) or as the expected number of migrants in a population (vertical axis), where all Chiricahua demes together count as one population.

*Microgeographic scale and behavioural observations*

In the 99 $m^2$ plot, we found 40 *H. opacior* nest chambers, that is, nest density was high with 0.40 nests / $m^2$. We genotyped 102 workers from 30 nests (mean number of individuals per nest = 3.4, range: 1 − 5) and found a high allelic diversity at our microsatellite markers even in this very small patch. Allele numbers ranged from six to nine alleles per locus and median allelic diversity was eight. Hence, genetic diversity was substantial, representing on average about 30 % of the alleles found in our much larger sample from the entire Chiricahua Mountains. Geographic distance and pairwise symmetrical relatedness values were not associated (Mantel test: $r = -0.045$, $p = 0.351$).

During the aggression tests, ants interacted rarely and biting was the most prevalent behaviour, which we consequently analysed statistically. First of all, our aggression tests could demonstrate well-developed nestmate recognition abilities in *H. opacior*. Nestmate workers interacted invariably peacefully, while non-nestmate workers attacked and bit each other much more frequently (Mann-Whitney U-test: $n_{1,2}$ = 14, 66, U = 234.5, $p < 0.003$). In addition, the Mantel test revealed a significant negative association between geographic distance and symmetrical relatedness values between these larger nests with more than 15 workers (Mantel test: $r = -0.270$, $p = 0.014$, Fig.

3a). Further, aggression (N of bites) in our behavioural trials slightly increased with geographic distance between the nests (Mantel test: r = 0.214, p = 0.057, Fig. 3b). Aggression and relatedness between nests were not associated (Mantel test: r = - 0.137, p = 0.229).

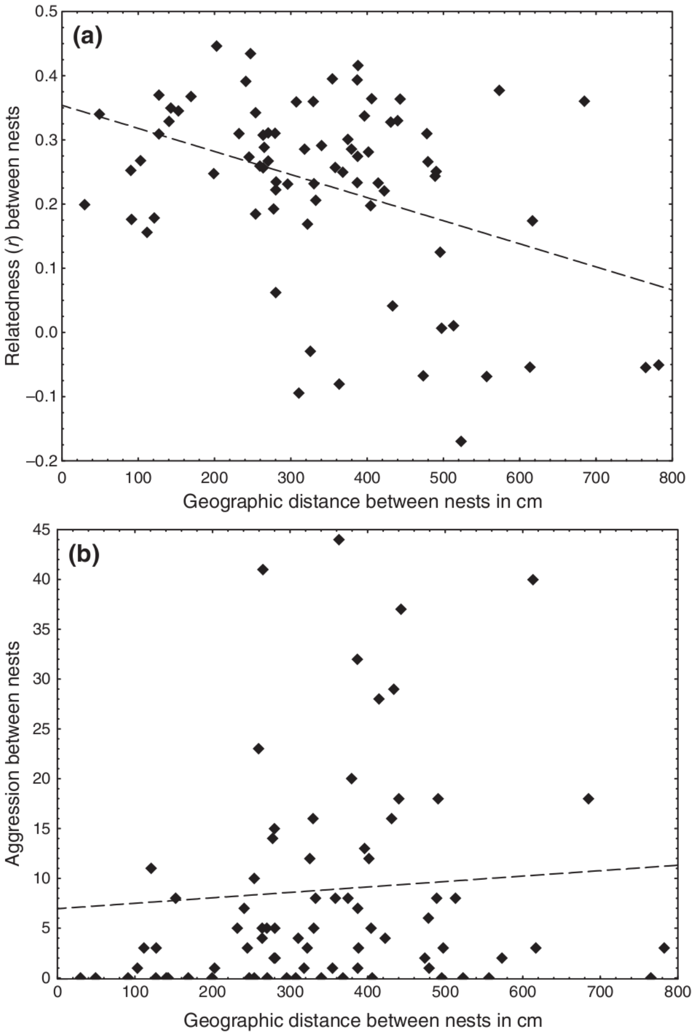


**Fig.3** Microgeographic structure and population viscosity in *H. opacior*. a) Between-nest relatedness decreases with geographic distance between nests. a) Aggression between ant nests slightly increases with distance. Only larger ant nests with more than 15 workers were included in these analyses, which contained enough workers for behavioural experiments.

# Discussion

This first genetic study on the population and colony structure of *Hypoponera* ants revealed significant geographic differentiation and high levels of inbreeding. This population genetic pattern can be explained by a combination of internal and external factors such as the dispersal capabilities of winged and wingless sexuals and the fragmented habitat on a larger scale.

On this macrogeographic scale, structure was pronounced between subpopulations on mountain ranges that lay about 100 km apart. Comparable genetic differentiation was shown for a number of ant species between subpopulations that are separated by several hundred up to a thousand kilometres (Sanetra and Crozier 2003; Brandt et al. 2007). Moreover, a significant $F_{ST}$-value between the seven Chiricahua sites reveals pronounced genetic differentiation between demes (Hartl and Clark 1997), which were only 4 – 24 km apart. However, the LAMARC analyses indicate higher migration rates within the Chiricahuas than between mountain ranges. The LAMARC estimates for the migration rates are high on a population genetic timescale, which is in accordance with the low $F_{ST}$ values (see Takahata 1983; Takahata and Nei 1984; Durrett 2008; but see also Whitlock and McCauly, 1999).

The unexpectedly high allelic diversity in all subpopulations may also be explained by historical habitat changes. Extant subpopulations of *H. opacior* in the “Sky Island region” might be remnants of a larger ancestral population. Indeed, several climate changes since the Pleistocene strongly reduced the areas with suitable habitats for *H. opacior* in the Southwestern United States. During the late Wisconsinan (22 000–11 000 years before now), the climate in this area was mesic and cooler. Woodlands spread across present deserts at lower elevations serving as links between mountain ranges (Van Devender and Spaulding 1979; Thompson and Anderson 2000). Yet, 11 000 years ago, the climate became warmer and dryer. Lowland desertification forced species adapted to more humid conditions to retreat to mountain slopes. Genetic data support such a scenario for populations of the ridge-nosed rattlesnake *Crotalus willardi* from the Sky Island region (Holycross and Douglas 2007). *Hypoponera opacior* has its centre of distribution in tropical Central America and the Southwestern United States represents the northern parts of its range. For such a species, at the ecological boundaries of its distribution, minor climate changes could have dramatic consequences. Nevertheless, we have no direct evidence how population sizes and the distribution of the species changed since the Pleistocene.

The LAMARC software, which we used to estimate gene flow, does not account for changes in population structure. Therefore, the actual long-distance migration rates could be considerably lower than estimated by LAMARC if genetic similarity between subpopulations is due to recent divergence from a joint ancestral population. We would need to relate the number of generations since the population split to the effective population sizes to infer how strongly a split 11 000 years ago would bias the migration rate estimates. For this, additional information on population sizes or ant microsatellite mutation rates are needed as we can only estimate the product of the two parameters from the data.

Another alternative scenario to explain the current distribution of *H. opacior* in Southeastern Arizona would be that subpopulations were established following a stepping stone model (Kimura and Weiss 1964) through multiple long-distance dispersal events over extended stretches of uninhabitable desert. Yet, this would have resulted in genetic bottlenecks, which are not supported by our genetic data.

Genetic structuring on a microgeographic scale was influenced by the reproductive tactic of wingless reproductives in *H. opacior* with their restricted dispersal abilities, intra-nest matings and regular polygyny and polydomy. We found significant relatedness among nestmates and, on a behavioural level, recognition and aggression towards non-nestmates. Hence, despite a very dense and highly connected nest organisation, *H. opacior* populations in Southeastern Arizona have a clear multicolonial local structure. Population viscosity was revealed by a decrease in relatedness between nests with geographic distance. This local genetic pattern is generally associated with low dispersal capabilities of founding queens (Hamilton 1964; Pamilo 1998), which is typical for polygynous ant species (Pamilo 1983; Crozier et al. 1984; Giraud et al. 2000). Population viscosity is rare in monogynous ants, in which queens generally disperse on wing, but there are a few exceptions of monogynous species with locally dispersing queens (Doums et al. 2002; Sanetra and Crozier 2003; Hardy et al. 2008). The low yet detectable viscosity at the fine scale in *H. opacior* can be explained by polydomy, polygyny and dependant colony foundation of wingless queens who disperse on foot accompanied by workers (Foitzik et al. 2002; Foitzik et al. 2010). It should be noted that population viscosity was apparent only in the sample including the larger nests (> 15 workers), that were also used for the behavioural experiments.

On the intra-nest level**,** relatedness estimates varied between the two reproductive seasons. These seasonal differences are supported by demographic analyses of ant nests (Foitzik et al. 2010). In fall, multiple wingless sexuals emerge and mate within their natal nest. Soon after their emergence they start to lay eggs, which develop into larvae before hibernation. In June/July winged sexuals and workers emerge from this fall brood, which was produced by many queens. Either in late fall or in spring, colonies split and are thus reduced to one or very few reproducing queens. Eggs laid in late spring to summer are then raised into the wingless sexual generation emerging in August/September. Because this generation is produced by few queens per nest, the relatedness among them is higher, which is evident from our genetic data.

High inbreeding coefficients were expected from our behavioural observations of habitual within-nest mating (Foitzik et al. 2002, 2010). Indeed, genetic analyses revealed extremely high $F_{is}$ values, which were only slightly lower than the highest values ever reported in ants. These were found in *Cardiocondyla batesii*, an ant with regular intranidal mating (Schrempf et al. 2005). Several generations of inbreeding should theoretically result in a nearly clonal nest structure as modelled for the ant *Technomyrmex albipes* (Tsuji and Yamauchi 1996). In stark contrast, *H. opacior* nest aggregations exhibit high allele numbers, which points towards frequent immigration of unrelated alate queens in suitable habitats. Inbreeding in haplodiploid Hymenoptera, with their typical complementary sex determination, can have a very detrimental impact on colony fitness caused by the production of sterile diploid males (Cook 1993). Under single locus complementary sex determination, predominant in ants, bees and wasps, hemizygous individuals on the sex locus will develop into males, while heterozygous eggs will become females (Whiting 1939; Whiting 1943). Fertilized eggs, which are homozygous at the sex locus, will develop into sterile diploid males (Agoze et al. 1994; Stouthamer et al. 1992). In the future, we plan to investigate the effect of inbreeding on colony growth, sexual investment and individual sizes and the production of diploid males. Diploid males are indeed occasionally produced and raised to adulthood in *H. opacior* colonies, as we uncovered in an ongoing study (see Chapter V). However, we have not studied whether diploid males are sterile, as it is known from other Hymenopteran species (Cook 1993; Agoze et al. 1994; Santomauro et al. 2004), and if they can be detected and killed by their colony members. We therefore plan to investigate frequencies of diploid males among pupae and adult individuals and study their reproductive success.

In conclusion, we can explain both the genetic structure and the high level of inbreeding in *H. opacior* by its two alternative mating and dispersal tactics. Winged reproductives are able to disperse over long distances and to discover and settle in new, unoccupied sites. Long distance dispersal is often unsuccessful, especially if it occurs in fragmented habitats, such as the Sky Island region of Southeastern Arizona. In contrast, wingless queens mate in the safety of the nest and disperse and found new nests accompanied by workers. This dependent colony foundation mode is certainly more often successful due to lower risks during dispersal (e.g. desiccation, predation) and a higher likelihood of nest establishment through the help of workers. The dual reproductive strategy of *H. opacior* with outcrossing winged sexuals in June and inbreeding wingless reproductives in August/September, resembles the mixed breeding system in Viola plants with cross-pollinating chasmogamous and self-pollinating cleistogamous flowers (Culley 2002). Outbreeding chasmogamous flowers occur in early spring (mid-April to May) and are typical pollinated by insects, whereas self-pollinating flowers are produced after the canopy has formed (May to September) (Culley 2002; Culley 2003). Such a dual strategy may combine several advantages: Outbreeding allows new combinations of the genome that might be advantageous for colonizing new habitats, while selfing (inbreeding) conserves favourable gene combinations for the local habitat.

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# Chapter III

# Wingless ant males adjust mate-guarding behaviour to the competitive situation in the nest

This chapter is based on a research article published in *Animal Behaviour:*

**Kureck IM**, Neumann A, Foitzik S (2011) Wingless ant males adjust mate guarding behaviour to the competitive situation in the nest. Anim Behav 82:339-346

# Abstract

We investigated whether wingless sexuals of the ant *Hypoponera opacior* adjust mate-guarding behaviour to the level of competition in the nest. Males mate with young nestmate females shortly before these emerge from the cocoon. Aggressive interactions among adult males have never been observed, but males embrace and guard the cocoons of their mating partners for up to 2 days. In laboratory experiments, the duration of pupal guarding increased with the number of adult males in the nest, but decreased with an increasing number of mating partners per male. These findings demonstrate that males are aware of the competitive situation in the nest and adjust their mating behaviour in an adaptive manner. Males also guarded and attempted to copulate with sterile worker and male pupae. These misdirected behaviours might be the result of identification errors, as we found that the cuticular hydrocarbon profiles of young individuals of the different castes were very similar. Copulatory behaviour towards sterile workers is certainly maladaptive, whereas interactions with young males may provide a fitness benefit: We found a high mortality rate of young males that were embraced and guarded by adult males. Adult male-male pupae interactions predominantly occurred when only a single male was present in the nest. In addition, single-male nests were found at unusually high frequencies. These findings suggest that wingless males try to kill their pupal rivals through embracing when there are few adult competitors in the nest, but switch to mate-guarding behaviour when male-male competition is high.

# Introduction

Sexual selection can select for diverse morphological, sensory or behavioural traits, such as ornamentation, weapons, sensory acuity and courtship or aggressive behaviour. The two main processes of sexual selection – female choice and male-male competition – select for different characters, that either make a male more attractive to females or help in fights among males (e.g. Wiley and Poston 1996; Sullivan-Beckers and Cocroft 2010). In some animal species, males do not openly fight for access to females, but guard their mating partner to prevent copulations of the female with other males. Mate-guarding is especially common if the last male that copulates with a female fertilizes most of her eggs (last male sperm precedence) and/or if the opportunities to find a second receptive female are low (e.g. Jormalainen 1998; Birkhead and Hunter 1990). The latter is the case if females have a short receptive period, are widely scattered or difficult to find. Mate-guarding behaviour is widespread in the animal kingdom and has been described both in vertebrates and invertebrates (e.g. Gilbert 1976; Watts 1983; Conner and Itagaki 1984; Fahey and Elgar 1997; Jormalainen 1998; Yamauchi et al. 2001; Morbey 2002; Stephenson and Verrell 2003; Parker and Vahed 2009; Nichols et al. 2010; Parga 2010; Estrada et al. 2010).

We can distinguish between non-contact mate-guarding, where males stay close to the females, but are not attached to them, and contact mate-guarding, also described as ‘passive phases’ of matings. During these phases, males are attached to females without genital insertion and either guard them until they become receptive (precopulatory passive phases) or stay associated with them after the copulation is terminated (postcopulatory passive phase) in order to reduce the chance of sperm competition (Parker 1974). Time investment during mate-guarding is costly, and therefore males should adjust their mate guarding behaviour to the current social environment. For example, males should guard their mating partner longer if male-male competition is high (Parker 1974). Indeed, mate-guarding duration was shown to be affected by the number of competitors and potential mating partners in several animal taxa (e.g. Cuadrado 2000; Komdeur 2001; Ward 1983; García-González and Gomendio 2006; Takeshita and Henmi 2010).

In contrast to the diverse sexual strategies found in many insect taxa, social Hymenoptera show little diversity and usually mate in large swarms during nuptial flights. Under these conditions, both male-male competition and active mate choice is difficult and social Hymenopteran males have not developed weapons or ornamentations or fighting/courtship behaviour. Instead, scramble competition occurs in mating flights which selected for sensory acuity (large eyes and sensitive antennae) and good flying abilities (Foitzik et al. 2002). However, some social Hymenoptera, such as bees of the genus *Perdita* or ants of the genera *Formicoxenus, Cardiocondyla, Technomyrmex* and *Hypoponera,* also produce wingless worker-like males that mate within the mother nest (Michener 1974; Hölldobler and Wilson 1990; Danforth 1991). Some of these males, driven by local mate competition, developed strong mandibles, which they use in deadly fights (Hamilton 1979; Danforth 1991; Heinze and Hölldobler 1993; Yamauchi et al. 1996) or to defend territories within the colony (Frohschammer and Heinze 2009). Yet, wingless males of other *Hypoponera* ant species do not fight, but guard their mating partner for hours. They embrace pupae (Fig. 1) and mate with very young queens before these emerge from the cocoon (Yamauchi et al. 2001; Foitzik et al. 2002). This guarding of pupal females has also been observed in butterflies (Gilbert 1976; Estrada et al. 2010) and mosquitos (Conner and Itagaki 1984) and allows the male to be the first mating partner of the emerging female.

We investigated matings and mate-guarding behaviour in wingless sexuals of the ant *Hypoponera opacior.* Ant colonies from a population in the Chiricahua Mountains, Arizona, USA, raise winged sexuals in early summer that mate during nuptial flights in July. During a second reproductive season in August–September only wingless sexuals are produced, which mate within the nest. Wingless males copulate with queens during the last stages of pupal development mate-guard their partners (Foitzik et al. 2010). Male genitalia are inserted at the rear end of the cocoons, which have been opened to facilitate the emergence of the young ants. The duration of mate-guarding with or without genital contact varies between a few minutes and several hours (up to two days), although it was shown that sperm can be transferred within minutes (Foitzik et al. 2002). Copulations are usually terminated by the emergence of wingless queens, which are invariably inseminated. Emerged queens have been observed to resist further copulations, so that the first mating partner of a young queen is in most cases also the last one. Our goal was to determine whether males adjust their guarding behaviour to the competitive situation in the nest as proposed by Parker (1974). We predicted that males should guard mating partners for longer, if more competitors are in the nest. However, in nests with many pupae per adult male, they should shorten their guarding behaviour to take advantage of additional mating opportunities.

Second, we investigated if the caste of pupae influences male behaviour. *Hypoponera* males were observed attempting to mate with worker and male pupae and we aimed at determining how common this behaviour is (Yamauchi et al. 2001; Foitzik et al. 2002). We were interested in whether males guard queens more often than expected by chance. Furthermore, we studied whether wingless males show different behaviours towards queen and worker pupae, such as different frequencies of genital insertion. There are only slight differences in body size between the wingless castes of *H. opacior* (Foitzik et al. 2010) and consequently cocoons of the different castes do not vary in size or shape. Hence, we assumed that males use chemical cues to identify the caste of a pupae and we therefore analysed the cuticular hydrocarbon profiles of young wingless queens, males and workers.

Third, it was previously observed that young *Hypoponera* males sometimes do not survive the embrace of other males (Yamauchi et al. 2001; Foitzik et al. 2002). If true, embracing might be a form of male-male competition. We found support for this idea in data from a sex ratio analysis of *H. opacior* nests (Foitzik et al. 2010), which showed a strong variance in the number of wingless males per nest. While some nests contained many males we found a high number of nests with only one adult male. This could be the outcome of adult males killing young emerging competitors and therewith lengthening their reproductive monopoly. We recorded the survival rate of embraced pupal males and investigated whether single-male nests occur more often than expected. Furthermore, we expected the killing of male pupae to occur predominantly in nests with few adult competitors. In multi-male colonies all males would benefit from the removal of new rivals, but the killer male would bear the costs of this behaviour.

## Material and methods

### *Ant collection and maintenance*

Nests of the ant *H. opacior* were collected in the Chiricahua Mountains in Arizona, USA, close to the Southwestern Research Station (N 31° 52.000′, W 109° 12.609′) in August and early September 2010. Ants occur in the upper soil layers, preferentially under stones. Therefore we turned over stones and collected complete nests with an aspirator. Nests were then transferred to our laboratory at the Southwestern Research Station where we kept them in three-chamber boxes (10 x 10 x 3 cm) with a moistened plaster floor. The plaster floor exhibited a circular cavity of approximately 3 mm depth and a radius of 1.5 cm, which was covered with a microscope slide. This cavity served as a nest chamber. Ants were fed with water and dead insects every 1 – 2 days.

*Mate-guarding and pupal development*

We checked our laboratory nests one to three times per day for mate-guarding behaviour. Males were recorded as showing mate-guarding behaviour if they embraced cocoons (Fig.1) and/or inserted their genitalia into the genital opening of the mating partner. Matings often ended when queens emerged from the cocoon and started walking around in the nest. They then dragged copulating males around until they detached. Occasionally males left guarded pupae after a while, without having established genital contact. As soon as guarding behaviour was detected, we recorded the time and counted the current number of males, females and potential mating opportunities (cocoons) in the nest. We added together the numbers of adult workers and wingless queens, hereafter named 'females', because it is difficult to discriminate between older individuals of these two castes. Wingless queens closely resemble workers in external morphology, and can only be recognised by comparing eye size, which is larger in queens (Foitzik et al. 2010), or by dissection, because only queens have ovaries (Foitzik et al. 2002). For our analysis, however, an exact caste determination of adult females was unnecessary, because males only mate with newly emerging individuals. Potential mating opportunities were recorded as the number of cocoons in the nest, because the determination of caste of young pupae is impossible. We furthermore noted whether the mating partner had already emerged from the cocoon and, if distinguishable, if there was insertion of genitalia or not.

After termination of mate-guarding, we isolated the mating pairs together with three adult workers and recorded both the caste of the emerging individuals and their survival rate within the first two days after being embraced. The isolated individuals were kept in the same type of three-chamber boxes and under the same conditions as the colonies. Workers were added because they occasionally help emerging individuals by removing parts of the cocoons. In cases in which we could not clearly discriminate between workers and queens by eye size, we dissected their ovaries at the end of our observation.

To determine which proportions of cocoons contained male, queen or worker pupae, we carefully studied the emergence of pupae in a subsample of 42 nests from the same season. Each day we counted the number of newly emerged individuals and determined their caste. It is easy to identify the caste of freshly emerged ants (callows) as these have a lighter cuticle than older individuals, so that the difference in eye size between queens and workers is more distinct. After caste determination we removed the callows from the nest. The obtained proportions were used to analyse whether males preferentially mate with queen pupae.

Statistical analyses were conducted using the program STATISTICA from StatSoft (version 6.0, Tulsa, OK, USA).

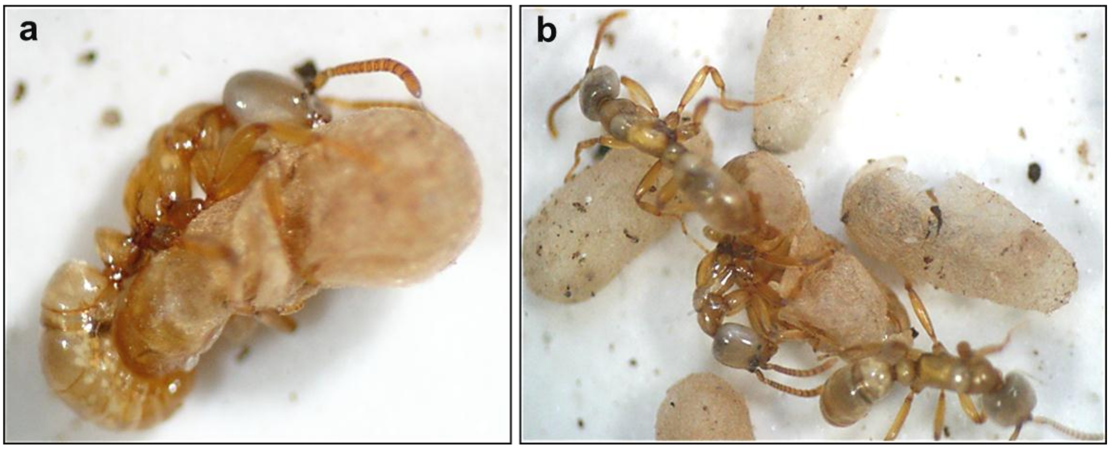

**Fig. 1** a) Male embraces a pupa that is still enclosed in the cocoon. b) Local mate competition: two males are still searching for mating partners while the third is already guarding a pupa

*Chemical analysis*

We extracted cuticular hydrocarbons from *Hypoponera* ants by submerging individuals in 30 µl hexane for five minutes. We removed the ant from the hexane with a sterile glass pipette and stored our samples at− 20°C. Samples were then analysed using coupled gas chromatography and mass spectrometry (GC-MS) on an Agilent Technologies 6890N GC and 5975 MSD which was equipped with a Restek Rxi-5MS column (30 m length, 0.25 mm ID, 0.25 µm film thickness). Analyses were conducted as in Foitzik et al. (2011). We only included cuticular hydrocarbons in our analysis, which were identified by retention time and their mass spectra with Wiley 7N spectral database. We standardized by the maximum peak area in order to detect differences in relative proportions.

Chemical data where analysed using the software PRIMER 6 (version 6.1.12, Primer-E Ltd., Ivybridge, U.K.) with the PERMANOVA+ add-in version 1.0.2 (Anderson et al. 2008). Nonparametric, permutational multivariate analyses of variance

(PERMANOVA) were conducted with 9999 permutations. To test for differences in profiles between castes we conducted pairwise tests for the three possible combinations of wingless castes (males, queens and workers). Bray-Curtis similarities were calculated for each analysis (Bray and Curtis 1957). For the graphic illustration of multivariate profile differences (see Results) we used multidimensional scaling (MDS).

In total, we extracted and analysed cuticular profiles from 23 wingless males, 61 workers and 51 wingless queens. We grouped individuals into three age classes: ‘Age class 1’ contained individuals up to six hours after their emergence from the cocoon ($N_{males}$ = 14; $N_{workers}$ = 19; $N_{queens}$ = 6), ‘age class 2’ contained individuals from six hours to two weeks after emergence ($N_{males}$ = 9; $N_{workers}$ = 24; $N_{queens}$ = 17) and ‘age class 3’ contained individuals that were older than one month ($N_{males}$ = 0; $N_{workers}$ = 18; $N_{queens}$ = 28). The reason for the absence of males in age class 3 is that none of the males in our artificial nests survived long enough to be grouped into this age class. Additionally we extracted hydrocarbons from 28 pupae which were still enclosed in cocoons. The samples stemmed from 48 nests with between one and 23 analysed individuals per nest.

*Analysis of the number of males within nests*

We noticed in the field that most *H. opacior* nests with males contained only a single male. In order to determine whether nests with a single male are indeed more common than expected, we counted the number of males in *H. opacior* nests at the time of collection. All nests contained at least one male and 6 − 255 adult females (mean number of adult females = 32) and were collected in the Chiricahua Mountains in late summer of 2004, 2005, 2009 and 2010. We then compared the distribution of the number of males per nest to the distributions of five other ant species with similarly small nest sizes: *Temnothorax longispinosus* ($N_{nests}$ = 58, number of adult females: 7−159, mean = 44), *Leptothorax acervorum* ($N_{nests}$ = 160, number of adult females: 6−328, mean = 84)*, Leptothorax muscorum* ($N_{nests}$ = 59, number of adult females = 7−201, mean = 63)*, Temnothorax crassispinus* ($N_{nests}$ = 53, number of adult females = 8−181, mean = 70) and *Temnothorax curvispinosus* ($N_{nests}$ = 34, number of adult females = 8−64, mean = 30). Only nests with at least one male were included in the analysis. All of these ant species have winged males, which mate in nuptial flights and we therefore did not expect competitive fights among nestmate males in these species. We admit that our selection is taxonomically biased, as the comparative species all belong to the subfamily

Myrmicinae, but these data were available and can give a first indication of whether single male nests are more common in *H. opacior* than in other ant species.

## Results

*Observation of development of pupae and measurement of mate guarding duration*

We observed mate-guarding and measured the guarding duration of 119 males from 41 nests. In 103 of the 119 interactions we recorded the caste of the mating partner and in 95 cases we noted the presence or absence of genital contact. Of the 103 mating partners, 89 were still in the cocoon at the beginning of our observation. The other 14 individuals were newly emerged callows. We presumably only observed the end of the interaction in those 14 cases and mate- guarding might, like in most other observations, have started before these individuals had fully emerged from the cocoon. Our estimates of mate-guarding duration are therefore always lower bound, but since we made this error systematically for all guarding interactions, meaningful comparisons should be possible. Additionally, we might have missed some very short interactions, as we did not observe the ant nests continuously. However, we did record 10 interactions that lasted shorter than 10 minutes.

To investigate whether males adjust their mate-guarding behaviour to the competitive situation in the nest, we tested if the number of adult males in the nest (hence the competition) and the number of available cocoons divided by the number of males (potential mating partners per male) is correlated with the length of the guarding behaviour. We found that guarding durations increased with the number of adult males in the nest (Spearman Rank correlation $r_s = 0.45$; $N = 119$; $p < 0.000001$; Fig. 2 a), but did not change with the nest size (i.e. number of females, Spearman Rank correlation $r_s = -0.09$; $N = 119$; $p = 0.30$). Yet, the length of guarding behaviour decreased with increasing mating opportunities, that is the number of cocoons per male (Spearman Rank correlation $r_s = -0.49$; $N = 119$; $p < 0.000001$; Fig. 2 b).

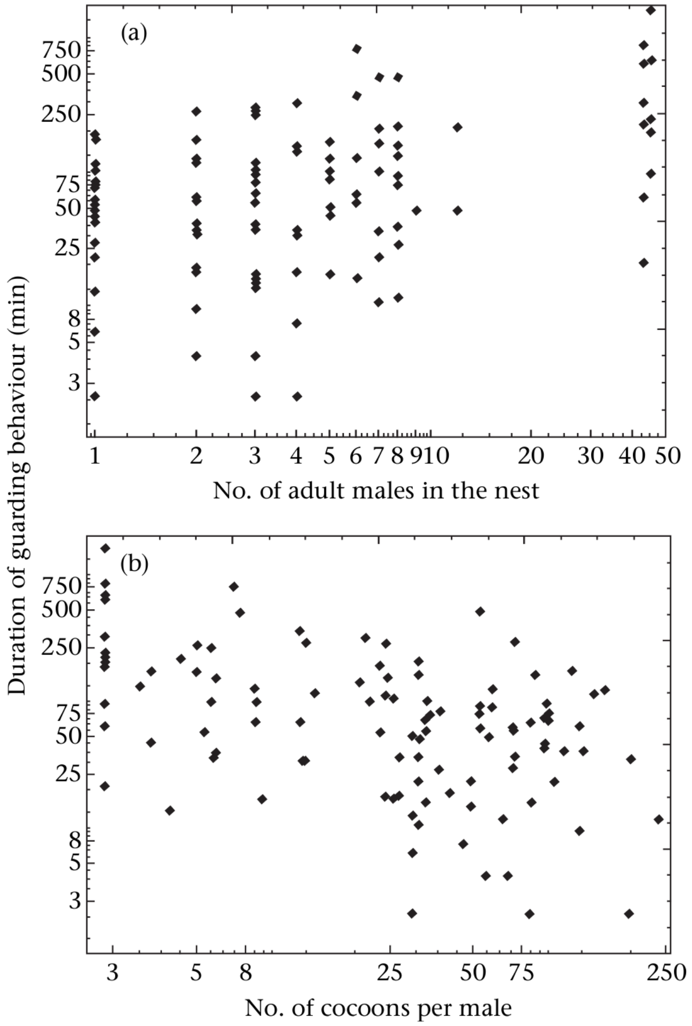

**Fig.2** Association between a) the number of adult males in the nest and b) the number of cocoons per males in the nest and the duration of mate-guarding behaviour (min). Axes are given in a logarithmic scale, because there were a few outliers. Excluding outliers did not change the significance in our findings.

Caste determination of guarded individuals showed that only 29 (28 %) of the mating partners were queens, whereas 61 (59 %) turned out to be workers, and 13 (13 %) were males. Even among the 14 already emerged mating partners, we found seven queens, six workers and one male. Taking only matings with queens into account, we found the same effects of an increase of guarding duration with the number of males and a decrease of guarding duration with increased mating opportunities (Spearman Rank correlations: number of males: $r_S$=-0.54; N = 29; p < 0.003; number of females: $r_S$ = -0.29; N = 29; p = 0.13; number of cocoons per male: $r_S$ = -0.58; N = 29; p < 0.001). Interactions (guarding, embracing and copulating) of males with pupae lasted longer than male interactions with emerged adult queens (Mann- Whitney U-test: U = 312; $N_1$ = 15; $N_2$ = 104; p < 0.0002).

In the subsample of nests (N = 42), for which we determined the caste of all newly emerged individuals, we found that on average 72 % of the cocoons contained worker pupae, 21 % male pupae and only 7 % queen pupae (Fig. 3 a). Next, we tested the observed distribution of castes among mating partners against the expected distribution of mating partners if males would guard pupae randomly. We found that males guarded queens more often than expected compared to males (Chi$^2$-test; $\chi_1^2$ = 13.84; $p < 0.002$) or workers ($\chi_1^2$ = 14.26; $p < 0.002$). We did not find a difference in the relative frequency by which males and workers were guarded ($\chi_1^2$ = 0.73; p = 0.39). *Hypoponera opacior* males therefore guarded and copulated with young queens more often than expected by chance, that is if they showed no choice, but they still guarded queens in less than 30 % of the cases. Moreover, we did not find a difference in the length of the guarding behaviour between interactions of males with queen pupae, male pupae or worker pupae (Kruskal-Wallis test: $H_{2,103}$ = 0.06; p = 0.97). Genital contact was observed not only in interactions between males and queens (N = 20; 80 %), but also between males and workers (N = 22; 38 %) or other males (N = 1; 8 %, Fig. 3 b). However, genital insertions were more frequent in male-queen interactions than in male-worker ($\chi_1^2$ = 12.37; $p < 0.004$) or male-male interactions ($\chi_1^2$ = 16.97; $p < 0.0001$). Males inserted their genitalia more frequently into worker pupae than into male pupae ($\chi_1^2$ = 3.95; $p < 0.05$). The length of the mate-guarding behaviour did not vary with whether or not we observed a male inserting its genitalia (Mann-Whitney U-test: U = 1173; $N_1$ = 64, $N_2$ = 45, p = 0.10).

Additionally, we analysed the demography of the ant colonies during pupal guarding events and studied whether the interactions of males with different pupal castes depended on the composition of the nest. We found that embracing events between males and male pupae took place in nests with fewer males than interactions with either queen or worker pupae (Kruskal-Wallis test: $H_{2,89}$ = 7.63; $p < 0.022$; Mann-Whitney U-tests: queen – male: U = 61.5, $p < 0.01$; worker – male: U = 197, $p < 0.03$, queen – worker: U = 490, p = 0.20). The median number of males present in the nest during male-male pupae interactions was one, while male-queen and male-worker interactions occurred at a median of 5.5 and 4 males, respectively.

Of the 103 guarded individuals for which we identified the caste, 17 could not be further observed after males detached, because they either got lost within the nest by walking away or, if still partly enclosed in the cocoon, by being carried away before we could separate them from the nest. Yet, for 86 of the pupae we recorded not only the caste, but also their survival within the first two days after being guarded by a male including the typical embracing behaviour (Fig. 1). Off these cocoons, 11 contained males, 25 queens and 50 workers. 8 of the 11 males (73 %) died within the first two days after being guarded and embraced while only five callow workers (10 %) and one (4 %) of the queens died (Fig. 3 b). Therefore, males survived guarding and embracing less often than workers ($\chi_1^2 = 21.16$; $p < 0.0001$) and queens ($\chi_1^2 = 19.24$; $p < 0.0001$). Almost all of these individuals died, while still enclosed in the cocoon, but one male and two workers managed to emerge from the cocoon and died shortly afterwards. Genital insertion did not appear to be the cause of male death as we observed genital contact between male and male pupae only once and in this case the male survived the interaction.

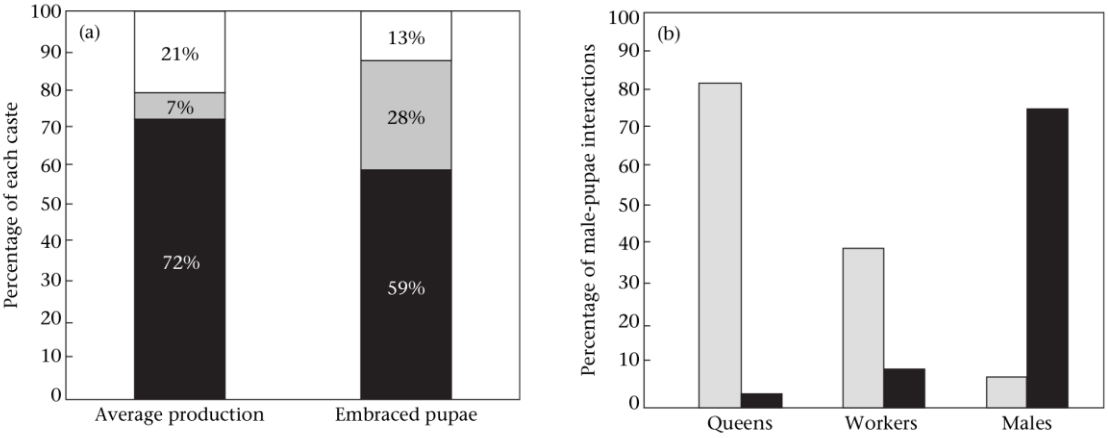

**Fig.3** a) Percentage of males (white), queens (grey) and workers (black) among newly produced individuals. Average production: the percentage of each caste among pupae in a subsample of 42 nests (N = 1204). Embraced pupae: the percentage of castes among pupae that were embraced by adult males (N = 103). (b) Percentage of male-pupa interactions with genital contact (grey bars; separated by caste; $N_{queens} = 24$; $N_{workers} = 59$; $N_{males} = 12$) and percentage of the three castes dying after being embraced by a male (black bars; $N_{queens} = 25$; $N_{workers} = 50$; $N_{males} = 11$).

### *Chemical analysis*

We detected the same cuticular hydrocarbons as in a previous analysis of older individuals of the *H. opacior* (Foitzik et al. 2011), with the exception of methyl-heptacosadien, 2-methyl-octacosane, nonacosene C and hentriacontene C. Instead, we found two additional hydrocarbons (dimethyl-hentriacontene and dimethyl-heptacosadien), which were not previously detected on the cuticle of *H. opacior*.

Chemical profiles varied with age ($p < 0.001$) and caste ($p < 0.001$). Furthermore, the interaction between both factors was significant ($p < 0.001$). We detected only few amounts of substances on extracted cocoons, which made differentiations between castes unlikely (Fig. 4). Therefore, we included only emerged individuals in our statistical analysis (Table 1). Pairwise tests revealed differences in profiles between all three castes, although those between workers and males were not significant after correction for type 1 error. This was done because the same data were used in the overall analyses and the separate analyses for each age class. For the young individuals of age class 1, which represent mating partners of pupal mating males at the time when copulations are usually terminated, we found no significant difference between castes. Caste difference approached significance for comparisons between workers and queens, and workers and males, in age class 2 and were highly significant in workers and queens that were at least 1 month old. The amount of cuticular hydrocarbons clearly increased in the hours after emergence from the cocoon (Fig. 4). This has been shown in other ants as well, in which freshly emerged callow individuals were described as chemically insignificant (Lenoir et al. 1999).

**Table 1** Differences in the hydrocarbon profiles of ants of different castes. 'Age class 1' contained individuals up to six hours after emergence from the cocoon, 'age class 2' contained individuals from six hours to two weeks after emergence and 'age class 3' contained individuals that were older than one month. Pairwise PERMANOVA were conducted with the factor 'caste'. Significant p-values after correction for type I error are given in bold.

| | Comparisons | t | p | Permutations | |
|---|---|---|---|---|---|
| All | Males, workers | 1.77 | 0.03 | 9949 | N of males = 23 |
| | Males, queens | 3.96 | **0.0001** | 9951 | N of workers = 61 |
| | Workers, queens | 4.02 | **0.0001** | 9957 | N of queens = 51 |
| Age class 1 | Males, workers | 0.87 | 0.45 | 9954 | N of males = 14 |
| | Males, queens | 0.65 | 0.70 | 5042 | N of workers = 19 |
| | Workers, queens | 1.32 | 0.17 | 8533 | N of queens = 6 |
| Age class 2 | Males, workers | 1.66 | 0.05 | 9959 | N of males = 9 |
| | Males, queens | 1.17 | 0.25 | 9921 | N of workers = 24 |
| | Workers, queens | 1.47 | 0.07 | 9935 | N of queens = 17 |
| Age class 3 | Workers, queens | 5.11 | **0.0001** | 9944 | N of males = 0 |
| | | | | | N of workers = 18 |
| | | | | | N of queens = 28 |

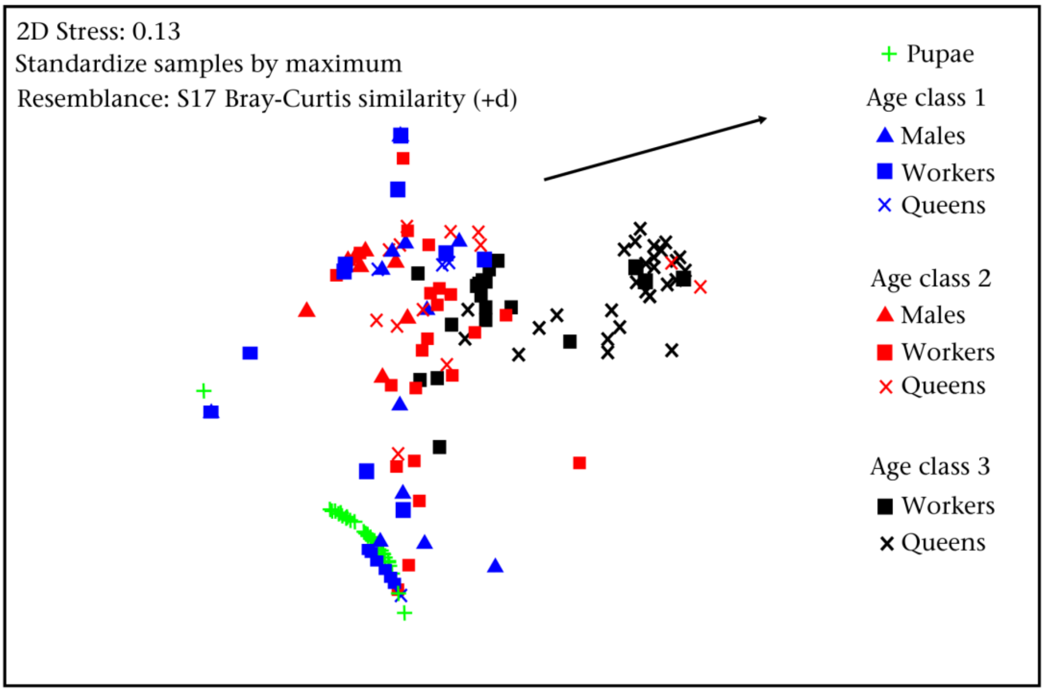

**Fig. 4** Multidimensional scaling (MDS) of cuticular hydrocarbon profiles of males, workers and queens from age class 1 (up to 6 h after emergence), age class 2 (6 h to 2 weeks after emergence) and age class 3 (older than 1 month). The direction of the black arrow indicates the increase in the total amount of cuticular hydrocarbons. Comparison of total amount of hydrocarbons: $p < 0.001$ for each pairwise comparison of age classes based on log-transformed data; ANOVA followed by post hoc least significant difference test: $F_{2,135} = 62.72$.

### *Analysis of the number of males per nest*

We found that about half of the 127 investigated *H. opacior* nests contained only one male while the percentage of single-male nests varied in the five other species between 27 % and 34 % (Fig. 5 a). The distribution of the number of males per nest differed significantly between *H. opacior* and all other species (Kolmogorov-Smirnov tests: $p < 0.05$), with the exception of *T. curvispinosus*, where we found only a trend in the same direction ($p < 0.10$). Moreover, the distributions did not differ between the other species (Kolmogorov-Smirnov tests: $p < 0.10$ for *T. crassispinus* − *L. acervorum*; $p > 0.10$ for all other comparisons). These analyses demonstratethat single-male nests occur in *H. opacior* at unusually high.

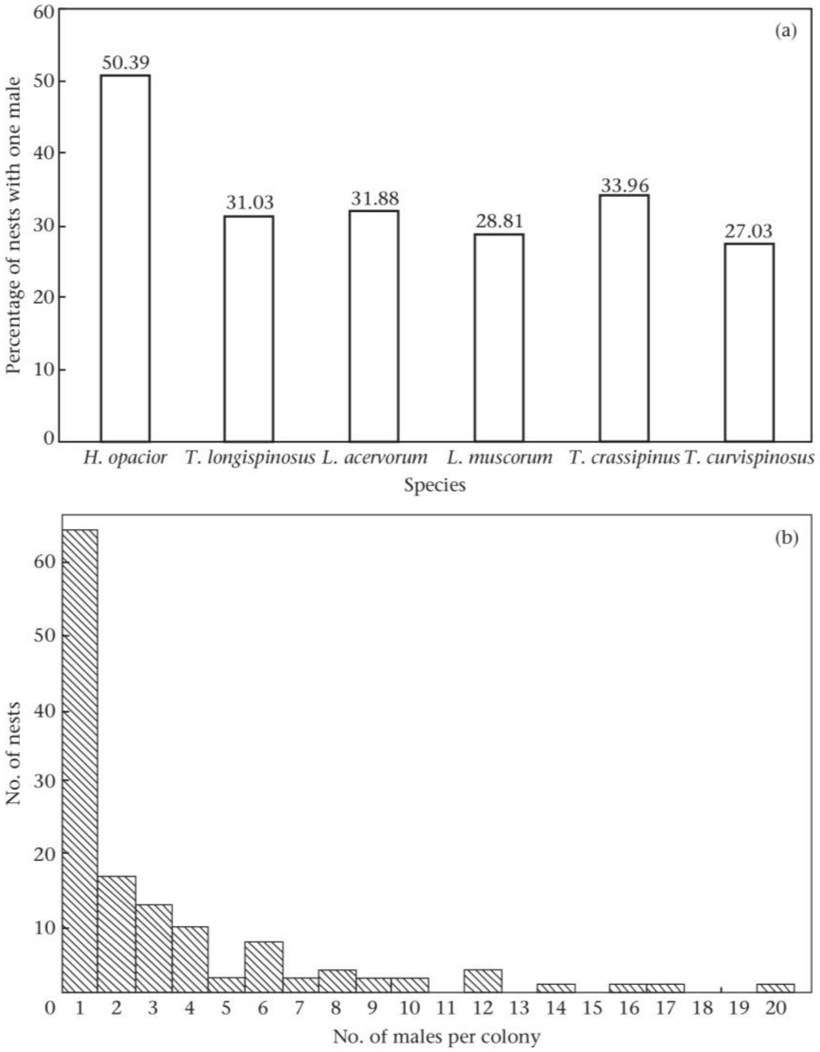

**Fig 5.** a) Percentages of nests containing only one male for the species *H. opacior, T. longispinosus, L. acervorum, L. muscorum, T. crassispinus, T. curvispinosus* (nests without males were excluded from the data set). b) Number of males per nest in *H. opacior* nests with males (N = 126) directly after collection. One nest with 45 males was left out of this figure.

# Discussion

The results of this study suggest that *H. opacior* males increase the length of mate-guarding with the number of rivals in the nest. Moreover, they guard their partners shorter if there are more mating opportunities, i.e., more pupae available per male. These findings are in line with Parker's (1974) prediction and with similar observations in crustaceans, reptiles, birds and other insects (Ward 1983; Cuadrado 2000; Komdeur 2001; García-González and Gomendio 2006; Takeshita and Henmi 2010). They demonstrate that wingless males correctly assess the competitive situation in the nest and that they adjust their mating behaviour in an adaptive manner. In a different *Hypoponera* species, males were already shown to lengthen their guarding behaviour if they detect rival males (Yamauchi et al. 2001). Yet, we can show here that males not only correctly detect the presence or absence of rivals, but that they alter their mating behaviour in response to the number of competitors in the nest and to the potential mating opportunities.

The mate-guarding behaviour of *H. opacior* males can be regarded as a combination of pre- and postcopulatory mate-guarding, because males start to guard pupae before making genital contact and often stay attached to them until the latter emerge from the cocoon. This behaviour is probably adaptive. First, if a young queen is guarded before she becomes receptive, the guarding male ensures to be her first mating partner. Second, adult queens are very reluctant to mate after emergence and most copulations end when young queens fully emerge from the cocoon. Hence a male that is still with a queen until this point, will most likely remain her only mating partner. On the other hand, it has been shown that *H. opacior* males can mate several times (Foitzik et al. 2002), which explains why mate-guarding durations are shorter when there are more mating opportunities available per male.

Although the adaptive adjustment of mate-guarding indicated that mating behaviour is under selection, we found that males did not exclusively mate with queens. Males preferentially guarded young queens, but there were an unexpectedly high number of males that guarded worker or male pupae. The guarding of workers in *H. opacior* can be regarded as maladaptive because workers have no ovaries and are completely sterile (Rüger et al. 2008). As mentioned before, cocoons of the three wingless castes do not differ in size or outer appearance and we therefore expected caste discrimination to be mainly based on olfactory cues. Our chemical analyses showed that caste differences in chemical profiles develop during early adulthood and that profiles of young individuals are still very similar. These findings explain the high rate of caste recognition errors by sexually active males. Males, which are better able to single out queen pupae, should have a higher reproductive success, and indeed we found that males did not guard pupae at random, but more often attended queen pupae than expected by the relative frequency of queen and worker pupae in the nest. This indicates that slight chemical or morphological differences are detectable at least by some males. Interestingly, the guarding duration did not vary with caste of the pupae.

Homosexual mountings have been described for several animal taxa (Aiken 1981; Bagemihl 1999; Harari et al. 2000; Maklakov and Bonduriansky 2009) and were interpreted as a practice for later heterosexual interactions, as dominance interactions due to rivalry (Vasey 1995; Dagg 1984) or, as they mainly occur in insects, as a result of perception errors (e.g. Parker 1968; Aiken 1981; Serrano et al. 2000; Estrada et al. 2010; Dukas 2010). In contrast to the mate-guarding of worker pupae, wingless *H. opacior* males might directly benefit from the guarding and embracing of male pupae. Similar to observations in *H. nubatama* (Yamauchi et al. 2001), we found that a high rate (73 %) of male pupae died after being embraced. Regarding the chemical similarity of young individuals one could argue that the guarding and embracing of male pupae is also simply due to recognition errors. Behavioural observations, however, suggest that this is not the case. Males embracing male pupae more rarely inserted their genitalia than those interacting with queen or worker pupae. This suggests that at some point males recognize that they are currently guarding a male pupa. Nevertheless, they continue to embrace these pupae, and the guarding duration did not differ from that of interactions with female pupae. *Hypoponera opacior* males are incapable of openly fighting against each other as it has been shown for the wingless ant males of other *Hypoponera* or *Cardiocondyla* species, which carry strong and sharp mandibles (Hamilton 1979; Heinze and Hölldobler 1993; Yamauchi et al. 1996). Moreover, adult *H. opacior* males behaved invariably peacefully towards each other. Most of the time, they were either occupied with guarding and embracing of pupae or with investigating pupae with their antennae. Squeezing male pupae with a very soft cuticle might be the only possibility to actively influence competition in the nest.

The killing of young males which are still enclosed in the cocoon could thus be a second strategy, besides mate-guarding, to deal with male-male competition. Besides the high mortality rate of previously embraced male pupae, this hypothesis is further supported by the analysis of the distribution of males within *H. opacior* nests: We found an unusual high fraction of nests containing just a single male compared to other ant species with similar nest sizes. For example, four times as many nests contained a single male as contained two males. On the other hand, several nests contained multiple males with up to 45 males per nest. We interpret these findings as the first males to emerge trying to lengthen their reproductive monopoly by killing pupal rivals, but when many males emerge at once, they give up and tolerate each other. Especially in larger nests with many pupae it is impossible for a male to eliminate all young competitors. Once several adult males have emerged, the time-consuming deadly embrace of other males is expected to be a less beneficial competitive strategy compared to mate guarding. All males would benefit from the death of their rivals, while only males that invest time and energy in killing male pupae would lose mating opportunities. In support of these predictions, we found male-male pupae interactions in nests with few adult males, while guarding of queen and worker pupae occurred more often in nests with many males. Furthermore, the guarding durations increased in nests with many males, pointing to a switch to a mate-guarding strategy under strong male-male competition. These behavioural variations of wingless *H. opacior* males can be regarded as two tactics of a conditional strategy (Gross 1996). Conditional strategies are usually status dependent, but a switchpoint adjustment to the demographic environment, including operational sex ratio, has also been shown in other insect species (e.g. Crespi 1988; Carroll and Corneli, 1995).

To verify this last assumption of rival killing through embracing of male pupae, further studies are planned. We intend to compare survival rates of embraced and non-embraced pupae, as it is still possible that the survival rate of male pupae is generally lower than that of queens and workers. Furthermore, we intend to study male-male interactions by carefully recording the adult males' behaviour before and during the interaction with male pupae. If males embrace male pupae to harm or kill them, we would expect their behaviour to differ from mating behaviour towards queens. For example, males might squeeze male pupae more tightly to indent the soft cuticle of male pupae. Indeed, we found males with indented heads; however, whether these injuries were caused by other males is currently unclear.

In summary our study illustrates a complex and adaptive mating behaviour of wingless ant males of the species *H. opacior*. They were shown to adapt their mate-guarding behaviour precisely to the competitive situation in their local environment as proposed by Parker (1974). The high fraction of mate-guarding behaviour towards sterile workers is most likely due to perception errors, while embracing of male pupae could reduce male-male competition and therewith carry a fitness benefit.

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# Chapter IV

## The early male catches the queen: Selection for early emergence in sib-mating ant males

This chapter is based on a research article that is currently under review for publication in the Journal *Behavioral Ecology and Sociobiology:*

**Kureck IM**, Nicolai B, Foitzik S (under review) The early male catches the queen: Selection for early emergence in sib-mating ant males. Behav Ecol Sociobiol

# Abstract

Sexual selection led to male morphologies and behaviours that either increase their attractiveness to females or their success in male-male competition. We investigated male traits under selection in an ant species, *Hypoponera opacior*, with wingless sexuals that mate within the natal colony. As a consequence of local mate competition, males mate with pupal females and mate-guard their mating partners for hours. Under these circumstances, the first males to emerge in the nest should have a higher mating success as they encounter a more female-biased operational sex ratio. We recorded the number and length of matings and tested whether these measures of mating success were associated with the order of emergence in the nest and with male body size. High variance in mating success indicated strong sexual selection on males although female choice and male-male aggression are absent in this mating system. Early emerging males mated more often and longer than their later emerging rivals. Besides, larger males obtained more matings, either because they have more sperm available or perform better in mounting females. We found no evidence for a trade-off between body size and emergence time. Moreover, male removal manipulations revealed that males quickly adapt their guarding behaviour to changes in the competitive environment. Under experimentally lower competition, wingless males guarded their mating partners for shorter time periods. In conclusion, these sib-mating ant males are under selection to develop fast, to be large and to be able to respond to the competitive situation in the nest.

# Introduction

In most species males are under strong sexual selection acting on different morphological, physiological and behavioural traits that either make them more attractive to females (female choice) or are beneficial in male-male competition. Two traits that were found to be under positive sexual selection in many animals are a large body size (Villabos and Shelly 1991; Haley et al. 1994; Stone et al. 1995; Bel-Venner and Venner 2006; Robbins et al. 2009) and early emergence/arrival on the mating site (Wiklund and Solbreck 1982; Carchini et al. 2005; Saino et al. 2012). In some species, there is a trade-off between these two traits (Zonneveld 1996), whereas in others body size and emergence time are positively associated, indicating a higher fitness of early and large males (Yoshimura et al. 2003). Male-male competition also led to the evolution of characteristic male behaviours such as fighting (McElligott et al. 1998, Brown et al. 2007), territorial defence (Jakobsson 1988; Frohschammer and Heinze 2009) or guarding of mating partners (Endo and Doi 2002; Foitzik et al. 2002).

Most social Hymenopteran males mate during a single annual mating flight, were they face scramble competition for the limited mating opportunities (Hölldobler and Wilson 1990). Weak sexual selection resulted in the termination of sperm production during the pupal phase, so that adult males have only a fixed amount of sperm available. Lives of these males are short and they live only a few days after leaving the nest to mate. In contrast, the ant *Hypoponera opacior* produces, as an alternative reproductive tactic to winged outbreeding sexuals in summer, wingless sexuals in fall that mate within their natal colony. Wingless males exhibit an extended lifespan and they mate multiply. Due to male-biased sex ratios in this wingless generation, local mate competition is strong for these wingless males. As a consequence, males copulate with pupal queens before the latter fully emerge from the cocoon and they guard their mating partners for hours by embracing their cocoons (Foitzik et al. 2002; Foitzik et al. 2010). Mate-guarding durations were found to co-vary with the number of pupae and rivals in the nest (Kureck et al. 2011). In this mating situation, females cannot choose their mating partner and males do not actively fight with their rivals. Hence, female choice and male-male aggression are absent and selection is expected to work on traits that are unconnected to attractiveness to females or fighting abilities. Rather, we here expected sexual selection to act on fast development in males. Early emerging males should suffer less from local mate competition as they arrive before their rivals emerge on the mating site. Further, as males only mate with pupal or newly emerged young queens, emergence at the beginning of the reproductive season should offer them more mating opportunities. Early in the season the operational sex ratio is much more female-biased and males respond to this seasonal shift by increasing guarding time over the season (Kureck et al 2011).

We investigated whether sexual selection shapes the emergence time of males in *H. opacior*. We also studied male body size as large males might perform better in embracing or mounting queen cocoons. Alternatively, larger males might be able to mate more often because they produce more sperm (Pech-May et al. 2012). Further, we analysed if mating behaviour is influenced by the relatedness among males in the nest. A lower relatedness would indicate stronger local mate competition, which should select for more competitive behaviour, for example longer mate-guarding durations. Finally, we investigated how fast males adjust their guarding behaviour to changes in the local competitive situation by manipulating the number of rival males in a nest. Males should have a higher mating success if they quickly respond to changes in the competitive environment.

## Material and methods

*H. opacior* colonies were collected in the Chiricahua Mountains located in the southeast of Arizona in August 2011. Ant nests were detected by turning stones. Once a nest site was detected we sifted the soil and collected all individuals and brood with an aspirator. Colonies were transferred to our laboratory at the Southwestern Research Station (31°52.0000 N, 109° 12.6090 W) and kept at room temperature in three-chamber boxes (10 x 10 cm and 3 cm high) with a moistened plaster floor. A circular cavity in the plaster floor (3 mm depth and 1.5 cm radius) that was covered with a microscope slide served as a nest chamber. Colonies were daily provided with water and dead insects.

Nests were scanned three to six times per day for newly emerged males and for matings. Each newly emerged male was colour marked and we recorded its day of emergence and death, as well as the number of its matings until death (N = 194 males from 49 nests). *H. opacior* ant males show a typical embracing behaviour, when copulating with queen pupae. Wingless males can remain in this posture for hours, thereby repeatedly loosing and re-establishing genital contact (Kureck et al. 2011).

Dissections after 5 min of copulation time demonstrated that sperm is transferred already within the first minutes of the interaction, so that the lengthy “embraces” can be seen as a mate-guarding behaviour. In most observations of mating behaviour, we directly observed genital insertion, but in some the position of the mating couple in the nest prohibited the direct observation of genital contact. As we did not want to disturb matings, we recorded all situations in which males showed the typical embracing and guarding behaviour of a cocoon as ‘matings’. Consequently, we noted down the entire period in that a male was embracing a cocoon – with or without genital contact – as ‘mating duration’. Albeit we checked each of our observation nests three to six times per day, we did not observe the beginning or end of all matings. We therefore noted the time of the first and last observation of a mating as starting or end point, respectively. If a mating was observed only once, we defined its duration to be five minutes as it has been shown before that sperm can be transferred within 5 min (Foitzik et al. 2002). Hence, our mating duration measurements are lower bound estimates. Further, our scan sampling procedure led us to miss some short matings. Therefore not only the duration but also the numbers of matings per male are minimum values. We used this observation method, because it allowed us to obtain data from a maximal number of males, albeit we had to compromise some accuracy in our measurements. However, we argue that comparisons between males are possible, as our sampling error was conducted systematically for all males (Kureck et al. 2011).

In total, 238 males emerged from 59 nests and we were able to track male mating activity and lifespan for 206 males from 49 nests. For the remaining males, we were unable to follow the mating behaviour over their lifetime, either because they died and were eaten by workers or they lost their colour marking and could not be clearly identified anymore. Males that were already present in the nest at the time of collection were also excluded from the analyses, because we could not correctly estimate their lifespan.

We ranked males that emerged in the observation nests by their emergence day on a per colony basis. Males of the same nest that emerged on the same day obtained the same ranking number. Males that emerged in a nest that already contained one or several males during the time of collection, obtained the ranking number ‘two’. The variable ‘emergence rank’, as used in our analyses, was calculated by dividing the emergence ranking number of a male by the number of males emerging in the nest, i.e. low numbers reflect a relatively early emergence in the nest.

Additionally we recorded the duration of matings, which are prolonged in this species and can be regarded as a mate-guarding behaviour (Foitzik et al. 2002), so that we expected them to be associated with paternity certainty (Knox and Scott 2006). Mating number and the mean mating duration per male were then tested using Generalized Linear Mixed Models (with a binomial error distribution), in which the emergence rank (ratio of the rank of emergence divided by the number of males in the nest) and body size (thorax width) were entered as fixed factors and nest as random factor. After males had died, we preserved them in 100 % ethanol. We measured thorax width under 40 x magnifications using a stereomicroscope coupled to a computer and the measuring software LAS (Leica Application Suite V.3.8 from Leica Microsoft). In total, we measured the thorax width of 94 individuals. We decided to focus on thorax width as a size parameter instead of the more typical used trait head width, as the heads of many dead males were deformed and correct measurement could not have been obtained.

### *Genetic analysis*

After the observed males had died, we genotyped all males on 11 highly variable microsatellite loci as described in (Kureck et al. 2012). Our analyses revealed that 12 males were diploid. Diploid males occur in haplo-diploid species with single / multiple locus sex determination under inbreeding (Cook 1993). We excluded these diploid males from all our analyses as diploid males are likely to differ in their mating behaviour, reproductive success and longevity, resulting in a sample size of 194 males.

### *Relatedness among males*

We used genetic data of males from 31 of the observation nests to calculate mean relatedness values within nests. Only nests with at least two males (mean number of males 4.678 ± SE 0.462) and only haploid males were included in this analysis. We used the program RELATEDNESS 5.0.8. for the calculation of relatedness (Queller and Goodnight 1989; Goodnight and Queller 1994). Mean nest relatedness values were used to investigate whether relatedness was associated with the mating behaviour of males. We calculated average relatedness values for all nestmate males and in addition for all sexually active males of a nest. In addition of testing for an association between relatedness and mating duration, we analysed whether mean male lifespan and / or mean number of matings per male was correlated to male-male relatedness.

*Manipulation of the competitive situation*

We manipulated the strength of male-male competition in 12 nests containing between four and 22 males (9.00 ± 1.34, mean ± SE). After recording at least two matings per nest, we removed half of the males and waited 10 hours (adaptation phase) before we continued our observations. Observations were terminated after a maximum duration of three days, so that the number of pupae at the beginning and the end of the experiment were similar despite the emergence of new individuals. We compared the average mating duration for each nest before and after manipulation in a paired design. Statistical analyses were conducted using the statistical software R 2.14.0 and STATISTICA from StatSoft (version 6.0, Tulsa, OK, USA).

## Results

Between one and 18 (4.78 ± 0.55; mean ± SE) males emerged per nest. Males lived up to 33 days (6.33 ± 0.40 mean ± SE,) and mated between zero and eight times during their lifetime (0.89 ± 0.01 mean ± SE, Fig 1). The the variance of mating success was 2.23. Mean mating duration was 52.778 ± 12.377 minutes with a maximum duration of 691.5 minutes for a single mating (11.5 h). 112 males (58 %) were never observed to mate during their lifetime.

Among the sexually active males, males that mated more often also mated longer (N = 87, S = 17334.56, $p < 0.001$). Emergence order was uncorrelated to body size, but earlier emerging males had a longer lifespan (‘Lifespan’, ‘Body size’; Table 1 a). Therefore, we excluded lifespan in from the analyses of traits associated with mating success. The total number of matings was negatively correlated to the emergence rank of a male and to a lesser extent to its body size (‘N of matings’, ‘Body size’; Table 1 b, Fig 2 a, c). Mean mating duration of sexually active males decreased with emergence rank, but not with body size (‘Mean mating duration’; Table 1 b; Fig 2 b).

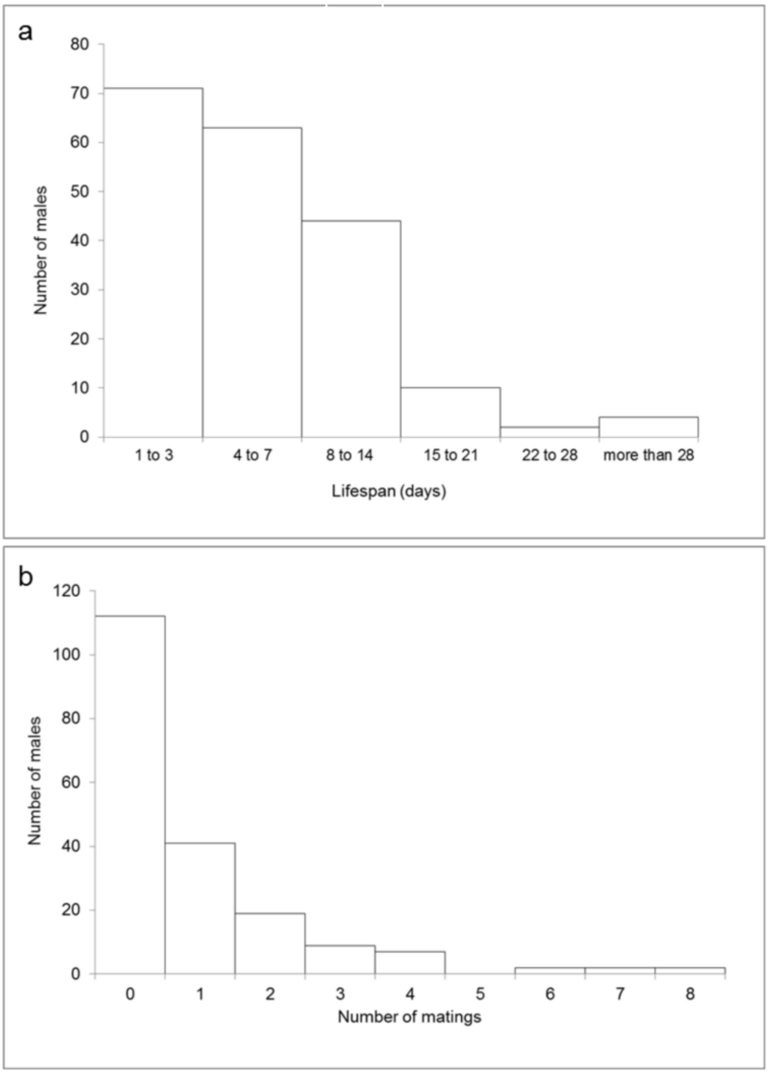

**Fig 1** Distributions of a) wingless male lifespan in days and b) the total number of matings per males. Males live up to 33 days and mated between zero and eight times.

**Table 1** a) Association between emergence rank and the variables lifespan and body size. b) Associations between emergence rank and body size on the number of matings and mean mating duration. Significant values are given in bold.

| a) | Lifespan | Body size |
|---|---|---|
| Emergence rank | $\chi_1^2$ = 6.40, **p = 0.011** | $\chi_1^2$ = 0.50, p = 0.480 |
| b) | N of matings | Mean mating duration |
| Emergence rank | $\chi_1^2$ = 14.74, **p = 0.0001** | $\chi_1^2$ = 5.58**, p = 0.018** |
| Body size | $\chi_1^2$ = 4.19, **p = 0.038** | $\chi_1^2$ = 0.03, p = 0.856 |

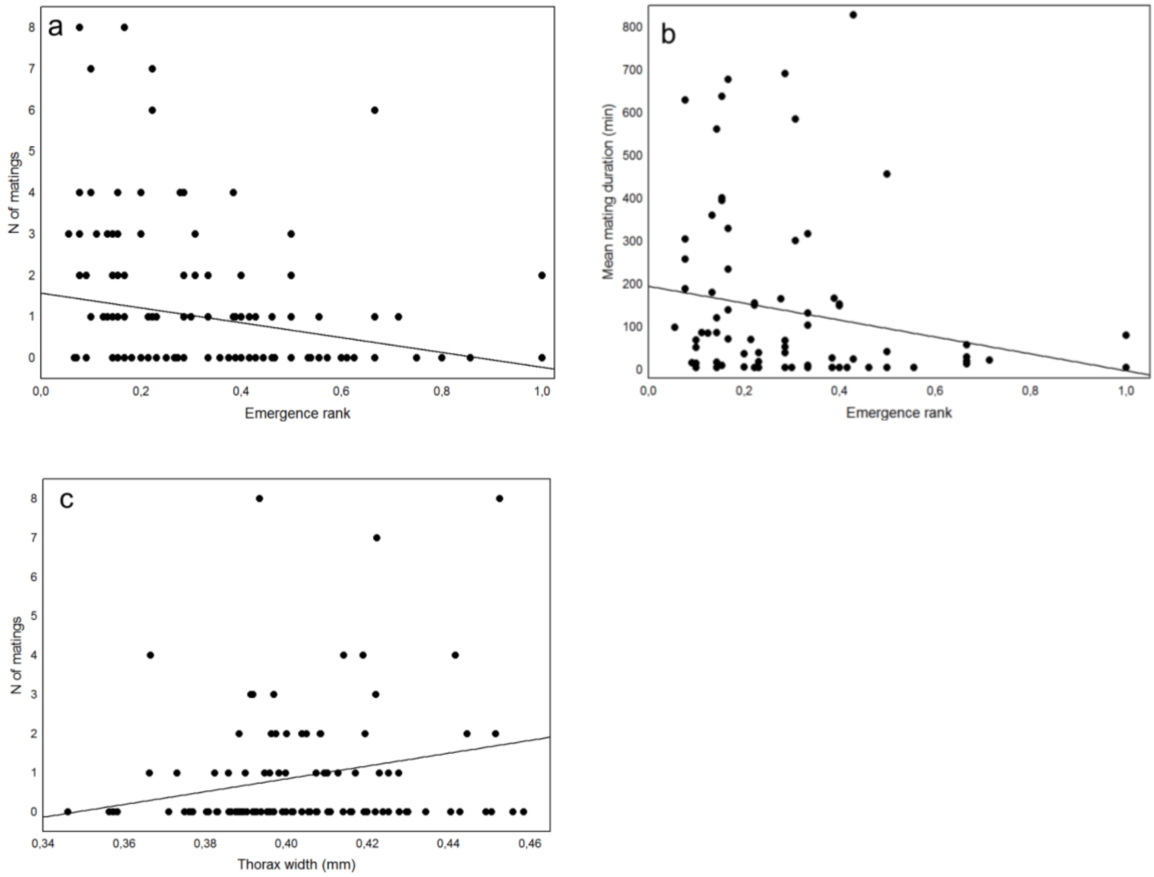

**Fig 2** Associations between a) mating duration of sexually active males and emergence rank, b) N of matings and emergence rank and c) N of matings and thorax width. Earlier emerged males mated more often and longer. Body size was positively associated with the number of matings.

Mean relatedness among males was 0.247 ± 0.042 (mean ± SE) with a minimum relatedness of r = -0.287 and a maximum of 0.842. Male-male relatedness was not associated with any of the mating behaviour variables tested (Table 2).

**Table 2** Spearman Rank correlations between mean male-relatedness (*r*) within nests and the mean nest values for mating duration, lifespan and number of matings.

| **Variable pair** | **N** | **$r_s$** | **t** | **p** |
|---|---|---|---|---|
| *r* x mean mating duration | 31 | 0.085 | 0.462 | 0.648 |
| *r* x mean mating duration (sexually active males) | 26 | -0.006 | -0.028 | 0.978 |
| *r* x mean male lifespan | 31 | -0.166 | -0.905 | 0.189 |
| *r* x mean N of matings | 31 | 0.243 | 1.346 | 0.189 |

After removal of rival males, males mated shorter in our manipulation nests (paired t-test: $t_{11} = 2.808$, $p = 0.017$, Fig 3). This finding cannot be explained by reduced mating durations later in life as our experiment was short (maximum duration of three days) and because matings generally become rather longer instead of shorter over the course of the mating season (Kureck et al. 2011).

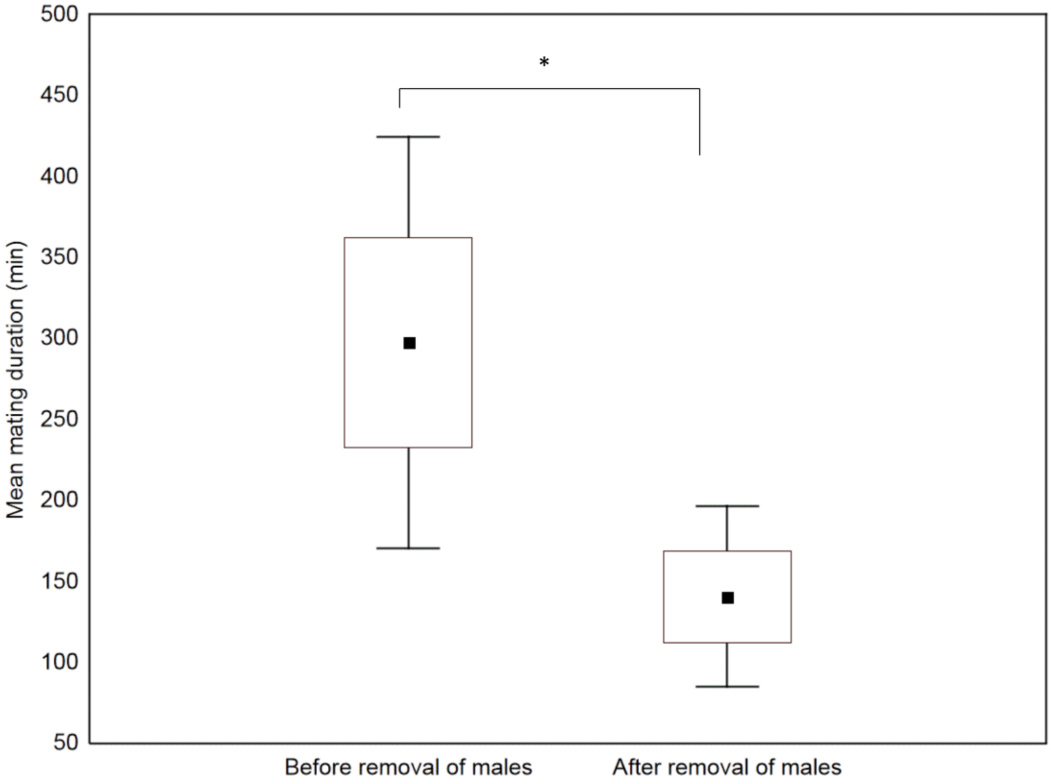

**Fig 3** Mean mating durations within nest before and after removal of males. Mating durations increased after removal of males. Point: mean, box = mean ± SE, whiskers = mean ± 1.96*SE.

## Discussion

Our results demonstrate that wingless *H. opacior* males are under sexual selection to emerge early: Low emergence rank (early emerged) males managed to mate more often and longer than their later emerging rivals. These males face a more female-biased operational sex ratio as more queen pupae are available the earlier a male emerges. Further, individuals that emerge before their rivals, are confronted with a lower competition in the nest. Since males can only mate if queen pupae or young queens are present in the nest, early emergence is more important than a generally long lifespan.

Indeed, we observed that workers kill males after all pupae emerged and the reproductive comes to an end. Extended mate guarding prevents post-copulatory competition on the level of sperm (reviewed in Birkhead and Hunter 1990) as a male that guards his partner until she has fully emerged from the cocoon has a good chance of remaining her only mating partner.

Male body size appeared to be also under sexual selection, albeit to a lesser extent. Larger males mated more often than smaller ones. A higher reproductive success of larger males has also been reported in other Hymenopteran species (Villabos and Shelly1991; Stone et al. 1995; Abell et al. 1999) as well as in many animal taxa in general. Depending on the mating system and ecology of a species, a higher reproductive success of larger males can have different reasons: First, females preferentially choose larger males as mating partners because body size signals a high fitness (female choice; e.g. Robbins et al. 2009). Second, larger males have a higher chance of encountering females because they are better dispersers (Stone at al. 1995, Abell et al. 1998). Third, large males perform better in fighting and in the defence of territories or mating partners (Haley et al. 1994; Bel-Venner and Venner 2005; Pitnick et al., 2009; Breuer et al. 2012). Finally, larger males are more successful in seizing and holding females (Villabos and Shelly 1991). Since wingless *H. opacior* males mate with immobile queens that are still enclosed in the cocoon, there is obviously no female choice. Also, wingless males neither disperse nor actively fight or defend territories. Hence, the higher mating success of larger individuals is most likely due to a better performance in mounting and holding on to female cocoons. Even though we never observed any active aggressive behaviour among males, males might occasionally be pushed off a cocoon by competitors. This interpretation is supported by the observation that sometimes several males try to mount and embrace a single cocoon (Kureck et al. 2011). However, the fact that wingless males are the smallest caste in the nest and carry tiny mandibles suggests that selection for fighter males is rather low. Additionally, as spermatogenesis is terminated in adult males of this species (Foitzik et al. 2002), the sperm amount of wingless males is limited. Larger males might produce more sperm which could allow them to copulate more often (Pech-May 2012).

The finding that early and later emerging males did not differ in body size indicates that there is no trade-off between body size and early emergence. It is yet unclear if the early emergence of some males results from a faster development in these individuals. Alternatively to a fast development, early emerging males might derive from earlier laid eggs, indicating that selection for early males could already act on the level of the mother queens. Indeed, *H. opacior* nests frequently contain several reproducing queens, so queens that lay male eggs early in the season might have a higher fitness.

Relatedness among males did not affect the competitive mating behaviour of wingless males although it influences their indirect fitness. Losing a competitive fight is less costly for a male, if it he is related to its competitor. Therefore males should reinforce their competitive behaviour if the relatedness level of males within a nest is low. The lack of such an adaptive behaviour indicates that males might be 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 Hymenopterans (Crozier 1988; Ratnieks 1991; de Heer and Ross 1997; Strassmann et al. 1997).

Our manipulation demonstrates that ant males mated shorter after we removed competitors. Indeed, the duration of mate-guarding behaviour was found to depend on the number of competitors in other animals (Komdeur 2001, Takeshita et al. 2010) indicating that males can assess the competitive situation in their local social environment. Earlier observations had shown that mating durations of *H. opacior* males co-vary with the number of pupae and rivals in the nest (Kureck et al. 2011). Our experiment now reveals that these behavioral changes can be seen as an active response of males, which are able to notice changes in the competitive situation and quickly adapt by altering their guarding behaviour. It has been suggested that changes in reproductive behaviour can be explained by the encounter rate with competitors and possible mating partners (Wada et al. 1999; Clark and Grant 2010). This means that a lower density of males decreases their competitive behaviour, in the case of *H. opacior* the mating duration, because they encounter each other less frequently.

In conclusion, we demonstrate here that males of a social insect are selected to emerge early, to be large and to be able to respond to the competitive situation in their local environment. Our study thus contributes to the understanding of sexual selection in social insects, a field that is largely unexplored (Boomsma et al. 2005).

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# Chapter V

## No inbreeding depression but increased sexual investment in highly inbred ant colonies

This chapter is based on a research article published in *Molecular Ecology:*

**Kureck IM**, Jongepier E, Nicolai B, Foitzik S (2012) No inbreeding depression but increased sexual investment in highly inbred ant colonies. Mol Ecol 21:5613-5623

# Abstract

Inbreeding can lead to the expression of deleterious recessive alleles and to a subsequent fitness reduction. In Hymenoptera, deleterious alleles are purged in haploid males moderating inbreeding costs. However, in these haplodiploid species, inbreeding can result in the production of sterile diploid males. We investigated the effects of inbreeding on the individual and colony level in field colonies of the highly inbred ant *Hypoponera opacior*. In this species, outbreeding winged sexuals and nest-mating wingless sexuals mate during two separate reproductive periods. We show that regular sib-matings lead to high levels of homozygosity and the occasional production of diploid males, which sporadically sire triploid offspring. On the individual level, inbreeding was associated with an increased body size in workers. On the colony level, we found no evidence for inbreeding depression as productivity was unaffected by the level of homozygosity. Instead, inbred colonies altered their allocation strategies by investing more resources into sexuals than into workers. This shift towards sexual production was due to an increased investment in both males and queens, which was particularly pronounced in the dispersive generation. The absence of inbreeding depression combined with increased reproductive investment, especially in outbreeding sexuals, suggest that these ants have evolved active strategies to regulate the extent and effects of frequent inbreeding.

# Introduction

Inbreeding often negatively affects the fitness of animals by reducing their survival rate, birth weight, morphology, immune system, fecundity, and offspring number and viability (Ralls et al. 1979; Packer and Pusey 1993; Keller et al. 1994; DeRose and Roff 1999; Keller and Waller 2002). Inbreeding depression generally is thought to be caused by the expression of deleterious recessive alleles or a reduction of heterosis (Wright 1977; Charlesworth and Charlesworth 1999). As a consequence, many species actively avoid mating with close kin (Hoogland 1992; Keller and Passera 1993; Pusey and Wolf 1996; Cooney and Bennett 2000; Bretman et al. 2004). However, inbreeding can also be beneficial, because it can fix successful genotypes within a habitat (Hamilton 1964, 1972; Frankham 1995), support social behaviour through an increase in relatedness between individuals within a population (Reeve et al. 1990; Chapman et al. 2000) and evade the risks associated with dispersal (Pusey and Wolf 1996). Furthermore, individuals that mate with their relatives can increase their inclusive fitness not only directly but also indirectly by improving the relative’s mating success (Waser et al. 1986; Kokko and Ots 2006). In addition, in social insects, inbreeding can compensate for the reduction of relatedness and thereby restore fitness benefits to altruistic workers in colonies with multiple reproductives (Thurin and Aron 2009; Thurin et al. 2011). Indeed, not only a lack of inbreeding avoidance (Dewsbury 1988; Keane et al. 1996; Keller and Fournier 2002) but even preferential inbreeding has been described in social and non-social species (Thünken et al. 2007; Thurin and Aron 2009).

In Hymenoptera, a fundamental cost of inbreeding is the production of diploid males, which are often non-viable or sterile. Sometimes, these males exhibit only a reduced fertility and succeed in siring triploid offspring (Petters and Mettus 1980; Cook 1993; El Agoze et al. 1994; Duchateau and Mariën 1995; Holloway et al. 1999; Krieger et al. 1999; Santomauro et al. 2004; de Boer et al. 2007, but also see Cowan and Stahlhut 2004; Elias et al. 2009). The production of diploid males instead of workers or queens can lower colony growth and survival in social Hymenoptera because unlike workers, males never contribute to colony maintenance (Plowright and Pallett 1979; Ross and Fletcher 1986; Tarpy and Page 2002). The sex of most Hymenopteran species is determined by a single-/multilocus complementary sex determination system: heterozygotes at the sex locus develop into females, whereas hemizygous individuals, derived from unfertilized eggs, develop into males. Diploid larvae turn into males if they are homozygous at the sex locus/loci (Crozier 1977; Cook 1993). This locus is generally under diversifying selection and therefore extremely variable (Yokoyama and Nei 1979; Hasselmann ad Beye 2004), so that homozygosity is rare in outbreeding generations. Besides the costs of diploid male production, inbreeding depression was shown to be weaker in haplodiploids, because deleterious alleles are removed from the population by haploid males (Werren 1993; Antolin 1999; Henter 2003). Indeed, in the regular inbreeding haplodiploid ambrosia beetles, positive effects of inbreeding were found (Peer and Taborsky 2005).

So far, only few studies report an impact of inbreeding on colony performance, sexual investment and body size in social insects that are not related to diploid male production. In the outbreeding ant *Formica exsecta*, the relative investment in young queens in single-queen colonies decreases with increasing homozygosity in workers. The number of males remained unchanged, but male size was reduced under inbreeding. These findings imply that workers in inbred colonies care less well for larvae as the size and female caste determination are controlled by larval nutrition (Vitikainen et al. 2011). Further, inbreeding reduces queen lifespan and hence colony survival in *Formica exsecta* (Haag-Liautard et al. 2009), just as in the ant *Cardiocondyla obscurior*, in which also brood mortality increased with inbreeding (Schrempf et al. 2006). Likewise, inbred *Bombus terrestris* bumblebee queens survived hibernation less often and were less successful during colony foundation (Gerloff and Schmid-Hempel 2005).

In this study, we investigated the effects of inbreeding in a regular inbreeding ant, *Hypoponera opacior*. The focal population in the Chiricahua Mountains in Southeastern Arizona exhibits two reproductive seasons during the year. In summer, colonies produce outbreeding winged males and queens that conduct large-scale mating flights. In a second reproductive phase in fall, exclusively wingless reproductives are produced that mate with their nestmates (Foitzik et al. 2010). Wingless males show mate-guarding behaviour and stay for hours in copula with young queens that have not fully emerged from the cocoon. Albeit *H. opacior* nests can contain several queens, most matings are between close kin leading to the second highest levels of inbreeding ever recorded in ants (Foitzik et al. 2011). However, the focal population does not appear to suffer from inbreeding on a population level, as consistently high nest densities were recorded over the last twelve years (Foitzik et al. 2002; Foitzik et al. 2010; Foitzik et al. 2011; Kureck et al. 2011).

We studied the costs and benefits of inbreeding on the individual and colony level in *H. opacior* including diploid male production, body size changes and colony productivity. As in other ants, detrimental effects of inbreeding on both levels are likely. Yet, it is also possible that this species evolutionary adjusted to high inbreeding levels due to habitual brother-sister matings. These adaptations could include an active response of highly inbred ant colonies by diverting resources to the production of outbreeding sexuals, which ensure lower levels of homozygosity in subsequent generations. We therefore investigated allocation strategies of *H. opacior* nests in both seasons with respect to colony inbreeding levels.

## Materials and methods

### *Ant collection, nest demography and productivity*

In 2010, we collected 43 *H. opacior* colonies with cocoons in early July (N = 12) and late August (N = 31) from their natural nest sites under stones in Southeastern Arizona, shortly before the emergence of the pupae. All colonies stemmed from the same population in the Chiricahua Mountains and were collected close to the Southwestern Research Station (N 31° 52.000′, W 109° 12.609′). *H. opacior* has a highly synchronized brood production. New workers and sexuals emerge twice per year over the course of several days in mid-July and early September. We kept ant colonies until all ants emerged from the cocoons (one to two weeks) in observation nests in the laboratory at the Southwestern Research Station (SWRS near Portal, AZ). Directly after collection, we counted the number of adult individuals and pupae. In addition, we noted down the number of young light-coloured callow individuals, which had already emerged from the cocoon. These individuals belong to the same generation as the ones still enclosed in the cocoons and we removed them from the nest and stored them for further analyses. Ant colonies were checked daily for the emergence of new queens, workers or males, which were immediately removed and stored in 100 % ethanol. Colonies were kept in three-chambered boxes (10 x 10 x 3 cm) with a moistened plaster floor and fed daily with dead insects. Newly emerged individuals (‘callows’) are easy to discriminate from older individuals by their soft and yellowish cuticle, compared to the dark brown colouration of older individuals. In our analyses, light coloured callows and emerging ants were recorded as ‘young generation’, whereas individuals with a darkened cuticle at the time of collection were grouped to the ‘old generation’. The latter individuals, workers and queens, were also used to estimate colony size (Kureck et al. 2011).

We carefully determined the number of emerging males, queens and workers of these field colonies. Total biomasses per colony and allocation ratios were calculated by multiplying the number of newly produced individuals of each caste with its average dry weight as previously determined for the same population (Mean dry weights ± SE: winged queens: 246 ± 9 µg; intermorphic queens: 203 ± 6 µg, workers: 179 ± 5 µg, winged males: 151 ± 9 µg, wingless males: 89 ± 2 µg; Foitzik et al. 2010). Number of pupae, biomass of newly produced individuals and the number of newly emerged individuals were used as measures of colony productivity.

*Morphologic measurements and microsatellite analyses*

After all ants emerged from the cocoons, the colonies were preserved in 100 % ethanol. From each colony we took a sample of five old generation workers and up to five old queens, young queens and young workers each for morphometric measurements and genetic analyses. In total, we measured and genotyped 441 individuals. For each of these individuals we measured the head and thorax width under 40 x magnifications using a stereomicroscope coupled to a computer and the measuring software LAS (Leica Application Suite V.3.8 from Leica Microsoft). Individuals were washed in dd$H_2O$ and homogenized in a ball mill (Retsch MM301 Ball Mill). DNA was isolated using the Puregene DNA extraction kit (Gentra Systems). We amplified the DNA on 11 microsatellite loci, four of which were already described (HoP26, HoP54, HoP60, HoP64; Rüger et al. 2005). Additionally, we designed seven new primer pairs (HoP4, HoP26, HoP28, HoP45, HoP65, HoP76, HoP89) for repeat motifs found in clones designed in 2005 (Rüger et al. 2005; for a detailed primer description and amplification conditions see table S1 of the supplementary material). The amplification products were detected in a capillary sequencer (Applied Biosystems GA 3130xl Genetic Analyzer) and analysed using the Program Genemarker (V1.97 Demo Version). Deviations from Hardy-Weinberg equilibrium, observed and expected heterozygosities, linkage disequilibrium and the inbreeding coefficient ($F_{is}$) were calculated using GenAlex v.6.4 software (Peakall and Smouse 2006).

In addition to the 441 queens and workers, we measured head and thorax width of 19 winged and 47 wingless males from 18 of the 43 nests. For the estimation of the frequency of diploid males we genotyped a total 416 (379 wingless and 37 winged) males from 112 nests collected in 2010 and 2011 in Chiricahua Mountains. 17 of these nests (including 83 males) belonged to the sample also used to estimate inbreeding effects.

In 38 of the nests collected in fall 2011, we genotyped all present males. These nests were used to analyse the frequency of diploid males among diploid brood ($N_{\text{diploid males}}$ / $N_{\text{pupae}} - N_{\text{haploid males}}$) and the frequency of diploids among males ($N_{\text{diploid males}}$ / $N_{\text{all males}}$).

### *Estimation of the level of inbreeding*

We estimated the level of inbreeding as the individual average homozygosity at loci weighted by the allelic diversity of each locus (*HL*), a method introduced by Aparicio et al. (2006). In order to estimate the level of inbreeding within a colony, we averaged over the *HL* values of diploid female individuals. Individual *HL* values were used to investigate the impact of homozygosity on the individual level, whereas we used the average *HL* values to investigate inbreeding effects on the colony level, including an association with the frequency of diploid males.

### *Statistical analysis*

The effect of individual level homozygosity on the size of newly emerged diploids was assessed in separate analyses for workers, winged and wingless queens, for which we used a set of linear mixed effect models (lme function in the nlme R package; Pinheiro et al. 2009), including either thorax width or head width as the dependent variable and individual *HL* as the predictor. Both season (as a covariate) and its interaction with individual worker *HL* were included in the analyses of worker morphometrics, to account for potential differences in worker morphology between seasons as well as test for season dependent effects of individual homozygosity. The effect of season on winged and wingless queen morphometrics could not be assessed since only one queen morph was produced each season. To circumvent the problem of pseudoreplication, we included nest identity as a random factor in the mixed-effect models. This takes the non-independence of data from individuals of the same colony into account. Male size was analyzed separately since individual *HL* scores could, by definition, not be assigned to haploid males and therefore the average colony *HL* was used to estimate the effect of colony level homozygosity on male morphometrics.

To test whether workers, winged and wingless queens differed in individual level homozygosity, individual *HL* was compared between castes using lme (Pinheiro et al. 2009) as residuals did not deviate from normality. Nest identity was included as random factor to account for non-independence of nest mate homozygosity scores. Potential differences between the individual *HL* of summer and fall generation workers as well as between the old and young (i.e. newly emerged) generation workers were likewise assessed while taking nest identity into account.

The colony-level effects of homozygosity on productivity were assessed with a set of general(ized) linear models. Hereto, the total number of pupae and newly emerged individuals were analysed using Poisson regression with log-link function. The total biomass produced was square-root-transformed to obtain normality of residuals and subsequently analysed using linear regression. The initial models assessing colony productivity included the average colony *HL*, season (as a covariate) and their interaction to test for potential main and season dependent effects of colony homozygosity. In addition, colony size and its second order component served as covariates to control for potentially confounding effects.

The reproductive allocation ratio (relative investment in sexuals vs. workers) and the sex allocation ratio (relative investment in males vs. queens) were analysed using logistic regression with a logit-link function and *HL*, season (the covariate) and their interaction as predictors. We repeated these analyses using numerical ratios, the results of which are reported in Table S2 of the supplementary material.

All generalized models were fitted by quasi-restricted maximum-likelihood estimation to account for overdispersion. For model selection, we used a backward-forward stepwise selection procedure ($\alpha = 0.05$). All analyses were performed using R (R Development Core Team 2010).

# Results

*Frequency of diploid males and occurrence of triploid workers*

In seven of the colonies used for the inbreeding analyses, we detected nine wingless diploid males. These seven nests also belonged to the subsample of 38 nests, for which we calculated the proportion of diploid males among diploid brood and diploids among males. Between zero and 4.2 % of the diploid brood developed into males (average frequency over nests: 0.56 % ± 0.20 %, mean ± SE). The proportion of diploid males among wingless males varied between zero and 50 % (average frequency over nests: 4.29 % ± 1.68 %, mean ± SE). The average proportion of diploids in the seven (18.4%) diploid male producing nests was 23.3% ± 5.1%. The frequency of diploid males did not increase with average colony *HL* (logistic regression on the N of genotyped diploid vs. haploid males per nest: $\Delta$ deviance = -0.36, N = 17, $\Delta$d.f. = 1, p = 0.55).

In total, among all genotyped 416 males, we detected 66 (15.9 %) diploid individuals from 43 (38.4 %) colonies and all of these diploid males were wingless. Hence, a higher frequency of diploid males was found among the wingless males (17.4 %), than among the winged males (0 %; Fisher exact two-sided: df = 1; $p < 0.002$). Observed heterozygosity ($H_O$) of diploid males was 0.117. Males were heterozygous on one (N = 60) to two (N = 6) loci.

Among the 441 genotyped queens and workers of these colonies, we detected five triploid individuals (1.1 %), including four workers and a wingless queen, which were all adult individuals (‘old generation’) in fall and in two of these nests we also found diploid males.

*Individual level effects of homozygosity*

Overall, expected heterozygosity ($H_E$) was 0.823 and overall observed heterozygosity ($H_O$) was 0.587. The $F_{is}$ as averaged over loci was 0.28 ± 0.027 (mean ± SE, Table S1, supplementary material). The *HL* of diploids ranged from almost completely heterozygous (*HL* = 0.068) to fully homozygous (*HL* = 1). Deviations from Hardy–Weinberg equilibrium were significant for all loci and we did not detect significant linkage between any marker pair ($p < 0.01$ after Bonferroni correction for multiple comparisons).

Newly emerged workers, winged queens and wingless queens did not differ in their individual *HL* score ($F_{2,207} = 0.395$, p = 0.674; $HL_{\text{worker}} = 0.401 \pm 0.018$, $HL_{\text{winged queen}} = 0.412 \pm 0.022$, $HL_{\text{wingless queen}} = 0.426 \pm 0.033$), neither did worker homozygosity differ between seasons ($F_{1,36} = 0.103$, p = 0.751), or generations ($F_{1,274} = 0.006$, p = 0.939).

Surprisingly, worker head width significantly increased with individual *HL* ($F_{1,112} = 5.72$, p = 0.019; Fig. 1 a), and this association did not vary with season ($F_{1,111} = 0.91$, p = 0.343). Contrastingly, worker thorax width only tentatively increased with individual *HL* ($F_{1,111} = 3.42$, p = 0.067), but we found a season-dependent relationship (*HL*–season interaction: $F_{1,111} = 5.36$, p = 0.023). Thorax width increased with individual homozygosity in fall (Fig. 1 b; $t_{111} = 2.70$, $p = 0.008$) but not in summer ($t_{111} = -1.21$, $p = 0.291$). In winged queens, thorax width tended to increase with individual level homozygosity ($F_{1,35} = 3.98$, $p = 0.054$). However, Fig. 1c indicates that this trend was heavily influenced by a single data point and it disappeared when we redid the analysis excluding this outlier ($F_{1,34} = 2.02$, $p = 0.165$). Also head width was unaffected by *HL* in winged queens ($F_{1,35} = 0.298$, $p = 0.588$). Similarly, we found no relationship between wingless queen morphometrics and *HL* (head width: $F_{1,31} = 0.227$, $p = 0.637$; thorax width: $F_{1,32} = 0.322$, $p = 0.575$). Male size was unrelated to the average homozygosity of diploids in the colony (head width: $F_{1,16} = 0.259$, $p = 0.618$; thorax width: $F_{1,16} = 0.173$, $p = 0.683$).

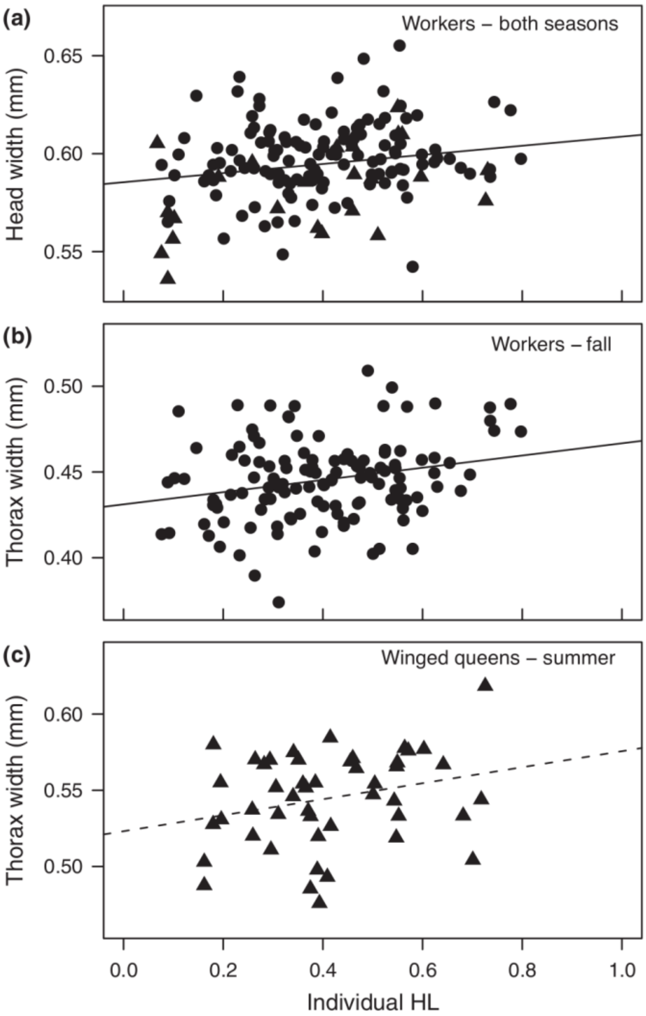

**Fig. 1.**Relationship between individual level homozygosity (*HL*) and morphometrics of newly emerged winged queens and workers. Estimates are derived from the linear mixed effect models as described in the result section. Solid lines: $p < 0.05$; dashed line: $p = 0.054$. Circles: newly emerged individuals in fall; triangles: newly emerged individuals in summer. Sample sizes: a) N = 151; b) N = 125; c) N = 47.

Worker size was subject to allometric changes between seasons (Fig. 2), suggesting that workers, like reproductives, consist of two seasonal castes. Workers of the summer generation had smaller heads ($t_{36} = 4.27$, $p < 0.001$), but larger thoraces ($t_{36} = -3.00$, $p = 0.005$) compared to workers produced in fall. Winged queens produced in summer were larger than wingless queens produced in fall, both with respect to head width (winged: 0.638 ± 0.004, wingless: 0.621 ± 0.003, $F_{1,28} = 9.50$, $p = 0.005$) and thorax width (winged: 0.545 ± 0.005, wingless: 0.469 ± 0.005, $F_{1,28} = 117.79$, $p < 0.001$). Likewise, winged males were larger than the wingless males of the fall generation (head width: winged: 0.549 ± 0.008, wingless: 0.510 ± 0.006, $F_{1,16} = 16.53$, $p < 0.001$; thorax width: winged: 0.526 ± 0.005, wingless: 0.391 ± 0.004, $F_{1,16} = 436.74$, $p < 0.001$).

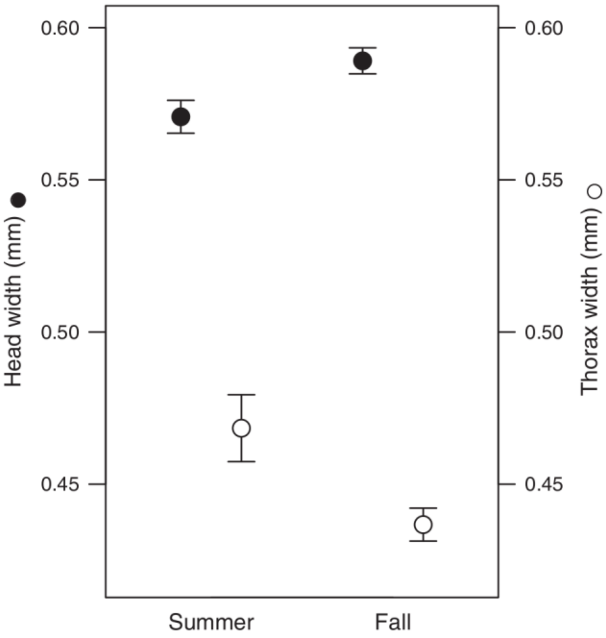

**Fig. 2** Allometric changes in worker morphology with season. Head and thorax width are represented by the mean ± SE, as estimated by the linear mixed effect model. N = 151.

*Colony level effects of homozygosity - productivity*

The 43 *H. opacior* nests contained on average 17.4 ± 2.6 workers and queens and produced between 0 and 233 pupae (mean ± SE: 65.7 ± 9.8). Colony size (i.e. the number of workers and queens) was not related to the average colony HL (quasi-Poisson GLM: $F_{1,41} = 0.04$, $p = 0.840$).

The average level of colony homozygosity was not related to the number of pupae, the number of newly emerged individuals or the total biomass produced, nor was there an interaction effect of season with colony *HL* on any of the productivity measures (Table 1).

Colonies produced more pupae in fall (mean ± SE: 79.58 ± 11.56) than in summer (29.75 ± 12.74), although there were no seasonal differences in either the number of or the total allocation into newly emerged individuals. Both the numeric and the biomass allocation increased asymptotically with colony size (Table 1).

**Table 1** Results of the general(ized) linear regression analyses on the relationship between colony productivity, colony homozygosity and several covariates. The number of pupae and the number of newly emerged individuals were analysed using quasi-Poisson regression. The (square-root-transformed) total biomass was analysed with a set of general linear models.

| | F | df | p |
|---|---|---|---|
| *a) Number of pupae* | | | |
| Colony size | 2.44 | 1,40 | 0.126 |
| Colony size$^2$ | 2.79 | 1,39 | 0.103 |
| Season | 8.43 | 1,41 | **0.006** |
| Average colony *HL* | 0.38 | 1,40 | 0.541 |
| Season x Average colony *HL* | 0.12 | 1,39 | 0.726 |
| *b) Number of newly emerged individuals* | | | |
| Colony size | 4.59 | 1,41 | **0.038** |
| Colony size$^2$ | 3.92 | 1,40 | **0.055** |
| Season | 3.16 | 1,40 | **0.083** |
| Average colony *HL* | 0.70 | 1,40 | 0.407 |
| Season x Average colony *HL* | 0.14 | 1.38 | 0.706 |
| *c) Total biomass ** | | | |
| Colony size | 6.60 | 1,40 | **0.014** |
| Colony size$^2$ | 4.64 | 1,40 | **0.037** |
| Season | 0.63 | 1.39 | 0.432 |
| Average colony *HL* | 0.97 | 1.39 | 0.330 |
| Season x Average colony *HL* | 0.04 | 1,37 | 0.840 |

* square-root transformed

*Colony level effects of homozygosity – allocation strategies*

The relative investment in sexuals was season-dependent and higher in summer. More importantly the reproductive allocation ratio increased with the average level of colony homozygosity ($p < 0.001$; Table 2). This effect was more pronounced in summer than in fall as the interaction between *HL* and season demonstrates ($p < 0.014$; Table 2). However, the inbreeding-dependent shift in the reproductive investment ratio was present in both seasons (winged summer generation: $t = 2.41$, $p = 0.021$; wingless fall generation: $t = 2.08$, $p = 0.044$; Fig. 3). Analysis of the numeric reproductive ratio showed similar, season-dependent investment biases in response to colony inbreeding (Table S2, supplementary material).

Neither the sex allocation ratio (Table 2), nor the numeric sex ratio (Table S2, supplementary material) was related to colony level homozygosity. This suggests that a shift towards higher reproductive investment in more inbred colonies resulted from both increased investment in males and queens. Indeed, closer examination of the relationships between the average colony *HL* and the relative investment in “males vs. workers” and “queens vs. workers” showed a shift towards both males ($F_{1,40} = 5.05$, $p = 0.030$), and queens ($F_{1,41} = 4.23$, $p = 0.046$) in more inbred colonies.

In accordance with previous reports of seasonal sex ratio shifts in *H. opacior* (Foitzik et al. 2010), the relative investment in sexuals became more male biased in fall (Table 2; est ± SE = 1.70 ± 0.41). To test whether the more male-biased sex allocation ratio in fall could be due to the higher frequency of diploids among the wingless males, we repeated the sex ratio analysis after randomly assigning 17.4 % (i.e. the fraction of diploid males among the wingless males, see above) of the wingless males to the wingless queen caste. This procedure was repeated 5000 times and we report the highest p-value. Under the highly stringent assumption that all presumed diploid, wingless males were destined to develop into queens (and not into workers), we still found a more male-biased sex ratio in the wingless fall generation compared to the winged summer generation ($p_{max} = 0.038$). This additional analysis demonstrates that the observed seasonal sex-ratio difference is not solely caused by an increased production of diploid males in fall, albeit the higher rate of diploid male production might contribute to the magnitude of the sex ratio difference.

**Table 2** Results of the logistic regression analyses of the relationship between colony inbreeding and season on the reproductive allocation ratio (rel. investment in sexuals vs. workers) and the sex allocation ratio (rel. investment in males vs. queens). Both allocation ratios were analysed using overdispersed logistic regression.

| | F | df | p |
|---|---|---|---|
| *Reproductive allocation ratio* | | | |
| Season | 21.96 | 1,41 | <0.001 |
| Average *HL* | 14.73 | 1,40 | <0.001 |
| Average *HL* x Season | 6.69 | 1,39 | 0.014 |
| *Sex allocation ratio* | | | |
| Season | 18.43 | 1,36 | <0.001 |
| Average *HL* | 0.13 | 1,35 | 0.716 |
| Average *HL* x Season | 0.41 | 1,34 | 0.528 |

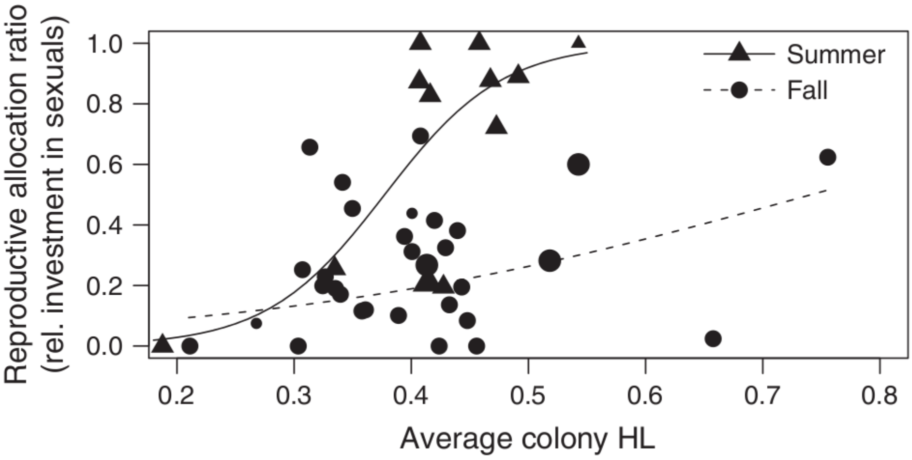

**Fig 3** Relationship between colony level homozygosity (*HL*) and the reproductive allocation ratio (rel. investment in sexuals vs. workers). Regression lines are derived from the overdispersed logistic regression model presented in Table 2. Character sizes reflect the total sample size (i.e. the weights in the logistic regression): small: <10 individuals, intermediate: 10 - 40 individuals, large: >40 individuals. Summer: N = 12; fall: N =31.

# Discussion

We demonstrate no effect of inbreeding on colony productivity in an ant species with regular sib-mating. Rather, inbred *Hypoponera opacior* ant colonies developed alternative investment strategies and allocated more resources into sexuals than into workers. On an individual level, body size increased with the level of homozgyosity in workers. The only detectable cost of inbreeding was a moderate production of diploid males, which were able to sire triploid offspring.

In contrast to the detrimental effects of inbreeding on the fitness of animals in general (Charlesworth and Willis 2009) and of ants in particular (Schrempf et al. 2006; Vitikainen et al. 2011), we found no decrease in colony productivity with inbreeding in *H. opacior* albeit colonies could reach mean *HL* values of up to 0.76. In the ant *Cardiocondyla obscurior*, which has the highest inbreeding level ever recorded in ants, inbreeding led to increased brood mortality (Schrempf et al. 2006), whereas there was no effect on any developmental stage in *H. opacior*. In the outbreeding ant *Formica exsecta*, colonies with a higher *HL* raised the same number of offspring, but offspring biomass was lower due to the production of fewer queens and smaller males (Vitikainen et al. 2011). In contrast, in *H. opacior* the size of workers increased with the individual level of inbreeding. The absence of inbreeding depression has also been reported in the regular inbreeding haplodiploid Ambrosia beetle *Xylosandrus germanus*, in which inbreeding did not reduce brood number, size or survival (Peer and Taborsky 2005). Rather, outbreeding had a detrimental effect on the hatching success of eggs. In line with this study we here present another regularly inbreeding species that is not negatively affected by its reproductive system.

Despite the high level of homozygosity, we detected a very low diploid male production as only 0.56 % of the diploid larvae developed into males. This resembles the situation in *Cardiocondyla obscurior*, in which multilocus complementary sex determination was suggested to explain the very low frequency of diploid males (Schrempf et al. 2006). Genomic imprinting is an alternative mechanism of sex determination that could explain low numbers of diploid males despite high levels of homozygosity. This mechanism has been found in the parasitoid wasp *Nasonia vitripennis* (Beukebook and van de Zande 2010). Here, paternally derived genes are necessary to activate the female developmental pathway. Diploid males emerge if unfertilized diploid eggs are produced. In *N. vitripennis* this is the case when triploid queens lay eggs. However, the finding that diploid males show a high degree of homozygosity ($H_O = 0.117$) rather points to a complementary sex determination system in *H. opacior* and to inbreeding as the cause of diploid male production. Alternatively to a generally low production of diploid males, they might have a low survival probability or be detected and killed by workers during the larval stage (Woyke 1963). It is surprising that we did not find a single winged diploid male because *HL* values did not change with season. We cannot be sure at the moment whether winged diploid males are viable at all in *H. opacior*. They are so in many other ant species such as *Solenopsis invicta* (Ross and Fletcher 1985) or *Lasius sakagamii* (Yamauchi et al. 2002). Possibly the development towards winged *Hypoponera* males is more sensible to disturbances as they have a more complex bauplan and show more complex behaviours as adults than their wingless counterparts. On the other hand, there might be stronger selection on workers to eliminate winged diploid males during larval development. Winged diploid males will never contribute to the fitness of the mother nest, as winged queens mated with a diploid male will only have sterile triploid female offspring and probably fail already during colony foundation. Instead, diploid wingless males might be less costly as wingless queens mate multiply (Foitzik et al. 2002) and triploid workers might be less of a burden in an already established nest. In future, we plan to genotype more winged males to determine whether diploid winged males are viable.

The detection of triploid workers and a triploid queen, demonstrates that diploid males are not entirely sterile. As triploid queens are expected to be sterile, diploid males are an evolutionary dead end. However, diploid males, that were originally meant to develop into females themselves, can indirectly contribute to the colony’s worker force by siring workers. *Hypoponera* workers are invariably sterile, and triploids appeared to be quite viable. Reproduction by diploid males has been reported in a number of Hymenopteran species; however, these males were generally found to be less fertile (Woyke 1973; Hung et al. 1974; Woyke 1974; Duchateau and Mariën 1995; Krieger et al. 1999; de Boer et al. 2007). In *H. opacior* the low number of at least partially fertile diploid males implies that the fitness costs through diploid male production are less severe than in other Hymenopteran species (Plowright and Pallett 1979; Ross and Fletcher 1986; Tarpy and Page 2002).

Inbreeding and its associated costs affect reproductive allocation decisions in numerous taxa. For instance, many species bias the sex ratio in favour of the most dispersive sex and inbreeding avoidance has been invoked as one of the major drivers of sex-biased dispersal (e.g. Pusey 1987; Perrin and Mazalov 1999, 2000). In *H. opacior*, wingless and winged sexuals strongly differ in their dispersal capabilities: The 2 to 3-mm-long wingless sexuals can only disperse short distances on foot, whereas the larger, winged reproductives mate during large-scale nuptial flights. Investment in winged sexuals result in outbred daughter colonies, while wingless sexuals probably cannot avoid inbreeding due to their low dispersal abilities. Our finding that elevated reproductive allocation is most pronounced for the outbreeding summer generation is thus in accordance with inbreeding avoidance-driven investment strategies.

Increased investment in sexuals is unlikely caused by the production of diploid males for several reasons. First, we found that, even after randomly assigning the fraction of diploid males among genotyped wingless males to the wingless queen caste, the sex ratio still remained male-biased. Second, the proportion of diploid males is unrelated to the average level of colony inbreeding. And third, the increase in reproductive allocation with colony inbreeding was due to an increase in the number and total allocation into both males and queens, where the latter could not be caused by the production of diploid males. The more male-biased sex ratios rather reflect a stronger impact of local resource competition among wingless queens than local mate competition among wingless males during this season (Foitzik et al. 2010). The fact that we found more pupae in fall than in summer colonies, but no difference in the number or total allocation into new individuals, points to a higher brood mortality in fall. However, this higher mortality could also be due to variable laboratory conditions, as fall nests contained more pupae and the air humidity was higher after the summer monsoon rains. Hence, it is unclear whether fall pupae show a higher mortality also in the field.

The higher sexual investment under inbreeding contrasts with studies on the generally outbreeding ant *Formica exsecta*, in which more homozygous colonies produced a lower sexual biomass (Haag-Liautard et al. 2009; Vitikainen et al. 2011). This was explained as a cost rather than an adaptive response to inbreeding. In *H. opacior*, the higher sexual investment did not result in an increased overall biomass production, as the increased investment in heavier queens was compensated by an increased investment in lighter males (Foitzik et al. 2010). Although we did not find effects of inbreeding on colony productivity, occasional outcrosses are thought to be important to restore heterozygosity and to control diploid male production (Crozier 1971, 1977).

Social insects are able to recognize and respond to the global genetic structure of their colonies based on the colony odour composition, which consists of cuticular hydrocarbons of all nestmates (Boomsma et al. 2003; Kellner and Heinze 2010). High levels of inbreeding might be perceived by workers as well, for example via a reduction in the diversity of chemical cues. Workers are less likely to detect the inbreeding level of individual larvae, similar to the absence of true kin recognition in most social insects (e.g. Keller and Fournier 2002). In most Hymenopteran species, castes of female individuals are determined by the amount or quality of nutrition that they receive during the larval stage. Better provisioned larvae develop into queens while less-well-provisioned larvae will become workers (Wilson 1971; Wheeler 1986). This mechanism of female caste determination has also been suggested for *Hypoponera opacior* (Rüger et al. 2008). Hence, workers that are able to detect high levels of inbreeding could actively “push” more diploid larvae over the developmental threshold of becoming a queen by providing them with more or higher quality food. This assumption is further supported by the increase in body size in more inbred workers. Because queens were not significantly more inbred than workers, we conclude that the homozgyosity level of a diploid larva does not influence its caste fate. Rather, we interpret our findings in a way that workers are able to detect high levels of inbreeding in the colony and react to the situation by allocating more resources into larvae (resulting in more queens and larger workers).

The allometric changes in worker morphology with season are likely a side effect of the developmental sensitivity to season in the species. Morphological differences between individuals raised in different seasons are common in various bi- and multivoltine insects. These variations are thought to develop as a consequence of proximate environmental effects such as variations in developmental phases, temperature, food amount and food quality, but also as adaptive responses to seasonal cues (Mousseau and Roff 1995; Teder et al. 2010; Hardersen et al. 2011; Quezada-Euán et al. 2011). In *H. opacior*, winged sexuals that are produced in summer clearly differ in their morphology from wingless sexuals produced in fall. The seasonal differences in morphology associated with distinct sexual behaviours can be regarded as an adaptation to the season: While winged females in summer have enough time to establish a new colony independently, wingless queens found new colonies with the start-up help of workers from the mother colony which facilitates colony survival during the first winter (Foitzik et al. 2010). This seasonal phenotypic plasticity is obviously also present in worker larvae and therewith leads to varying morphometrics between workers produced in summer and fall. We cannot rule out that these differences in worker morphology are adaptive, as fall workers face different conditions than the possibly short-lived summer worker generation.

In conclusion, we found that inbreeding does not seem to have negative effects on *H. opacior* colonies although they exhibit very high levels of homozygosity. We further found evidence for an active response of ant colonies to high inbreeding rates by increasing reproductive investment. This way, colonies ensure regular outbreeding in combination to unavoidable inbreeding events in fall and thereby manage to regulate the extent and effects of inbreeding. Such a shift of resources towards sexual reproduction as an adaptive response to inbreeding has never been reported before.

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**Data Accessibility:**

Microsatellite data, morphologic data, R script and input files: DRYAD entry doi:10.5061/dryad.60750.

# Supplementary material

## *Microsatellite analysis*

A single PCR amplification was carried for locus HoP54 (reaction solution: 10 µl PCR $H_2O$, 3.34 µl 5x Taq polymerase buffer, 0.166 µl Phusion® Hot Start Taq, 0.33 µl of 10 mM dNTPs, 1.5 µl DNA template DNA and 0.66 µl of HoP54 Primer). Three multiplex amplifications with two primer pairs in each reaction were conducted for HoP76 and HoP45, HoP65 and HoP4 and with HoP74 and HoP64 (reaction solution: 10 µl PCR $H_2O$, 3.34 µl 5x Taq polymerase buffer, 0.166 µl Phusion® Hot Start Taq, 0.33 10 µl of 10 mM dNTPs, 1.5 µl DNA template DNA and 0.36 µl of HoP76/HoP4/HoP64 and 0.3 µl of HoP45/HoP65/HoP74 Primer). A multiplex amplification with four primer pairs in one reaction was carried out with the loci HoP26, HoP28, HoP60 and HoP89 (reaction solution: 21 µl PCR $H_2O$, 6.68 µl 5x Taq polymerase buffer, 0.33 µl Phusion® Hot Start Taq, 0.67 µl of 10 mM dNTPs, 2 µl DNA template DNA, 0.32 µl of HoP26 and HoP60 Primer, 0.3 µl of HoP28 Primer and 0.4 µl of HoP89 Primer). The PCR reactions were carried out with as starting temperature of 95°C for 15 min, followed by 33 cycles of 1:15 min at 94°C, 0:45 min at $T_a$ and 0:45 at 75°C. Deviations from Hardy-Weinberg equilibrium were significant for all loci and we did not detect significant linkage between any marker pair ($p < 0.01$ after Bonferroni correction for multiple comparisons). Overall $H_E$ was 0.823 and overall $H_O$ was 0.587. The $F_{is}$ as averaged over loci was 0.28 ± 0.027 (mean ± SE). For some individuals we obtained no or only very weak amplification products on some loci (missing amplification products in 9 % of 11 loci times 440 individuals). This might be either due to failures in the PCR or to the presence of null alleles. The inbreeding coefficient per locus ($F_{is}$) did not co-vary with frequency of missing data (Spearman Rank correlation: $r_s = -0.041$, $p = 0.905$, $N = 11$). Hence, missing products were most probably due to multiplexing the PCRs. The conclusion that high levels of homozygosity are due to true inbreeding and not to the presence of null alleles is in line with a previous study that showed that null alleles cannot explain the observed deviation from Hardy-Weinberg equilibrium in the loci HoP26, HoP54, HoP60 and HoP64 (Foitzik et al. 2011) and with the observation of frequent sib-matings within the colonies.

**Table S1** Primer sequences and characterization of eleven microsatellite loci in the ant Hypoponera opacior: 1: previously described in (Rüger et al. 2005); Ta: annealing temperature; NA: number of alleles; HO: observed heterozygosity; HE: expected heterozygosity; mca: most common allele; Fis: inbreeding coefficient per locus, *: Forward (F) primer was FAM, HEX or NED-labeled.

| Locus | Primer (5’-3’) | Repeat motif | $T_a$ (°C) | $N_a$ | Size of mca (bp) | Frequency of mca | $H_O$ | $H_e$ | $F_{is}$ |
|---|---|---|---|---|---|---|---|---|---|
| HoP4 | F: *TTTGCTCGCGAGAGTAGG<br>R: CGCTCTAAATAGGCGAGGAG | $(CT)_{27}$ | 56 | 22 | 118 | 0.382 | 0.589 | 0.824 | 0.284 |
| HoP26[1] | F: *TCGCGTTAAGTTCCGTTAAGC<br>R: TCGCGCGAAGCGTCTAACTCG | $(CA)_{16}CGCACG(CA)_2TA(CA)_8$ | 52 | 27 | 144 | 0.157 | 0.703 | 0.920 | 0.235 |
| HoP28 | F: *TAACGAGAGACGCACTAC<br>R: CACACGTAGCCCTTTGATCC | $(GT)_{17}$ | 52 | 22 | 145 | 0.273 | 0.486 | 0.851 | 0.428 |
| HoP45 | F: *GCGGTCCGTCTCCTTTTT<br>R: GCGGAGTGTCCTGGTATAAAT | $(CT)_8$ TT $(CT)_9$ | 57 | 10 | 132 | 0.461 | 0.619 | 0.692 | 0.102 |
| HoP54[1] | F: *TCCGCACGTGGCAAATAGC<br>R: AGCCTGTAGTCCAACTTATCG | $GTGC(GT)_4GC(GT)_{11}GC(GT)_2$ | 52 | 13 | 98 | 0.119 | 0.641 | 0.933 | 0.312 |
| HoP60[1] | F: *GATAACGGGACGTGATCTAGC<br>R: GCAAATCTAAACAGCGAACG | $(CA)_{26}CG(CA)_6CG(CACG)_2(CA)_2$ | 52 | 35 | 125 | 0.1661 | 0.632 | 0.931 | 0.321 |
| HoP64[1] | F: *CGATTTACAAACGATAATGC<br>R: ATACAAGGTGACTCCACTCG | $(TG)_{12}GG(TG)_8GGTG$ | 54 | 29 | 146 | 0.343 | 0.682 | 0.847 | 0.194 |
| HoP65 | F: *GGCGATACTGTCGTCTCT<br>R: GGTAGTATCGAGCGAACGTC | $(CA)_{28}$ CGTC $(CACG)_2$ | 56 | 22 | 128 | 0.440 | 0.465 | 0.700 | 0.338 |
| Hop74 | F: *CAGTCAACCCCGCAAGTTAT<br>R: CGAGATTCCCTAGGGCTGTT | $(GT)_8(GCGT)_2(GT)_5$ | 54 | 16 | 118 | 0.597 | 0.481 | 0.613 | 0.215 |
| HoP76 | F: *ATTTGTAGCCGTCCCTGA<br>R: GCCACCAGATTTTTCTCCTC | $(CA)_{52}$ | 57 | 39 | 65 | 0.155 | 0.603 | 0.942 | 0.361 |
| HoP89 | F: *GCAGCCTATTCACGCATA<br>R: GCAGATTGCATCGGTAAC | $(GC)_2(AC)_{23}$ | 52 | 20 | 76 | 0.166 | 0.559 | 0.802 | 0.301 |

### *Analyses of numeric ratios*

**Table S2** Results of the logistic regression analyses on the influence of colony homozygosity (*HL*) and season on the numeric reproductive ratio (proportion of sexuals vs workers), numeric caste ratio (proportion of queens vs. workers), numeric sex ratio (proportion of males vs, queens) and numeric ploidy ratio (proportion of haploids vs. diploids). All ratios were analysed using overdispersed logistic regression. Parameters depicted in bold were included in the final models which were selected using backwards-forward stepwise procedure.

| | F | df | p |
|---|---|---|---|
| *a) Numeric reproductive ratio* | | | |
| Season | 7.578 | 1,41 | **0.008** |
| Average *HL* | 13.406 | 1,40 | **<0.001** |
| Average *HL* x Season | 5.032 | 1,39 | **0.031** |
| *b) Numeric caste ratio* | | | |
| Season | 23.164 | 1,41 | **<0.001** |
| Average *HL* | 10.667 | 1,40 | **0.002** |
| Average *HL* x Season | 4.755 | 1,39 | **0.035** |
| *c) Numeric sex ratio* | | | |
| Season | 22.998 | 1,36 | **<0.001** |
| Average *HL* | 0.122 | 1,35 | 0.729 |
| Average *HL* x Season | 0.342 | 1,34 | 0.563 |
| *d) Numeric ploidy ratio* | | | |
| Season | 0.188 | 1,40 | 0.667 |
| Average *HL* | 6.986 | 1,41 | **0.012** |
| Average *HL* x Season | 0.366 | 1,39 | 0.549 |

# Chapter VI

# Similar performance of diploid and haploid males in an ant species without inbreeding avoidance

This chapter is based on a research article that is currently under review for publication in the Journal *Ethology:*

**Kureck IM**, Nicolai B, Foitzik S (*under review*) Similar performance of diploid and haploid males in an ant species without inbreeding avoidance. Ethology

# Abstract

Under haplodiploidy, a characteristic trait of all Hymenoptera, females develop from fertilized eggs, and males from unfertilized ones. Males are therefore typically haploid. Yet, inbreeding can lead to the production of diploid males, that often fail in development, are sterile or 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 their natal 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 in respect to relatedness to their partners, 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.

# Introduction

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 and Wolf 1996), but their restricted dispersal capabilities 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 polygynous ant species, wingless females can attract unrelated winged males by releasing sexual pheromones, a behaviour that has been termed ‘female calling’ (Peeters 1991; Passera and Keller 1994; Berghoff et al. 2008). Inseminated queens then return to their nest to reproduce or to take part of the worker 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 male morph is much less common than winged males, but has been found in nine genera (Heinze and Tsuij 1995).

Because 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 (Danforth 1991; Heinze and Hölldobler 1993; Kinomura and Yamauchi 1993; Yamauchi et al. 1996), defend territories within the colony (Frohschammer and Heinze 2009) or show courtship or guarding behaviours towards females (Yamauchi et al. 2001a; Foitzik 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: Unfertilized and therewith 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 fertilized eggs will develop into females because sex loci are highly variable (Yokoyama and 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 and Pallet 1979;

Ross and Fletcher 1986; Cook 1993; Tarpy and Page 2002). In honeybees, diploid male larvae are recognized by workers and killed to lower the cost they can exert on the colony (Woyke 1963). In species, in which diploid males survive to adulthood, they often exhibit a shorter lifespan (de Camargo 1982; Duchateau and Mariën 1995), but apparently normal sexual behaviour (Naito and Suzuki 1991; Krieger et al. 1999; de Boer 2007). In evolutionary terms, diploid males are a dead end because 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 2007; Cournault and Aron 2009, but see Cowan and Stahlhut 2004; Elias et al. 2009). In a variety of Hymenopteran species, diploid males have been reported to be larger and/or heavier than haploid males (Petters and Mettus 1980; Ross and Fletcher 1985; Packer and Owen 1990, El Agoze and Periquet 1993; Armitage et al. 2010), while they are smaller in the bumblebee *Bombus terrestris* (Duchateau and Mariën 1995; Gerloff et al. 2003).

In this study we compare sexual performance, lifespan and body size of diploid and haploid wingless males of the ant *Hypoponera opacior*, a species that 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; Foitzik et al. 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. Males can mate multiply, but spermatogenesis does not continue into adult life so that sperm is limited (Foitzik et al 2002). The absence of an ongoing spermatogenesis is a typical feature of ant males, which usually have short lives and few mating opportunities (Heinze and Hölldobler 1993; but see wingless *Caridocondyla* males: Forbes 1954; Hölldobler 1966). Sexual selection on these wingless males was found to act on fast development and body size, with earlier emerging and larger males showing a higher mating success (Kureck et al. *under review*). 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 these wingless males was indicated by the occurrence of triploid workers and queens (Kureck et al. 2012), but it is unclear how the ploidy level affects morphology, behaviour and life history of wingless males. In contrast to many other studies on Hymenopteran males, the nest mating behaviour and short reproductive season of about three to four weeks gave us the unique opportunity to observe the mating success over their entire male lifetime.

The traits we compared between the two wingless male types were lifespan, number of matings and the average duration of matings. Mating durations are important because they are associated with paternity certainty in this species with extensive postcopulatory mate-guarding behaviour (Kureck et al. 2011). Further, we investigated the emergence time and body size of wingless males as these traits are under strong sexual selection (Kureck et al. *under review*). Moreover, we studied whether wingless males actively try to avoid inbreeding by choosing less related queens as mating partners. Since nests usually contain more than one reproductive queen, relatedness values vary between nestmates. The ability of males to choose more distantly related queens would lower the inbreeding rate.

## Material and methods

### *Ant collection and maintenance*

Ant nests were collected in the Chiricahua Mountains of Southeastern 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°52.0000 N, 109° 12.6090 W) and kept them at room temperature in three-chamber boxes (10 x 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*

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.

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 the order of emergence within a nest influences the mating success of a male, we calculated an emergence rank for each male. We 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, rank 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, i.e. low numbers reflect an early emergence in the nest (Kureck et al. *under review*). 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 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 female 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 generalised Linear Mixed Models (with a binomial error distribution), in which each of the variables was entered as a fixed factor and nest as random factor. Because of a strong asymmetry in the sample size of haploid and diploid males (see results) the model was based on a cloglog-link function (Zuur et al. 2009). Analyses were conducted with the statistical software R 2.14.0.

### *Genetic analysis*

All males from the observation nests and 48 males and queens each from the mate choice experiments (see below) 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) and analysed using the Program Genemarker (V1.97 Demo Version).

### *Morphometric comparisons*

We measured the head width and thorax width of ETOH preserved males under 40 x magnifications using a stereomicroscope coupled to a computer and the measuring software LAS (Leica Application Suite V.3.8 from Leica Microsoft).

In order to enlarge our sample size for the morphometric comparisons, we genotyped additional 194 males from colonies collected in the same population, season and year (Kureck et al. 2012). We were able to add the data of 28 diploid males from 19 nests. For some individuals the measurement of head or thorax width was impossible, because the ETOH preserved individuals were deformed. We were able to measure head width of 21 diploid and 69 haploid males and the thorax width of 38 diploid and 112 haploid males.

### *Mate choice experiments*

In order 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 RELATEDNESS 5.0.8, and compared with a paired t-test.

# Results

Microsatellite analyses revealed that 12 of the 133 genotyped males were diploid and these males stemmed from 10 nests. Diploid males did not differ from haploid ones in lifespan (Table 1; Fig. 1 a), emergence rank (Table 1; Fig. 1 b), and the number of matings (Table 1; Fig. 1 c). Further, mean mating durations of sexually active males did not differ in the two male types (Table 1; Fig. 1 d). We also found no difference in the frequency of sexual activity between haploid and diploid males, as five of the diploid and 52 of the haploid males were sexually active ($\chi^2$ - test: $p = 0.920$, $N_{haploid} = 121$, $N_{diploid} = 12$). Diploid males were observed to mate up to two times, their matings lasted up to 360 min and they lived up to 10 days. Diploid males did not differ from haploid males in head with (Table 1; Fig 1e) or thorax width (Table 1; Fig 1f).

Male to queen relatedness did not differ between mating pairs and control pairs (male → queen: $r_{mating\ pair}$: 0.232 ± 0.083, $r_{control\ pair}$: 0.258 ± 0.0933; mean ± 95 % CI; t-test: $t_{23} = 0.268$, $p = 0.523$), demonstrating an absence of inbreeding avoidance in wingless males.

**Table 1** Comparison between haploid and diploid males. None of the analysed male traits differed between the two male types.

| | $\chi^2$ | p | N | **Haploid males**<br>median; min - max | N | **Diploid males**<br>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) | 0.34 | 0.558 | 69 | 0.54; 0.49 - 0.59 | 21 | 0.52; 0.46 - 0.58 |
| Thorax width (mm) | 1.56 | 0.211 | 112 | 0.340; 0.29 – 0.46 | 38 | 0.41; 0.35 – 0.46 |

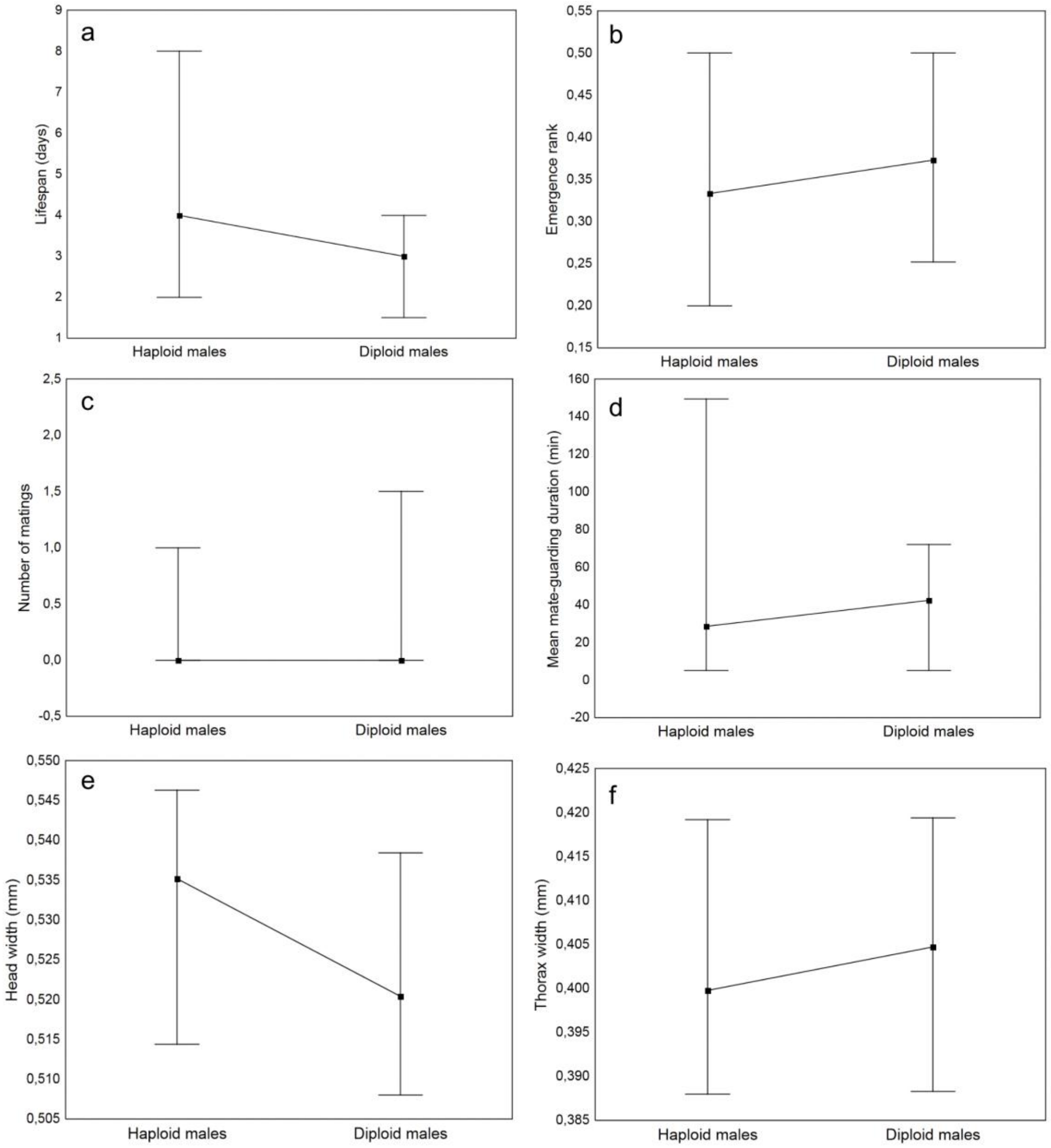

**Fig. 1** Comparison of the male traits a) lifespan, b) emergence rank, c) number of matings, d) mating duration (only taking sexually active males into account), d) head width and e) thorax width. None of the traits differed between haploid and diploid males. Points: median, whiskers: 25th and 75th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

# Discussion

Our study demonstrates that the nest-mating wingless *H. opacior* ant males did not select less related queens as mating partners; hence they did not show active inbreeding avoidance. Furthermore, our behavioural observations and morphometric analyses revealed that diploid males resemble haploid males in lifespan, mating success, time of emergence and body size.

Sexually active diploid males were found to sire triploid offspring under inbreeding in a few Hymenopteran species (Ayabe et al. 2004; Liebert et al. 2004; de Boer 2007). In two regularly inbreeding wasp species, *Euodynerus foraminatus* (Cowan and Stahlhut 2004) and *Cotesia glomerata* (Elias et al. 2009), diploid males are even able to sire fertile diploid offspring. In these so far exceptional cases diploid males appear to have evolved a functional reduction division that is usually aborted in Hymenopteran males. Alternatively one chromosome set from the sperm cell might be eliminated at some point of the fertilization process resulting in a diploid zygote (Cowan and Stahlhut 2004). We recently detected viable triploid workers and queens in the ant *H. opacior* (Kureck et al. 2012) and this study demonstrates that diploid males show normal sexual behaviour. 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 and 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. Also, it is so far unclear whether triploid workers exhibit the same work performance and lifespan as their diploid nestmates (Kureck et al. 2012).

Cell volume usually rises with genomic content (Olmo 1983). In nematodes body size increases with cell size and polyploidy, but studies on plants and vertebrates indicate that larger cells of polyploids do not necessarily result in larger bodies as developmental mechanisms seem to 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 and 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 and Mettus 1980; Packer and Owen 1990). However, this is not invariably the case: diploid males were found to be smaller in the bumblebee *Bombus terrestris* (Duchateau and Mariën 1995; Ayabe 2004). In this case, it is unclear whether the reduced body size is due to internal mechanisms or if diploid male larvae obtain less food during larval development. In our study, diploid *H. opacior* males did not differ from haploid males in body size. Hence, the ploidy level did not 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; body size of wingless sexuals and workers is very similar (Foitzik et al. 2010).

The comparison of relatedness between mating pairs and control pairs revealed that males do not actively choose less related queens as 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; de Heer and Ross 1997; Strassmann et al. 1997). Some social Hymenoptera can actively avoid incest by choosing non-nestmates over nest-mates (Gamboa 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 and Jaisson 2002; Keller and Fournier 2002). As a consequence, not only the extraordinary mating system, with nest-mating wingless sexuals in fall, but also the absence of active inbreeding avoidance on a local scale lead 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 stronger into outbreeding sexuals if the level of inbreeding is high. Second, diploid male production is moderate which might be due to multi-locus-CSD instead of single-locus-CSD levels (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, wingless *H. opacior* sexuals do not actively avoid inbreeding. Moreover, the species appears to be able to deal with high levels of inbreeding without suffering from inbreeding depression. Indeed, we could recently show increased investment in sexuals in highly inbred colonies, but no reduction in productivity (Kureck et al. 2012). Diploid males are moderately produced and, due to their incapability to sire fertile offspring and a potential lower fertilization success, they exhibit a lower fitness than haploid males. Yet, their fitness is greater than zero as they are able to sire viable workers that contribute to the productivity of the colony, so that diploid male production might be less costly than in many other Hymenopteran species.

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# Discussion

Dispersal is a widespread behaviour that ensures outbreeding, spreading of successful genotypes and reduces competition on a local scale. The ant *Hypoponera opacior* exhibits alternative reproductive tactics with winged, dispersing sexuals in summer and wingless, non-dispersive sexuals in fall. These two alternative reproductive tactics evolved in response to seasonal variations in weather conditions in their local habitat. In my thesis, I mostly focussed on the second reproductive season of the year when wingless sexuals mate within their natal colonies. The reduction of dispersal abilities in these wingless individuals leads to three main consequences that I will review in the following paragraphs:

1.) Wingless queens found dependent colonies in the vicinity of their mother nest and compete for the help of workers and for nesting sites (LRC).
2.) Wingless males compete for the access to females within their nests (LMC) and are affected by sexual selection.
3.) Frequent sib-matings within colonies lead to high levels of inbreeding and the production of diploid males.

As presented in Chapter I, *H. opacior* colonies produce - besides workers - mostly winged males and queens in early summer that conduct nuptial flights during the season with the highest precipitation rates of the year, while exclusively wingless individuals emerge in fall. In ants, winged dispersal is the ancestral state (Heinze and Tsuji; Boomsma et al. 2005) and the timing of the nuptial flight is often linked to recent rainfalls (Kaspari et al. 2001). Especially in Arizonian ant species, sexuals have been reported to conduct their mating flight at the beginning of the summer rainy season in July (Bartz and Hölldober 1982; Hölldobler 1976; Nagel and Rettenbeyer 1973). During this season, dispersal flights are not as risky as for example earlier in the year because the air is more humid and the small insects are better buffered from desiccation (Hood and Tschinkel 1990; Kaspari 1993). Further, conditions for the foundation of a new colony are favoured because rain softens the soil and therewith facilitates excavation of soil nests (Hölldobler and Wilson 1990). At the same time, rainfall leads to a large food supply because warm and moist soil triggers the multiplication of small invertebrates as a consequence of enhanced plant growing (Yanoviak and Kaspari 2000). In addition to these factors is the onset of the rainy season an easy recognisable cue that allows synchronisation of nuptial flights within a local area (Kaspari et al. 2001). A nuptial flight later in the year would lower the likelihood of a successful independent colony foundation before the winter starts. Hence, *H. opacior* colonies rather use the favourable conditions still present in late summer/fall to expand on a local scale. An association of dispersal with seasonal variations in weather conditions has been found in many taxa (plants: Gutterman 1994; mammals: Molteno and Benett 2002; birds: Walls et al. 2005; insects: Nardi et al. 2008).

Unlike the strong seasonal pattern we found in *H. opacior*, the ant *Cardiocondyla obscurior* only produces winged sexuals under adverse conditions such as low temperatures, starvation or a sudden decrease of colony size, whereas ergatoid males are constantly produced throughout the year. *C. obscurior* is an invasive tramp species that is adapted to live in isolated, patchy habitats. Hence, the species invests in local expansion rather than in risky dispersal as long as conditions are good (Cremer and Heinze 1993). Dispersal can here be regarded as a strategy to escape from unfavourable conditions. Similar patterns have been found in other animals were enhanced competition due to higher densities of individuals or lower food availability is positively associated with dispersal (insects: reviewed in Harrison 1980; birds and mammals: reviewed in Matthysen 2005).

In general, dispersal seems to be favoured under two, sometimes contradicting conditions: First, if environmental conditions lower the risk of dispersal (despite good conditions in the local habitat) and second, if conditions in the local habitat become worse. Regarding the seasonal weather conditions in the Chiricahua Mountains, dispersal in *H. opacior* is rather favoured by good conditions than a reaction to adverse conditions in the local habitat. However, I reported in Chapter V that colonies enlarge their investment in outbreeding sexuals relative to workers under strong inbreeding. This finding can be interpreted as a form of investment in dispersal under unfavourable conditions.

### *Wingless queens: Local resource competition and dependent colony foundation*

Through the production of wingless sexuals in fall, risks associated with mating flights and independent colony foundation are avoided. On the other hand, reduced dispersal abilities can lead to strong intraspecific competition. The production of wingless instead of winged queens often results in polygynous nest structures, because many queens start to reproduce in their natal colony. The queens that do not remain in the natal colony compete with each other for nest sites in the vicinity and for the help of workers that they need during dependent colony foundation (LRC; Chapter I).

The term ‘local resource competition’ was first introduced by Clark (1978) and can be regarded as an extension of the concept of local mate competition introduced by Hamilton (1967). While Hamilton explained sex ratios towards one sex by the competition for mating opportunities (usually affecting the male sex), Clark extended this model by the competition among relatives within a population for resources such as food or nesting sites (usually affecting the female sex). He reported male-biased sex ratios in the primate *Galago crassicaudatus* and hypothesized that LRC among a philopatric sex might generally shift sex ratios towards the more dispersive sex. This hypothesis was confirmed by several studies reporting male-biased sex ratios in species with LRC among females and male dispersal (mammals: Johnson 1988; Hewison and Gaillars 1996; insects: Walin and Seppa 2001). Generally, optimal sex ratios should depend both on LMC and LRC and be biased towards the sex that suffers less from local competition (Antolin 1993; cited from Perrin and Mazalov 1998). In the case of *H. opacior* both wingless sexes compete on a very local scale, but local competition appears to have a stronger impact on the female sex than on the male sex. This was concluded from the male-biased sex ratios we found in fall nests (Chapter I, Chapter V). Especially the number of workers seems to be the limiting factor here, because *H. opacior* colonies are rather small and the sex ratio became more female-biased in nests with larger colony sizes (Chapter I).

Reduced dispersal abilities are also reflected in the population genetic structure on the microgeographical scale (Chapter II). We here found population viscosity as relatedness between nests gradually decreased with geographic distance. Such a genetic pattern within a local patch is typical for ant species with reduced dispersal abilities of queens and can be explained by colony budding, i.e. dependent colony foundation (Hamilton 1964; Pamilo 1998). Population viscosity was also reflected in the aggression tests. Workers were more aggressive towards conspecifics from more distant colonies. A reduced aggression towards members from neighbouring colonies allows high population densities as we found it in most collection sites in the Chiricahua Mountains (Foitzik et al. 2002; Chapter II; Chapter V).

### *Wingless males: Local mate competition and sexual selection*

As mentioned in the introduction, mating biology and sexual selection have so far only rarely been investigated in social Hymenoptera (Baer 2003; Boomsma et al. 2005). But especially species with wingless males that mate inside their nest offer the opportunity to investigate these traits, because mating behaviour is here easier to observe than the behaviour of winged individuals that mate on nuptial flights. Further, the investigation of traits under selection is especially interesting in these species because wingless males have to compete with their nestmate rivals for the access to queens (LMC), while winged males rather face a scramble competition and have little opportunities to influence their reproductive success.

As reported in the Chapters III and IV, ergatoid *H. opacior* males copulate with and mate-guarded queen pupae in response to local mate competition. Males adapt mate-guarding durations to the competitive situation in the nest (Chapter III, Chapter IV), which indicates that they are able to correctly assess their competitive environment. This adaptation of mate-guarding durations to the competitive situation is in line with theoretical predictions (Parker 1974) and findings in other animal taxa (Ward 1983, García-González and Gomendio 2006; Komdeur 2001). Furthermore, the purposeful embrace of male pupae in order to eliminate future rivals (Chapter III) can be seen as an aggressive act that forgoes further adaptations for fighting. Mate-guarding adaptation and deadly embraces are two male behaviours that were somewhat unexpected for the wingless males whose small brains and rather slow activity patterns indicated low cognitive abilities. In Chapter IV I reported that males are selected to emerge early and develop a relatively large body size. Early emerging males face a higher number of not yet emerged queen pupae and a lower number of adult competitors (lower LMC). Hence, they have a higher chance of fertilizing multiple queens and eventually reducing the number of future competitors by embracing their cocoons. In line with our results, similar findings have been reported for the gregarious parasitoid wasp *Nasonia vitripennis*, a species with a comparable mating system. Here, short-winged (brachypterous) males compete for matings with virgin females at their natal site and sexual selection similarly acts on early emergence (Moynihan and Shuker 2011). But also in many other taxa sexual selection works on an early appearance (in terms of emergence or arrival at the mating site) of males. This often results in a generally earlier appearance of males relative to females– a condition termed as ‘protandry’ (insects:

Wiklund and Solbreck 1982; Carchini et al. 2005; fish: Morbey 2000; birds: Saino et al. 2012). Since *H. opacior* males almost exclusively mate with pupal queens, an emergence before their mating partners is essential for their mating success. Several hypotheses have been put forward to explain the evolution of protandry (reviewed in Morbey and Ydenberg 2001). The mating system of *H. opacior* best matches the 'mating opportunity hypothesis' proposed by Wicklund and Fagerström (1977). This hypothesis explains the evolution of protandry as being driven by a higher mating success of earlier males as these face more mating opportunities.

I interpret the finding that also body size appears to be under selection in a way that a large body size might be beneficial during mate-guarding (i.e. better performance in mounting and holding on to cocoons). Further, larger males might produce more sperm, enabling them to fertilize more queens (Chapter IV). Body size however seems not to be of major importance as males are still the smallest caste in this species. In contrast we find a different outcome of sexual selection in the Japanese sister species *Hypoponera bondoiti*: Here selection resulted in the evolution of two ergatoid male morphs: Major males that aggressively fight each other until one of the rivals is repelled and minor males that are not attacked by majors as they most probably mimic females (Yamauchi et al. 1996). Selection here strongly works on body size and consequently major wingless males are the largest caste in the colony. In *H. opacior* the production of more or less peaceful – despite the fact that they might kill male pupae – males that mate-guard their partners can be seen as an alternative competitive tactic to the production of more costly large fighter males.

### *Inbreeding effects*

In the Chapters V and VI of this thesis I focused on the effects and consequences of inbreeding. Chapter II already revealed that inbreeding levels in *H. opacior* are extremely high, which can be explained by frequent sib-matings among wingless sexuals in fall. The study presented in Chapter V revealed no inbreeding depression but an increased investment in sexuals in more inbred colonies. This increased production of queens and males was more pronounced in the winged summer generation. We interpreted this finding as an active response to high inbreeding levels: By investing more strongly in the production of dispersing sexuals, colonies ensure outbreeding events in the summer generation and can thereby moderate inbreeding levels in future generations. Further, we found that colonies occasionally produce diploid males that can sire triploid offspring. The follow-up study presented in Chapter VI revealed similar performance of diploid and haploid males. Diploid male production is thus not as costly as in most other Hymenopteran species. The combination of these two studies demonstrates that *H. opacior* does not suffer severely from regular inbreeding indicating that the species is well adapted to the season-dependent mating biology.

Another result reported in Chapter VI was the absence of active inbreeding avoidance within nests. The finding that males did not choose less related males as mating partners indicates that males are unable to distinguish close kin from less related individuals. Absence of inbreeding avoidance among nestmates also occurs in other social Hymenoptera in that males and females mate within their colony (Blatrix and Jaisson 2002; Keller and Fournier 2002). This can be explained by a general absence of true kin recognition in social Hymenoptera (Ratnieks 1991; de Heer and Ross 1997; Strassmann et al. 1997). In contrast, social Hymenoptera are usually very well able to discriminate nestmates from non-nestmates by discriminating between colony specific odours (Gamboa et al. 1986; Foster 1992; Rosset et al. 2007; Sturgis and Gordon 2012). As demonstrated in the aggression tests (Chapter II) this is also true for *H. opacior*. Selection might work against the development of true kinship recognition, because this could reduce general altruism in polygynous and/or polyandrous colonies and lead to numerous kin conflicts (Keller and Fournier 2002).

As mentioned in the introduction, inbreeding negatively affects the fitness of many organisms (e.g. Ralls et al. 1979; DeRose and Ross 1999; Packer and Pusey 1993; Keller and Waller 2002). However, to a certain extent inbreeding can also be beneficial because it maintains locally adapted genotypes within a habitat (Hamilton 1964, 1972; Frankham 1995). Furthermore, individuals that mate with close kin can increase their inclusive fitness not only directly, but also indirectly by improving their relative's mating success (Kokko and Ots 2006; Waser et al. 1986). In the case of polygynous and/or polyandrous social insect colonies, inbreeding can compensate for the reduction of relatedness and therewith restore fitness benefits for altruistic workers (Thurin and Aron 2009; Thurin et al. 2011). Additionally, negative effects of inbreeding are expected to be generally lower in haplodiploid species because deleterious alleles are purged in haploid males (Werren 1993; Antolin 1999; Henter 2003). Studies on several animal taxa reported the absence of inbreeding depression (mammals: Keane at al. 1996; insects: Facon et al. 2011) and even preferential inbreeding (birds: Choen and

Dearborn 2004; fish: Thünken et al. 2007; insects: Thurin and Aron 2009) and outbreeding depression (insects: Peer and Taborsky 2005; nematodes: Dolgin et al. 2007; fish: Houde et al. 2011) have been reported. This leads to the conclusion that inbreeding does not necessarily have to result in a fitness reduction and our findings in the highly inbred ant *H. opacior* support this conclusion.

Many species use dispersal as a mechanism to avoid inbreeding (Pusey and Wolf 1996). However, the here reported investment shift towards dispersing individuals as an active response to high inbreeding rates has, to the best of my knowledge, so far never been reported in any other animal species.

### *Future directions*

Since *H. opacior* occurs from southern North America to Central America (www.antweb.org), a broad region with different climate zones, it would be interesting to study how the species has adapted its reproductive seasons and morphs to annual climate variations in other geographic regions. Two suitable regions for a comparative study of annual reproductive cycles would for example be the tropics (e.g. Costa Rica) and Southern California. While we find constant warm temperatures and a long rainy season in the tropics, colonies from Southern California face dry, hot summers and high precipitation rates in winter. Hence, if dispersal of winged individuals depends on rainfalls, the timing of their production should vary between these regions and the population from Arizona described in this thesis. It is possible that the production of wingless sexuals is even completely absent in the tropics were dispersal and independent colony foundation are less risky. This in turn would mean that also inbreeding levels are lower in this region.

Another yet unexplored question is how male-male competition in wingless males continues after fertilization on the level of sperm. The finding that males invest a lot of time in guarding their mating partners, suggests that sperm competition exists. Seminal fluid was found to have a positive effect on a male’s sperm survival but a negative effect on sperm survival of competing males in polyandrous ants and bees. The same was not found in monoandrous species (den Boer et al. 2010). If an incapacitation of competing sperm has also evolved in *H. opacior* remains to be investigated. Further, genetic analyses of multiply mated queens, their mating partners and their offspring could reveal whether the first or the last male sires most of the offspring or if both males contribute equally to offspring production. So far, we only studied male behaviour and variations in *mating success*, that is, the number and the duration of matings. An investigation of sperm competition and offspring production will reveal how mating behaviour actually influences the *reproductive success* and therewith the fitness of wingless males.

A major result of my PhD-project was the finding that *H. opacior* colonies do not suffer from inbreeding depression in terms of colony productivity. Instead, they seem to be well adapted to their inbreeding lifestyle as the costs of inbreeding and diploid male production are lower than in other species. However, further studies are essential to reveal the actual costs of inbreeding. For instance, the performance of more and less inbred colonies should further be investigated. Inbred individuals might have a shorter lifespan or more inbred colonies might less well be able to cope with stress such as starvation or unfavourable climate conditions. A greater inbreeding depression under stressful conditions has been found in a variety of plant and animal taxa (reviewed in Armbruster and Reed 2005). Also the reproductive success of diploid males should further be studied. We found that diploid males show a normal mating behaviour and that they can sire triploid offspring, but it is yet unclear if sperm amount and fertilization success differs between haploid and diploid males. Diploid males might sire fewer offspring despite similar mating performance (Duchateau and Mariën 1995; de Boer et al. 2007). Finally, lifespan and work performance of triploid individuals should be investigated in order to find out if these individuals are of the same value as diploid workers.

The enhanced investment of inbred colonies in the production of winged, outbreeding sexuals has been interpreted as an active response to high levels of inbreeding. A follow-up study should examine the mechanism of this response. So far it is not known if more inbred workers change their feeding behaviour and thereby raise more sexuals into adulthood, or if the higher investment into sexuals depends on the level of homozygosity in the brood. One possibility to find out what causes the altered sexual investment would be to exchange workers and brood from more and less inbred colonies and investigate sex allocation and body sizes in the new generation.

In combination with the findings reported in this thesis, the here suggested future studies will contribute to the general understanding of how environmental conditions can shape the development of alternative phenotypes. Moreover, these studies will lead to a better understanding of sexual selection in a local mate competition situation. In addition to the here reported effects of sexual selection on emergence time, external morphology and behaviour, the study of sperm competition will focus on the smallest level of male-male competition. Last, the more detailed investigation of inbreeding effects and active responses will give further insight into one of a minority of mating systems that keep up regular inbreeding.

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