# Alternative reproductive tactics in the ant genus *Hypoponera*

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# Erklärung

Diese Dissertation wurde im Sinne von § 12 der Promotionsordnung von Frau Prof. Dr. Susanne Foitzik betreut. Ich erkläre hiermit, dass die Dissertation keiner anderen Prüfungskommission vorgelegt worden ist und dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

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# General Introduction

Humans at all times were intrigued by the seemingly infinite diversity of species on our planet. The current number of described species on earth range between 1.5 and 1.8 million, and over 70 percent of them belong to the animal kingdom (Pearse, 1987; Wilson, 2000). Although complex and difficult to calculate, estimations on the total number of species on earth range from five up to 50 millions and above (Erwin, 1988; Erwin, 1997; May, 1988). Others question these very high estimates of species beta diversity due to the applied methodology (Bartlett et al., 1999). Historically, myths and theories were used to explain species richness, many with a religious background, but this topic was investigated with a more and more scientific approach from the enlightenment on. Nowadays the generally accepted explanation of the origin of biodiversity is the evolutionary theory by (Darwin, 1859). In his book “The origin of species by means of natural selection” he suggests, that diversity is the outcome of a continuous process, which can be traced back to a common ancestor right at the bottom of the “tree of life”. In short, there are three factors, which are working together: Mutation and recombination generate a high variance between individuals and selection then acts on an excess of offspring. Individuals with beneficial heritable traits may have a higher probability to reproduce and consequently such traits may accumulate over time – ultimately generating new species.

All sexual species have two sexes and sexual dimorphism between males and females is widespread. However, the fact that males sometimes possess conspicuous traits such as large antlers or long and/or colourful feathers, which seem to reduce male survival, was at first puzzling to Darwin. Yet, years later, he discussed his solution “sexual selection theory” in-depth in “The descent of man and selection in relation to sex” (Darwin, 1871). Its central points are inter-male-competition over access to females and female choice. Stabilising selection generally maintains only a single phenotype per sex, yet disruptive selection can lead to the evolution of diverse reproductive strategies within a single sex. Those strategies may occur on the behavioural level, but can also involve morphological, physiological and life history adaptations. Typically, larger males tend to achieve mating success by dominance and aggression, whereas physically weaker ones apply “sneaking-tactics” or mimic females. For example, large males in some ungulates attract females on leks, whereas younger or weaker males, the so called satellite males, gather on the edges of the mating arena, and try to sneak mating opportunities (Appolino et al., 1992; Isvaran, 2005).

A theoretical review by (Gross, 1996) broadly classified alternative reproductive strategies and tactics into three categories. First, alternative strategies, that are genetically determined polymorphisms, which have equal average fitness and are maintained by negative frequency-dependent selection, i.e., the rarer phenotype achieves on average a higher reproductive success than the common one. Alternative strategies are rare in nature, but were found in several vertebrates and also in two ant species, *Harpagoxenus sublaevis* and *Leptothorax* spec. A. In the latter species a single locus polymorphism controls the development of winged and wingless females (Buschinger, 1978; Heinze and Buschinger, 1989). Second, mixed strategies (with alternative tactics) are genetically monomorphic. Here, again alternative tactics should have equal average fitness and should be retained via frequency-dependent selection. No example for a mixed strategy is known so far. Third, conditional strategies (with alternative tactics) are also genetically monomorphic, but tactics have unequal average fitness and are maintained by status-dependent selection (with or without frequency-dependent selection). The vast majority of described intrasexual variations belong to these conditional strategies and are found predominantly among the males. This was explained by a stronger intrasexual selection within the male sex, because competition for access to females and variation in mating success is higher (Gadgil, 1972; Trivers, 1972). Alternative reproductive phenotypes in male animals have been described for vastly different taxa such as insects (scarab beetle, *Onthophagus taurus* (Moczek and Emlen, 1999)), arachnids (mite, *Sancassania berlesei* (Radwan et al., 2002)), crustaceans (shrimp, *Paracerceis sculpta* (Shuster and Wade, 1991)), fish (blennies, *Salaria pavo* (Oliveira et al., 2001)), amphibians (woodhouse toad and great plains toad (*Bufo woodhousii* and *Bufo cognatus* (Leary et al., 2005)), reptiles (side-blotched lizard, *Uta stansburiana* (Sinervo and Lively, 1996)), birds (ruff, *Philomachus pugnax* (van Rhijn, 1973)) and mammals (blackbuck, *Antilope cervicapra* (Isvaran, 2005)).

Within social Hymenoptera, alternative reproductive tactics are much more common in queens than in males contrary to the above described general findings for other taxa. Indeed, different queen morphs or at least a bimodal distribution of queen size has been described in roughly ten percent of all ant species and nearly in all ant genera (Heinze and Keller, 2000). Various morphs of queens have been described in ants, from fully-winged ones to primarily wingless queens, alate queens are assumed to be the ancestral state, yet in several species queens have reduced wings (brachypterous queens) or are completely wingless (apterous queens). In some ant species workers have the opportunity to mate (gamergates) or to reproduce parthenogenetically and thus can replace the queen morph entirely (Heinze and Keller, 2000; Heinze and Tsuji, 1995). The morphological variation in queens is often associated with changes in the social organisation of their colonies. Whereas alate queens typically mate on the wing, disperse, found independently and head their colonies alone (monogyny), apterous queens tend to mate in or close to their nest, found dependently and reproduce along side their sisters in a colony (polygyny). Mounting evidence points towards a common evolutionary scenario, where natural selection discriminates against dispersing and independent founding alate queens, when dispersal costs are high. This is the case, when predation is high, habitats fragmented or close to saturation without available nesting sites, mating opportunities rare or under unfavourable (cold or dry) climates. Than natural selection favours queen adoption and consequently secondary polygyny with dependant nest founding (i.e. budding). In the long term, this in turn leads to a reduction in general queen size and especially of wings and flight muscles, which are not anymore needed for dispersal (Bourke and Franks, 1995; Heinze and Keller, 2000; Heinze and Tsuji, 1995).

Ant males are commonly winged, possess a pronounced thorax with strong flight muscles combined with large eyes, three ocelli and sensitive antennae. Further, their sperm production usually terminates during pupal development, but at the latest a few days after eclosion. All these features are explained by the fact, that mating usually occurs in large swarms, where males are unable to dominate several females and rarely mate more then once (Hölldobler and Wilson, 1990). In contrast to apterous queens that are at least present in a few genera of almost all subfamilies, wingless (ergatoid) males are only present in nine ant genera (Heinze and Tsuji, 1995).

The genus *Hypoponera* belongs to the Ponerini tribe of the Ponerinae ants (Bolton, 2003), which are regarded as primitive, as they are grouped into a very basal branch of the ant phylogeny. Roughly 170 species and subspecies are recognized of this almost worldwide distributed genus (www.ento.csiro.au/science/ants/ponerinae/hypoponera/hypoponera .htm). Genus-wide detailed research on the biology of the various species is still at the beginning, albeit this genus is a well-known hot spot for alternative reproductive strategies in males and females. An extension of a first grouping regarding the presence of different reproductive morphs and social structures in *Hypoponera* by Yamauchi (1998-1999) is shown in Table 1. The three types comprise possible steps in the evolution of wing loss and causal or concomitant circumstances. The first category contains species with for ants typical winged reproductives like *H. nippona* and *H. sauteri*. In the second, one find species with winged and wingless female and male morphs (*H. opacior*, *H. opaciceps* and *H. nubatama*). The third group contains species with the two queen morphs, but with only wingless males (*H. schauinslandi* and *H. gleadowi*).

My main study species, *H. opacior* exhibits alate and apterous reproductives of both sexes and thus belongs to the intermediate category (Table 1 and Figure 1). The queen morphs and the associated social forms were described as follows (Foitzik et al., 2002): alate reproductives conduct mating flights and mated queens found, after shedding their wings (now called dealate queens), new colonies haplometrotically or rarely pleometrotically (by one or a few queens), resulting in rare monodomous colonies (Figure 2). In contrast, apterous reproductives typically mate intranidally (within their mother colony). Such inseminated apterous (intermorphic) queens found new nests dependently by budding. One or several young queen/s leave the mother colony together with a part of their worker force and found a new nest in the neighbourhood, since dispersal abilities of wingless individuals are restricted.

**Table 1:** Overview over possible causes and concomitant circumstances, which correspond with the occurrence of alate and apterous reproductive morphs in different *Hypoponera* species (modified after Yamauchi (1998-1999)). Species are grouped into three types. Factors given in italics remain to be verified.

| | **Type 1** | **Type 2** | **Type 3** |
|---|---|---|---|
| **Queen** | winged | winged<br>wingless | winged<br>wingless |
| **Male** | winged | winged<br>wingless | wingless |
| **Ecology** | continuous habitat | patchy, favourable habitat | patchy, favourable habitat |
| **Social structure** | Monogyny | w : Monogyny<br>wl: Polygyny | Polygyny |
| **Mating** | mating flight | w : mating flight<br>wl: intranidal | intranidal |
| **Male behaviour** | mating flight | w : mating flight<br>wl: mate guarding | fighting |
| **Spermatogenesis** | *terminated* | terminated | *ongoing* |
| **Colony founding** | independent | w : independant<br>wl: dependant | dependant |
| **Relatedness** | high | w : high<br>wl: low | low |
| **Sex ratio** | *3:1* or *1:1* | w : 3:1 or 1:1<br>wl: LMC and LRC | *LMC* and *LRC* |
| **Inbreeding** | low | w : low<br>wl: high | high |
| **Examples** | *H. nippona*<br>*H. sauteri* | *H. opacior*<br>*H. opaciceps*<br>*H. nubatama* | *H. schauinslandi*<br>*H. gleadowi* |

**Figure 1:** Four different morphs of *H. opacior* pupae (cocoons were artificially removed). From left to right: ergatoid male, worker, intermorphic queen and alate queen.

**Figure 2:** On the left: young, alate *H. opacior* queen on a twig. Its still pale cuticula will harden and considerably darken within a few days. On the right: dealate *H. opacior* queen.

This results in a polydomous nest structure of colonies headed by intermorphic queens. The mating behaviour of *H. opacior* wingless sexuals is among the most exceptional ones in ants. Ergatoid males mate with their sisters, which are still enclosed in their cocoon for up to 40 hours (Figure 3). Those matings are described as mate guarding and are terminated when the intermorphic queen finally emerge from their cocoon and starts moving around. However, (Foitzik et al., 2002) never collected alate reproductives during two collection trips in August and September of 1997 and 1998, respectively, although those are present in museum collections.

**Figure 3:** Mate guarding behaviour of an ergatoid *H. opacior* male, which is mating with a intermorphic queen, still enclosed in the cocoon.

In this study, the appearance of both reproductive morphs was investigated in a population in Southeastern Arizona (USA) during three consecutive years from 2003 to 2005. The vast majority of all colonies were collected in the Chiricahua Mountains, which is a small mountain range with an approximate expansion of 30 km times 120 km and a maximum elevation of almost 3000 m. The Chiricahuas are part of the “Sky Islands”, that are mountain ranges separated by dry flatlands similar to islands in the sea, which are located where the Rocky Mountains and the Sierra Madre meet (Heald, 1951). The Chiricahua Mountains, with their exceptional geographic position at the border of the Nearctic and the Neotropical region belong to the northernmost extension of the “Madrean pine-oak woodland”. This is one of the 34 biodiversity hotspot regions worldwide, in which “…75 percent of the planet’s most threatened mammals, birds and amphibians survive within habitat covering just 2.3 percent of the Earth’s surface…” (www.biodiversityhotspots.org). Not surprisingly, the Chiricahua Mountains ant fauna diversity is also exceptionally high with around 200 described species (pers. com. “Ant Course 2003”). As a comparison, from the well-studied Central European fauna only 175 ant species have been described of 11,000 world wide described ant species and careful estimations of 17,000 species (Seifert, 2007). Species richness in the Chiricahuas has been also attributed to the presence of five vertical life zones, which gradually change with elevation and provide a wide spectrum of different habitats. The “Sonoran (west) and Chihuahua (east and south) Desert” communities inhibit the flats and foothills, and as available precipitation rises with elevation and temperatures decrease, the life zones change till one find “Mixed coniferous forest” on the mountain tops. *H. opacior* colonises suitable habitat patches throughout all vertical life zones except desert habitats. However, it prefers patches with sufficient soil humidity at intermediate elevations around 1700 m, which are found e.g. besides creeks (Figure 4). Their nests are preferentially established under stones and rocks, but they also use cavities in the upper soil layer as nest sites, which they only slightly modify. Workers forage in the soil and leaf litter, usually alone. Only rarely they were observed in tandems. The life cycle of *H. opacior* and the proximate causes for the development of reproductives are investigated in CHAPTER 1. In addition, I explore seasonal variation in demography and sex allocation strategies during the two reproductive seasons.

**Figure 4:** Views of a typical pine-oak-juniper woodland habitat of *H. opacior* in the Chiricahua Mountains (Arizona, US).

The second study species, *H. schauinslandi* (formerly *H. bondroiti*, revised by (Seifert, 2004)) produced alate and intermorphic queens and ergatoid males during each reproductive period. At least the laboratory colonies exhibited two reproductive periods per year, one in spring and a second in autumn. Interestingly, there are two morphs of the ergatoid males; large supposedly “aggressive” ones with a thick dark-brown cuticule and small “docile” ones with a thin amber coloured cuticula, which often occur simultaneously within the same nest (Yamauchi et al., 1996). Consequently, this species is grouped in the third category (Table 1). *H. schauinslandi* is a tramp species, which occurs in temperate zones only in anthropogenic surroundings like greenhouses, which are heated during the cold season. This and a few other species of the genus *Hypoponera* are almost cosmopolitan (Delabie and Blard, 2002; McGlynn, 1999; Seifert, 2004). However, due to their cryptic lifestyle their presence is often only noticed by the identification of alate queens caught during their mating flights (Delabie and Blard, 2002). Although these tiny ants manage to establish themselves in new areas on the long-term, they do not become detrimental to other species. Therefore, they generally lack the potential of invasive species to become a pest and are rather referred to as tramp species. Key features for invasive- and tramp species are a widespread geographical distribution, nests with several queens (polygyny), sometimes in combination with intranidal mating, multiple connected nests (polydomy), sometimes even unicoloniality, low levels of intra-species aggression and superior foraging strategies (Delabie and Blard, 2002; Holway et al., 2002; Passera, 1994).

A further characteristic of tramp species is their often reduced genetic diversity, which is partly linked to above described factors like reduced inter-species aggression. This can be traced back to the introduction of only a few founder colonies in new habitats, as it may occasionally happen e.g. by human transport of goods. Such genetic bottlenecks can be studied since the investigation on the protein- and genetic-level became possible with the advent of molecular methods.

(Hunter and Merkert, 1957) described in 1957, how enzymes can be separated via gel electrophoresis. Their different structures represent alleles of the same gene and are called allozymes. This quick, cheap and easy technique is still in use today. However, it suffers from a weak resolution, which is overcome by modern techniques such as DNA sequencing and microsatellites. The latter are segregated, neutral markers with codominant alleles consisting of short tandem repeats. As no suitable microsatellite primers for the ant genus *Hypoponera* were available, I developed five highly polymorphic markers for *H. opacior* (CHAPTER 2). Microsatellites became broadly available after the invention of the polymerase chain reaction (PCR) by Kary Mullis in 1983. PCR enables the amplification of small DNA amounts and rose subsequently to a standard method in genetically working laboratories. The growing application of microsatellites from the late 1980’s on allowed to address a large variety of behavioural ecological questions on the mating strategies, dispersal and kin structure (Queller et al., 1993). The latter became very important for social insects with their altruistic castes. Hamilton (1964) developed the “kin-selection theory” as a modification of Darwin’s “natural selection theory” to explain the evolution of altruism. Kin-selection theory “…specifies the conditions under which an organism is selected to perform an altruistic act toward a related individual” (Trivers and Hare, 1976). Although it is “the only acceptable evolutionary explanation of altruistic behaviour” (Oli, 2003), kin structures of haplodiploid eusocial insect colonies deviate regularly from classical assumptions for monogynous colonies (headed by a single queen), which is only singly mated (monandrous). Additionally, it was thenceforward possible to investigate the population structure on different hierarchical levels. Also dispersal abilities, in this study of both alate and apterous reproductives of *H. opacior*, which formerly could only be investigated by tedious mark and recapture experiments, are now estimated by the spatial structure of varying allele frequencies between demes or the relatedness between nests within a single plot (CHAPTER 3). Furthermore the relatedness structure within the colony can affect other decisions, such as sex ratio strategies, which dependent on the relatedness asymmetries between workers to young queens and to males (also within CHAPTER 1).

Let us go to back to the loss in genetic diversity caused by inbreeding and founder effects. Sibmating, as it occurs regularly in both *Hypoponera* species, reduces genetic diversity (Tsuji and Yamauchi, 1996), and may carry severe costs due to the production of usually sterile diploid males onto colonies (Ross et al., 1993; Stouthammer et al., 1992). Sex determination in Hymenopterans is not dependant on sex chromosomes, but instead is determined by their ploidy level. Males are usually haploid and females diploid. However, sterile diploid males are produced, when fertilized diploid eggs are homozygous at the sex determining locus. Those males are especially detrimental during the period of colony founding, as ant males never work (Hölldobler and Wilson, 1990). In case of sibmating, singly mated queens produce either zero or 50 percent diploid males from fertilized eggs. Rarely, diploid males are able to reproduce, yet, costs are only postponed to sterile triploid offspring (Agoze et al., 1994; Oishi et al., 1993; Stouthammer et al., 1992). To reduce costs due to sterile diploid males to a minimum, it is necessary that workers recognize them as early in development as possible, either already as egg or during the early larval stages. Males in the honey bee *Apis mellifera* are reared in special cells, which allow an easier detection and elimination of diploid male larvae by workers, as they develop in regular worker cells (Woyke, 1965). It is known, that also ants, e.g. *Formica,* which tend their brood collectively in brood chambers are able to detect such diploid male cues (Pamilo et al., 1994). A recent review describes over 40 species producing diploid males (van Wilgenburg et al., 2006).

This peculiarity is attributed to the mode of sex determination by haplodiploidy (also known as arrhenotoky or arrhenotokous parthenogensis), which is present in approximately 20 percent of all animal species (Bell, 1982; Cook, 1993). It was first described from (Dzierzon, 1845) for *A. mellifera*, and around 100 years later, (Whiting, 1939; Whiting, 1943) suggested single locus complementary sex determination (sl-CSD) as the genetic concept behind haplodiploidy in the parasitic wasp *Bracon hebetor*. Workers and queens develop from fertilized eggs which are diploid and heterozygous at the sex determination locus ($A_iA_j$ or $A_jA_i$). Different to that, males usually develop from unfertilised, haploid eggs which are hemizygous at the sex determination locus ($A_i$ or $A_j$). But in cases when fertilized diploid eggs are homozygous at the sex determination locus ($A_iA_i$ or $A_jA_j$) they will develop into diploid males. Recently, once again *A. mellifera*, which is one of the most important model organisms in Hymenoptera, was in the centre of interest as (Beye et al., 2003) described their sex determination locus and the mechanism behind, the sl-CSD, on the molecular level. Indeed, a single locus, called *complementary sex determiner* (*csd*), with highly variable alleles was found to be the primary signal governing sexual development by its allelic composition. Since diploid males have zero fitness positive selection act in favour of rare alleles and diversifying selection is expected to increase allele number (Hasselmann and Beye, 2004).

However, sex determination within the Hymenoptera may be diverse and supposedly strongly depends on the life history of the respective species. A further model proposed for this order, relaxing negative effects of inbreeding, is the multi-locus complementary sex determination (ml-CSD), which is based on the same concept as sl-CSD, but with two or more sex determining loci (Crozier, 1971; Snell, 1935). Models, which completely overcome the possibility of diploid male production, are the “genetic balance model” from (da Cunah and Kerr, 1957) and the “genomic imprinting model” (Beukeboom, 1995; Poirié et al., 1992). While the former “model is now considered invalid due to lack of evidence (e.g. it cannot explain diploid males)” (Beukeboom, 1995), there is twofold evidence for the second one in the regularly inbreeding wasp *Nasonia vitripennis* (Dobson and Tanouye, 1998; Trent et al., 2006).

Sociality describes the habit of animals of living in groups or colonies. But social insects like ants, some bees and wasps are more than that, they are eusocial (truly social) (Bourke and Franks, 1995). These species are characterized by a reproductive division of labour, overlapping generations of adults and cooperative brood care (Wilson, 1971). Tasks in which different castes cooperate are further extended to e.g. foraging for food, nest maintenance and colony defence. Workers of those species are expected to act altruistically, that is individuals act in ways that decrease their own fitness in favour of the fitness of related individuals, usually the queen. However, systematically overlooked in the past, there is more and more evidence for social individuals that act selfishly with the goal to increase exclusively or predominantly their own fitness. Although cooperation is prominent in social insect societies, conflicts and their resolution came more and more in the focus of scientific interests during the last three decades. Currently five different kinds of conflicts are under investigation, by name, there is queen-worker conflict over the sex ratio, conflicts over male parentage, conflicts over female parentage and conflict over reproductive dominance (Bourke and Franks, 1995; Crozier and Pamilo, 1996; Wenseleers et al., 2003). Just recently the conflict over caste determination was added (Bourke and Ratnieks, 1999; Ratnieks, 2001; Strassmann et al., 2002; Wenseleers et al., 2003). Bourke and Ratnieks (1999) theoretical work, and preceding preliminary articles (Bourke and Franks, 1995; Nonacs and Tobin, 1992; Ratnieks and Reeve, 1992), on conflict over caste determination predict potential conflict between female larvae and adult individuals, usually workers. The conflict may occur during economical periods of colony growth followed by a reproductive phase and especially when workers and reproductives are reared simultaneously. It arises, because a diploid larva which develops into a queen instead of a worker gains more inclusive fitness (Bourke and Ratnieks, 1999; Ratnieks, 2001; Wenseleers et al., 2003). Selection acts within this conflict scenario on two levels. Whereas larvae are selected on the individual level, benefiting from selfishly choosing the pathway of queen development, adults are selected on the colony level, displaying their antagonistic behaviour for the good of the colony. Prerequisite is a high self-determination potential of female larvae due to a low queen-worker size dimorphism and control of their own food intake (Bourke and Ratnieks, 1999). Several lines of evidence for the above described conflict come from detailed studies on swarm founding *Melipona* stingless bees, which vastly overproduce worker-size queens and from the genera *Nannotrigona*, *Plebeia* and *Schwarziana*, where a second caste of dwarf queens is regularly produced besides “normal” queens (Ratnieks and Wenseleers, 2005; Ribeiro et al., 2006). However, workers kill the majority of those excess queens upon emergence (Wenseleers et al., 2004; Wenseleers et al., 2005).

In three *Hypoponera* species, *H. opacior*, *H. schauinslandi* and *H.* sp., I observed low size differences between worker and queen caste, cannibalism between sibling larvae and a for ants untypical active separation of larvae by workers. This is discussed in CHAPTER 4 in terms of caste conflict and further possible interpretations.

# Chapter I

# Alternative reproductive tactics and sex allocation in the bivoltine ant *Hypoponera opacior*

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# Abstract

The ant *Hypoponera opacior* exhibits alternative reproductive morphs in both sexes associated with distinct sexual behaviours. Our long-term study reports strong seasonality in sexual production with two separated mating seasons in early and late summer. Alate reproductives emerge in June, swarm during the monsoon season and establish new colonies independently. In contrast, wingless reproductives appear in late August, mate within their natal or adjacent nests and either do not disperse or reproduce by budding. The divergent dispersal patterns allow to analyse the impact of local factors on investment strategies by comparing sex allocation between the two reproductive phases. The optimal allocation ratio for wingless reproductives should be influenced by both competition for matings between brothers (local mate competition) and rivalry among young queens for workers, nest sites or food (local resource competition). A more male-biased allocation ratio for wingless reproductives demonstrates the greater importance of local resource competition.

# Introduction

During the last three decades alternative reproductive strategies or tactics within a single sex were shown to allow flexible reactions to environmental and social conditions in many animal and plant taxa (Gross, 1996; Lovett-Doust and Lovett-Doust, 1990). The ability to vary morphological, behavioural, physiological and life history traits with external parameters can enable an individual, or in the case of social insects a colony, to maximise its reproductive success.

According to a theoretical review by Gross (1996) alternative reproductive strategies and tactics can be broadly classified as (i) alternative strategies that are genetically determined polymorphisms, which have equal average fitness and are maintained by frequency-dependent selection. (ii) Mixed strategies (with alternative tactics) are genetically monomorphic, and tactics should have comparable average fitness and should be retained via frequency-dependent selection. (iii) Conditional strategies (with alternative tactics) are also genetically monomorphic, but tactics have unequal average fitness and are maintained by status-dependent selection (with or without frequency-dependent selection). The vast majority of described intrasexual variations belong to this category (Gross, 1996).

The majority of alternative reproductive phenotypes in animals is found among males. This was explained by a stronger intrasexual selection within the male sex, because competition for access to females and the variation in mating success is higher (Gadgil, 1972; Trivers, 1972). Alternative reproductive phenotypes in male animals are described for vastly different taxa such as insects (scarab beetle, *Onthophagus taurus* (Moczek and Emlen, 1999)), crustaceans (shrimp, *Paracerceis sculpta* (Shuster and Wade, 1991)), fish (blennies, *Salaria pavo* (Oliveira et al., 2001)), reptiles (side-blotched lizard, *Uta stansburiana* (Sinervo and Lively, 1996)), birds (ruff, *Philomachus pugnax* (van Rhijn, 1973)) and mammals (blackbuck, *Antilope cervicapra* (Isvaran, 2005)).

In contrast to the general greater diversity in male reproductive strategies, in social Hymenoptera different female reproductive morphs are much more common than morphological or behavioural variation in males. The ancestral state in ants is alate male and female reproductives that conduct mating flights. Males in those large swarms undergo intense scramble competition and are mostly unable to monopolise potential mating partners. Consequently, intrasexual selection is low in males leading to general adaptations as strong flight muscles and excellent olfactory and visual capabilities (Hölldobler and Wilson, 1990). Yet, divergent selection frequently occurs in queens and primarily wingless (apterous or intermorphic) queens, mostly in addition to alate queens, were shown in all ant subfamilies. In contrast worker-like, wingless males (ergatoid males) are only present in nine ant genera (*Anergates*, *Aporomyrmex*, *Cardiocondyla*, *Crematogaster*, *Formicoxenus*, *Hypoponera, Pheidole, Plagiolepis* and *Technomyrmex*) (Heinze and Tsuji, 1995). The highest variation, both in male and in female reproductive morphs, occurs in the tiny ants of the genus *Hypoponera*.

In *H. opacior*, distinct differences in the social structure of colonies were shown to be associated with specific tactics of a conditional strategy in alate and apterous queens (Foitzik et al., 2002). Alate queens mate in nuptial flights presumably with alate males and shed their wings after dispersal. These dealate, inseminated queens found their colonies independently (haplometrotic) in low nest-density areas. In contrast, colonies with apterous queens occur typically in dense aggregations, and here mating takes place in or close to the natal nest. The extraordinary mating behaviour has been described as mate guarding (Foitzik et al., 2002): ergatoid males copulate with intermorphic queens for hours even before the latter completely emerge from the cocoon. New colonies are later founded dependently by these young inseminated queens by budding accompanied by a fraction of the worker force. Some young intermorphic queens become adopted by their mother nest and consequently nests are frequently polygynous. Furthermore, colony structure was found to be highly polydomous, with many colonies occupying multiple nest sites in the soil connected by tunnels.

Here, we analysed the annual cycle of reproduction in *H. opacior* with its two sets of alate and apterous reproductives. In the following we document two seasonally separated reproductive phases in early and late summer. Competition between related offspring of one sex should reduce the value of this sex for its parents and in social insects also for related adult workers (Alexander and Sherman, 1977; Hamilton, 1967). Clearly, for ergatoid males, which predominantly mate within the mother nest, the prerequisites for local mate competition (LMC) are met (Alexander and Sherman, 1977; Hamilton, 1967). Competition among brothers for access to females should lower their value and consequently sex allocation should be strongly female-biased. However, local factors similarly or possibly even stronger reduce the relative value of apterous queens (local resource competition, LRC) (Clark, 1978). Successful nest foundation of these queens in the vicinity of the mother nest, leads to strong competition over nest sites, resources and especially workers that accompany queens and help during nest foundations. The varying importance of local parameters on optimal investment strategies during the two reproductive phases makes *H. opacior* ideally suited to investigate the competing impact of LMC and LRC on sex allocation in apterous reproductives. Additionally, the sterility of workers excludes their direct influence on the primary sex ratio.

We show that sex allocation ratios were over years consistently more male-biased for apterous reproductives in August compared to the production of alate reproductives in June. This indicates that contrary to the expectation for a species with nest mating and consequently local mate competition other factors, in our case competition among females, can have a stronger impact on sex allocation strategies.

## Material & Methods

### *Ant collections and maintenance*

*H. opacior* ant colonies were collected in May 2003, June 2004 - 2005 and in August 2003 - 2005 in oak-juniper forest habitats in the Chiricahua Mountains AZ, USA. Additionally, in 2004 and 2005 *H. opacior* colonies were collected at different mountain ranges, the so called “Sky Islands” throughout Southeastern Arizona (Huachuca Mountains, Pinaleno Mountains and Santa Catalina Mountains).

*H. opacior* ants dwell in the uppermost soil layer, preferentially directly under stones. We turned stones and smaller rocks and collected ants and brood with an aspirator. The surrounding soil was carefully searched for possible existing side chambers. All ant colonies were transported to the laboratory either at the Southwestern Research Station (SWRS) or to Munich and were accurately censused. Small nests were frozen and stored in 100 % ethanol for genetic studies, while larger nests were kept in three-chamber-boxes (10 cm x 10 cm x 3 cm) with a moistened plaster floor. Small artificial nest chambers were created and covered by a microscope slide. At the SWRS, ant colonies were fed every other day with pieces of dead insects and kept at room temperature. In the Munich laboratory, ants were offered three times a week freshly killed fruit flies ad libitum and were kept either at room temperature or in a climate chamber at 24 °C, 60 % humidity and a 12/12 h night/day-rhythm.

To analyse the seasonal production of reproductives, we either closely monitored the production of reproductives and workers, or we stored entire *H. opacior* colonies in 100 % ethanol. We determined the caste of ethanol preserved adult individuals under the stereo microscope and noted the number of alate and dealate queens, intermorphic queens, workers, alate and ergatoid males, pupae (small or large) and larvae. In order to verify our morphological caste determinations we completely dissected all female ants from ten nests. Further, cocoons were carefully opened under the microscope and the metamorphosing ants were classified as alate queen, intermorphic queen, worker, alate or ergatoid male or "prepupa" (small or large). To allow the classification of prepupae into either alate reproductives or workers, we measured the cocoon length and -width of live, cocooned pupae in a single, large nest in June 2004 under a stereo microscope (NIKON SMZ 800) and observed their caste fate. Detailed caste determination was carried out in a random sample of ant nests in June and August 2004 and August 2005 and for the entire brood of all ant nests collected in June 2005.

### *Genetic analysis*

For genetic analysis 572 individuals from 92 *H. opacior* nest were used. August samples with 89 % of the individuals and 82 nests prevailed clearly those from June. 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 relatednesses were calculated by weighting colonies equally and the standard errors (SEs) were estimated by jackknifing over colonies (or in cases with sample sizes smaller then the number of loci, over loci) with the program RELATEDNESS 5.0.8 by Goodnight and Queller (1994; 1989). Life-for-life relatednesses were used throughout.

### *Definitions and statistics*

The differentiation between the castes in *H. opacior* nests poses unusual problems. There are four different types of females: alate queens, dealate queens, intermorphic queens and workers. Dealate queens are primarily winged (alate) queens, which are morphologically very distinct from workers (possess a structured thorax, large eyes and three ocelli), and which had shed their wings after the mating flight. In contrast, the external morphology of intermorphic queens resembles that of workers closely. They differed from workers only slightly in size and by having slightly larger eyes. In living ants, discrimination was only possible in freshly eclosed (callow) individuals, in which the pigmented eyes contrasted with the yellow cuticle. The considerably darkened cuticle of older individuals made this slight difference in eye size only unambiguously detectable in dead individuals under the microscope. Contrary to the small external differences, the internal morphology varied strongly between these two castes: intermorphic queens had fully functional ovaries consisting of 2 x 3 ovarioles and a spermatheca, while workers completely lacked reproductive organs. In cases where we did not discriminate between intermorphic queens and workers we referred to them as worker-like individuals.

The highly polydomous colony structure with interconnecting tunnels between nest chambers (Foitzik et al., 2002) and free ranging workers made a nest definition necessary. For this study 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-like individual.

To assess the different investment in the three apterous and the two alate morphs, samples were killed by freezing, dried at 60 °C for 48 h and weighed subsequently with 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 females * cost ratio + n of males). We note that alate queens were predominantly virgins at the time of collection, while most intermorphic queens were supposedly mated and had started ovary development.

For statistic calculations we used the program STATISTICA 6.0 from StatSoft. The Mann-Whitney U test is indicated by U value, the Chi-square test by $\chi^2$ value.

# Results

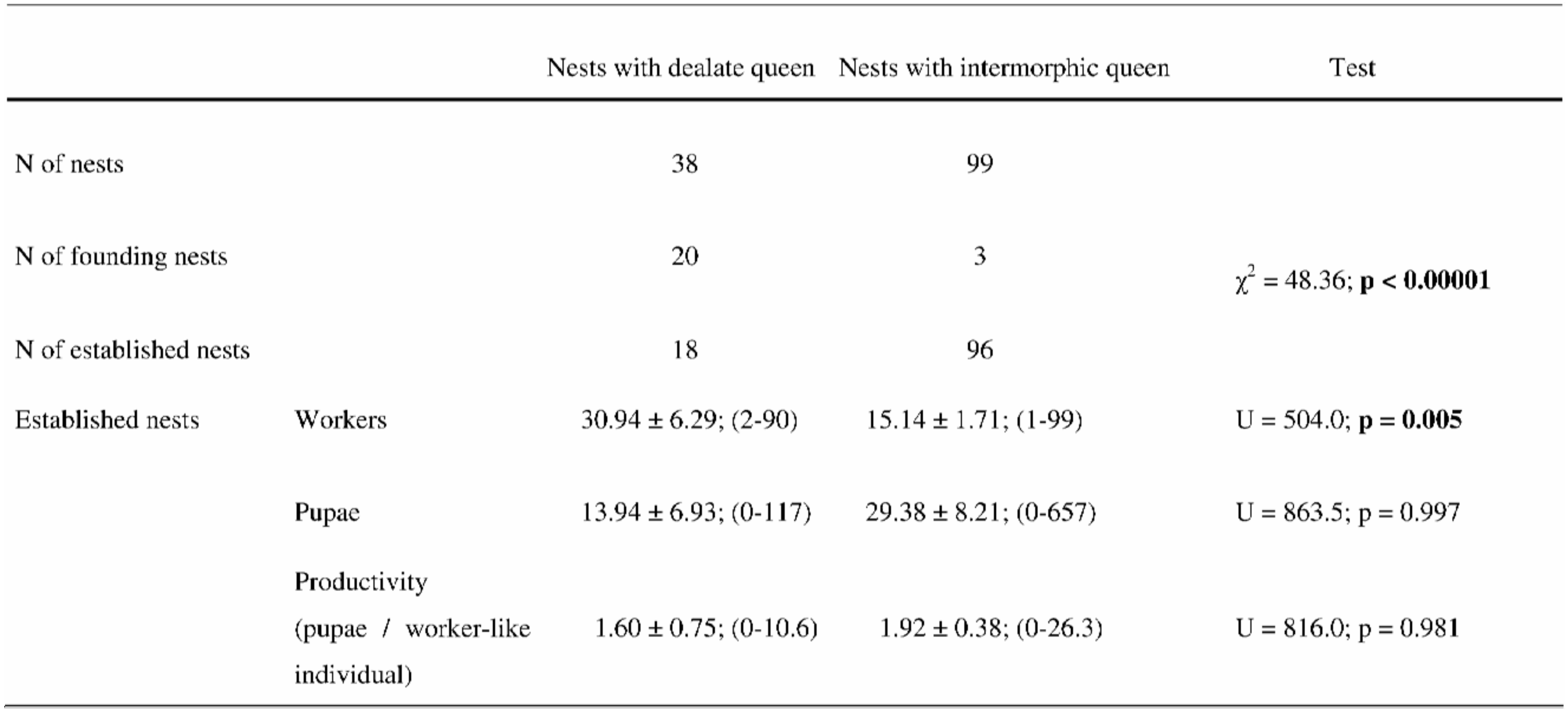
### *Nest demography*

From 2003 - 2005, we collected a total of 724 *Hypoponera opacior* nests, from which 81 % were queenless, 10 % were headed by a single intermorphic queen, 4 % by more then one intermorphic queen and 5 % were headed by dealate queen(s). Over all years, only 2.6 % of the 38 dealate queen colonies were polygynous, while 30 % of the 99 nests with intermorphic queens were polygynous. The frequency of polygynous nests was significantly higher for intermorphic queen nests than for dealate queen nests over all years (Fisher's exact test: $p < 0.002$). In queen-right nests the mean number of intermorphic queens was 1.52 (range 1 - 7) in spring and 4.15 (range 1 - 21) after eclosion in August 2005. This significant difference ($U = 777.0$, $n_{1,2} = 62, 53$, $p < 0.00001$) suggests budding before hibernation or in early spring. The number of intermorphic queens per nest did not vary between Mai 2003 and June 2004 and 2005 (Kruskal-Wallis ANOVA: $H = 1.14$, $p = 0.566$).

A high fraction of dealate queen nests were founding nests without workers (53 %), while only 3 % of intermorphic queen nests were collected during the founding stage (Table 1). Yet, established colonies with dealate queen contained more workers than nests with intermorphic queens (Table 1). The number of pupae and the productivity per worker-like individual did not differ significantly between the two types of colonies (Table 1). These data indicate independent nest foundation by alate queens and dependant nest foundation by intermorphic queens.

We found a strong seasonality, in that founding dealate queen nests were much more frequent in August than in May and June (Fisher's exact test: $p < 0.008$). Furthermore, nests with intermorphic queens contained more pupae and exhibited a higher productivity in August compared to June during the three study-years (number of pupae: $U = 14540.5$, $p < 0.00001$; productivity: $U = 13989.5$, $p < 0.00001$). This could not be shown for established dealate queen nests, for which our sample size is considerably smaller. As an exception to the relative constant seasonal production over years, we found an unusually high productivity in August 2005, after an extraordinary high precipitation in July and August 2005 compared to previous years (Kruskal-Wallis ANOVA: $H = 28.21$, $p = 0.00001$).

**Table 1:** Demographic comparisons between nests with dealate and intermorphic queens. Results are depicted as N or mean ± SE; (range). Significant p-values are in bolt letters.



| | | Nests with dealate queen | Nests with intermorphic queen | Test |
|---|---|---|---|---|
| N of nests | | 38 | 99 | |
| N of founding nests | | 20 | 3 | $\chi^2$ = 48.36; **p < 0.00001** |
| N of established nests | | 18 | 96 | |
| 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; (0-117) | 29.38 ± 8.21; (0-657) | U = 863.5; p = 0.997 |
| | Productivity (pupae / worker-like individual) | 1.60 ± 0.75; (0-10.6) | 1.92 ± 0.38; (0-26.3) | U = 816.0; p = 0.981 |

The investment in reproductive females in June was significantly higher than in August (U = 1252.0, p = 0.001), while the investment in males did not differ between June and August in both years (U = 1946.0, p = 0.848). Nests from our collections in August 2004 and 2005, which invested in ergatoid males, were significantly smaller then those that invested in intermorphic queens, or both apterous reproductives (Kruskal-Wallis ANOVA: $H_{2,86} = 12.60$, $p = 0.001$). The production of ergatoid males was observed in detail in August 2005. Apterous reproductives were produced in 85 from 131 nests with cocoons. However, only 47 % contained both sexes, whereas 18 % produced only intermorphic queens and 35 % only ergatoid males. Between one and a maximum of 18 ergatoid males coexisted within a single nest (mean ± SE: 4.86 ± 0.59). The number of ergatoid males and number of pupae per nest were clearly positively correlated (Spearman’s rank correlation: r = 0.406, p < 0.00001).

### *Sex allocation and reproductive seasonality*

Dry weights for the different castes are given in Table 2. Generally, castes differed in weight (ANOVA: $F_{4,55} = 73.36$, $p < 0.00001$) and the alate reproductive morphs were clearly heavier then the apterous ones. To allow the calculation of the investment ratio we computed the cost ratio for alate and apterous reproductives separately. Alate females weighted on average 1.63 times more than alate males, intermorphic queens were 2.28 times heavier than ergatoid males.

**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: $F_{4,55} = 73.36$, $p = 0.00001$). Asterisks indicate the p-value of the Fisher LSD post hoc test, ** = p < 0.005, *** = p < 0.0001.

| | N | Mean ± SE (µg) | Range | Fisher LSD (p) | | |
|---|---|---|---|---|---|---|
| Alate queens | 10 | 246 ± 9 | 190 - 291 | *** | | *** |
| Intermorphic 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 | | | |

In June 2004 and 2005 98.6 % of all produced reproductives were alates, whereas in August always 100 % were apterous. The population-wide numerical sex ratio (queens / males + queens) was 0.617 and 0.684, in June 2004 and 2005, respectively (Table 3). The population-wide investment ratio over both years was 0.766, which was not significantly different from 3 : 1 ($\chi^2 = 0.55$, $p = 0.459$). The mean proportional investment did not differ between both years (Kolmogorov-Smirnov two-sample test: $n_{1, 2} = 18, 11$, $p > 0.1$). The population-wide numerical sex ratio for apterous reproductives was 0.444 and 0.406 in August 2004 and 2005 (Table 3) and the population-wide investment ratio was 0.645 and 0.609. There was no difference in colony-level investment between years (Kolmogorov-Smirnov two-sample test: $n_{1, 2} = 32, 85$, $p > 0.1$) and over both years the female to male investment ratio in August was 1.60 : 1.

Due to the absence of annual variation in the colony investment ratios within each season, we pooled the data over both years for the graphical representations (Figure 1) and further analyses. The colony investment ratios of alate and apterous reproductives differed during the reproductive period in June (Kolmogorov-Smirnov two-sample test: $n_{1, 2} = 29, 10$, $p < 0.005$). Likewise, colony investment ratios for alates in June differed from the ratios for apterous reproductives raised in August (Kolmogorov-Smirnov two-sample test: $n_{1, 2} = 29, 117$, $p < 0.005$). However, we did not observe seasonal differences in the colony investment ratios of apterous reproductives either raised in June or August (Kolmogorov-Smirnov two-sample test: $n_{1, 2} = 10, 117$, $p > 0.10$; Figure 1).

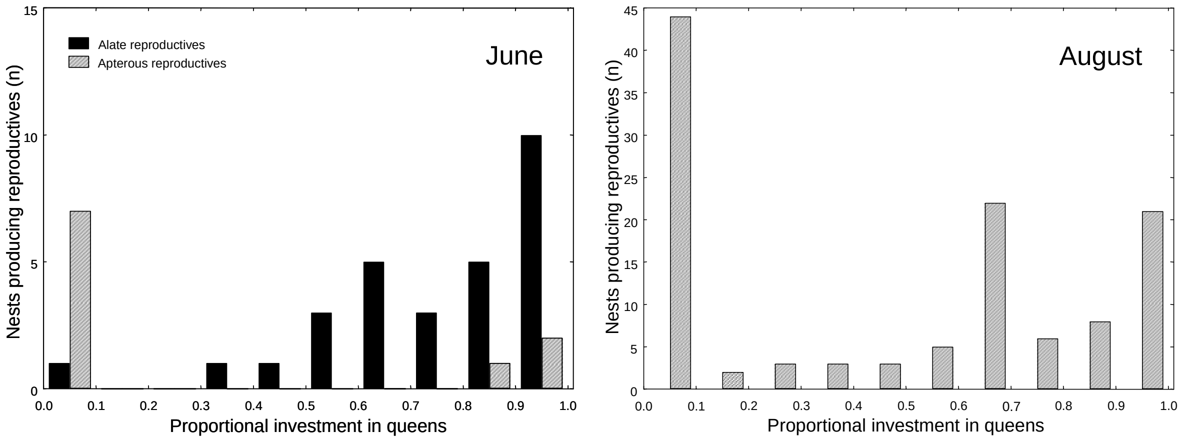


**Figure 1:** Proportional investment for both queen morphs in *H. opacior*, given as proportion of investment in young queens from total sexual investment, including the cost ratio values for alate and apterous morphs. Proportional investments within each reproductive morph did not differ between 2004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

**Table 3:** Sex allocation in a *H. opacior* population from Southeastern Arizona from 2003 - 2005. The numerical sex ratio was calculated as queens / (males + queens).

| Year | Nest (n) | Nests producing pupae (n (%)) | Reproductive morph | Nests producing reproductives (n) | Reproductives (n) | | Population-wide numerical sex ratio | Population-wide proportional investment |
|---|---|---|---|---|---|---|---|---|
| | | | | | male | female | | |
| Mai 2003 | 177 | 0 | apterous | 0 | 0 | 0 | 0 | 0 |
| | | | alate | 0 | 0 | 0 | 0 | 0 |
| August 2003 | 80 | 45 (56.3 %) | apterous | 5 | 5 | 5 | 0.500 | 0.690 |
| | | | alate | 0 | 0 | 0 | 0 | 0 |
| June 2004 | 83 | 33 (40.8 %) | apterous | 4 | 4 | 0 | 0 | 0 |
| | | | alate | 18 | 49 | 79 | 0.617 | 0.724 |
| August 2004 | 168 | 113 (67.3 %) | apterous | 32 | 64 | 51 | 0.444 | 0.645 |
| | | | alate | 0 | 0 | 0 | 0 | 0 |
| June 2005 | 59 | 24 (40.7 %) | apterous | 6 | 7 | 5 | 0.417 | 0.62 |
| | | | alate | 11 | 125 | 271 | 0.684 | 0.779 |
| August 2005 | 157 | 131 (83.4 %) | apterous | 85 | 328 | 224 | 0.406 | 0.609 |
| | | | alate | 0 | 0 | 0 | 0 | 0 |

In order to study which parameters influence the colony-level investment ratios we analysed its association with the total sexual investment and nest size (number of adult worker-like individuals) for all nests that produced at least one reproductive individual. Investment ratios in alate reproductives - a high value indicate investment predominantly in queens - were negatively correlated with the total alate sexual investment, but showed no association with nest size (Table 4). Nest size had no influence on the total sexual investment in alates (Spearman's rank correlation: $r_s = 0.10$, $n = 29$, $p = 0.614$). In August investment ratios were positively associated with total sexual investment and nest size (Table 4). Furthermore, the latter two parameters were also correlated (Spearman's rank correlation: $r_s = 0.29$, $n_{1,2} = 117, 117$, $p = 0.001$). In summary, *H. opacior* nests in June with a high investment in reproductives raised mainly alate males, while large nests in August invested mainly in intermorphic queens. Furthermore, nest size was only positively associated with a female-biased investment ratio in August (Table 4).

**Table 4:** Correlation of proportional investment in queens (alate, apterous) per *H. opacior* nest and total sexual investment and nest size. Correlations were conducted with pooled data from 2004 and 2005. Still significant p-values after correcting α–values according to the sequential Bonferroni method (Rice, 1989) were given in bolt letters.

| | Month | Reproductive morph | Spearman's rank correlation ($r_s$) | P-value | N |
|---|---|---|---|---|---|
| Total sexual investment | June | Alate | -0.45 | **0.015** | 29 |
| | August | Apterous | 0.52 | < **0.00001** | 117 |
| Nest size | June | Alate | -0.02 | 0.919 | 29 |
| | August | Apterous | 0.33 | **0.0003** | 117 |

New workers were produced in the two reproductive periods in June and August. In June 2005 all but one nest with brood produced new workers independently of their participation in the production of reproductives.

These results show a clear seasonality for the production of alate and apterous reproductives, which emerged in early and late summer. Yet, the social organisation of the nests did not influence which reproductive morphs a nest produced. All types of nests, queenless ones and those headed by dealate or intermorphic queen(s) raised reproductive offspring in the characteristic seasons. Furthermore, the frequency of nests with the three social organisations did not differ between June and August both in 2004 and 2005 (Figure 2).

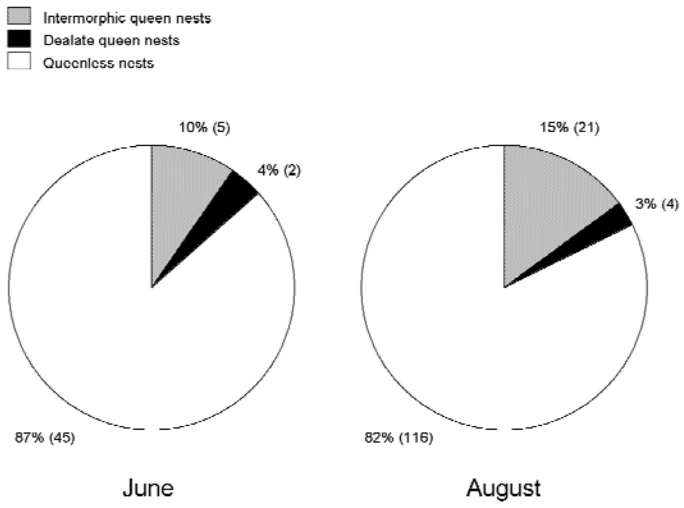


**Figure 2:** Proportion and n (in brackets) of reproductives producing nests headed by a dealate queen, by intermorphic queens or without a queen in June and August (pooled data from 2004 and 2005). Proportions did not differ between both reproductive periods (Fisher's exact tests (sequential Bonferroni corrected (Rice, 1989)): $p > 0.05$).

Clearly, not social organization but the reproductive periods influenced the production of the two sexual forms. Interestingly, the onset of the North American Monsoon in Arizona, USA, coincides with the emergence of the alate reproductives (Figure 3; precipitation data from 1965 – 2005 by courtesy of the "Western Regional Climate Center", http://www.wrcc.dri.edu).

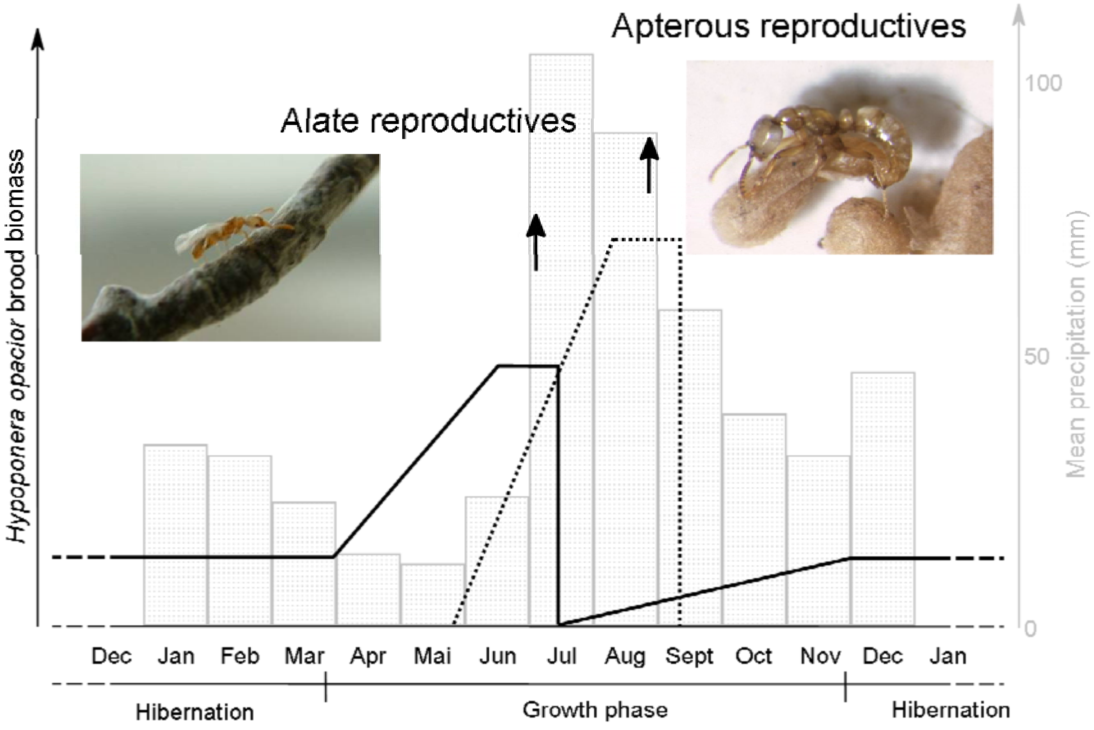


**Figure 3:** Schematic graph of the annual cyclic offspring production in *H. opacior* (the solid line indicates the brood-biomass of predominantly alate reproductives; the dashed line indicates the brood-biomass of apterous reproductives) as observed from 2003 to 2005. The grey dotted bars represent 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 intermorphic queen.

### *Genetics*

The life-for-life relatedness of workers as calculated from the entire data set, differed with a mean of 0.194 ± SE 0.082 in June only tendentiously from the mean in August with 0.427 ± 0.032 (t-test: $t = 1.73$, $n_{1,\,2} = 4, 58$, $p = 0.062$). Similarly, life-for-life relatedness values of workers to queens and workers to males did not vary between both reproductive periods (t-test: $t = 0.328$, $n_{1,\,2} = 5, 39$, $p = 0.745$; $t = 0.184$, $n_{1,\,2} = 5, 19$, $p = 0.856$). Relatedness coefficients (based upon 22 colonies) of workers toward reproductive females were significantly greater than those of workers towards males. Yet, relatedness asymmetry was not different between June and August (Figure 4).

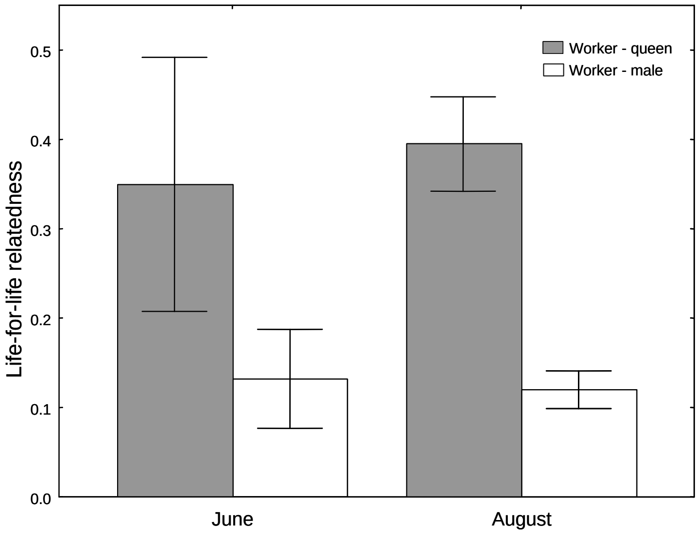


**Figure 4:** Mean life-for-life relatedness values ± SE. The relatedness coefficient of workers to queens was significantly greater than that of workers to males (repeated-measures ANOVA: $F_{1,20} = 33.62$, $p = 0.00001$). Relatedness asymmetry of workers to queens and males was not different between June (ratio = 2.64 : 1) and August (ratio = 3.29 : 1; $F_{1,20} = 0.04$, $p = 0.844$).

# Discussion

Why does *H. opacior* pursue a two-fold strategy with regular production of two reproductive morphs of both sexes? In ants reproduction by alate queens and males is assumed to be the ancestral state (Hölldobler and Wilson, 1990). *H. nippona* and *H. sauteri* produce only alates (Yamauchi et al., 2001), suggesting that nuptial flights may also be ancestral in this genus. While ensuring dispersal, mating flights followed by independent nest foundation are exceedingly risky endeavours. The production of apterous reproductives allows mating in the safe haven of the nest and dependant nest foundation with a start-up help of workers. Thus, rapid expansion and exploitation of favourable habitats become possible. However, this form of reproduction has the problem of inbreeding and restrains dispersal, as budding nest fragments disperse on foot. Thus, reproduction by both reproductive forms allows settlement of new habitats through alates, followed by rapid expansion by apterous reproductives in suitable patches.

In contrast to *H. opacior* where alates are produced regularly each year, in *Cardiocondyla obscurior*, a phylogenetically distant ant species with alternative reproductive tactics in males, alate males are only produced under adverse environmental conditions for the colony, e.g. extreme temperature changes or food shortage (Cremer and Heinze, 2003). In *H. opacior* the production of alate and apterous reproductives coincides with seasonal changes in the environment. Periodic environmental cues can be low winter temperatures - our study site is located above 1500 m in the Chiricahua Mountains - and high precipitation rates during the North American Monsoon in summer. As a proximate factor, the cold temperatures could influence the developmental avenue of hibernating larvae and the feeding activity of workers. Indeed, this was shown for the ant *Myrmica rubra*, where hibernated (vernalised), large, third instar, female larvae developed mainly into queens, while smaller ones developed into workers (Brian, 1955; Brian, 1975).

Ultimately, nuptial flights followed by independent colony foundations in the wet monsoon season are certainly favoured, because of a decreased risk of desiccation, facilitation of nest building and increased food abundance. Many ant species from Arizona, such as the harvester ants *Pogonomyrmex* and the honey pot ants *Myrmecocystus* conduct nuptial flights a few days after the beginning of the monsoon (Bartz and Hölldobler, 1982; Hölldobler, 1976; Nagel and Rettenmeyer, 1973), typically after a heavy rain. Although, precipitation varies in Arizona greatly during the year, the North American Monsoon (Mexican Monsoon), which starts in Arizona in early July (Higgins et al., 1999), causes the highest annual precipitation rates in this area (http://www.wrcc.dri.edu/cgi-bin/cliMAIN.pl?az6716). Mating flights are very risky for young queens, causing tremendous mortality rates through predation and dehydration (Hölldobler and Wilson, 1990). Facilitation of nest digging is certainly less important in *H. opacior* than in larger desert ant species, which construct deep, elaborate nests. Yet, the increased humidity in the soil and in the leaf litter layer, where *Hypoponera* ants typically forage, leads to a general proliferation of the soil fauna and thus to an increased food supply for the young founding nests. A correlation between the breeding success of birds in warm, arid climates and the amount of precipitation was shown in Li and Brown (1999) and papers within. This might also be the ecological reason for the second reproductive period in late summer with the fast developing apterous reproductives. Indeed, in our study we found a generally higher productivity of *H. opacior* nests in August than in the comparatively dry June. Productivity was highest in August 2005 following a monsoon with exceptionally high precipitation rates. Also other animals respond to the high food supply after the monsoon rain. The Ash-throated Flycatcher (*Myiarchus cinerascens*) depart from their breeding grounds in arid lowland habitats in the northern Southwest and Mexico to feed and moult in the region of the Mexican Monsoon shortly after the onset of the monsoon rains (Butler et al., 2006).

After the *H. opacior* alate queens have successfully started a colony, they have to raise their first workers. Dealate founding queens were quite common in August, while we found in spring only few established nests with dealate queens. Indeed, survival during the first winter is low and probably depends on the worker force. This might be another important reason for an early mating flight of the alates in *H. opacior* as it extends the time to set up a colony and rear the first workers.

The occurrence of alate and apterous reproductives of both sexes were also described in the congeneric species *H. eduardi* and *H. nubatama* and in the dolichoderine ant *Technomyrmex albipes* (Le Masne, 1956; Yamauchi et al., 1991; Yamauchi et al., 2001). In contrast to *H. opacior*, the reproductive cycles were reversed in *H. nubatama*. Apterous reproductives appeared in this Japanese *Hypoponera* species mainly in summer, whereas alate reproductives conducted nuptial flights in autumn (Yamauchi et al., 2001). *T. albipes* with its huge polydomous colonies produced alate reproductives in the first reproductive period in late spring, whereas apterous reproductives were produced acyclic throughout the year, except in winter. In this dolichoderine ant species dealate queens were even rarer, but the number of intercastes exceeded those of intermorphic queens in *H. opacior* by far (Yamauchi et al., 1991).

Each of the two queen phenotypes in *H. opacior* exhibits an alternative reproductive tactic of a conditional strategy, which is also reflected in the social structure of their colony (Foitzik et al., 2002). Our larger sample corroborates these earlier data, as all established dealate queen colonies but one contained a single queen, whereas nests with intermorphic 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 exceedingly rare and represented only less then three percent of all nests. As high numbers of alates were produced by *H. opacior* colonies, this low fraction of dealate nests certainly reflects the low rate of independent colony establishment. The number of intermorphic queens was highest shortly after their emerging and mating in late summer, while fewer queens per nest were consistently found in spring, reflecting nest budding either before or directly after hibernation.

Contrary to the majority of alternative reproductive tactics described from other animal species (Gross, 1996), we found in *H. opacior* that the production and mating season of alate and apterous reproductives are almost completely temporally separated.

Only a few nests reared ergatoid males in June, which may have the opportunity to copulate with freshly emerged alate queens. Copulation of alate males within the mother nest was never observed and it is therefore likely that these alate males need the mating flight to trigger sexual behaviour, as in many other ant species (Hölldobler and Wilson, 1990). Intrasexual conflict over reproduction between two male morphs should therefore be rare or totally absent, 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).

Intranest worker relatedness was found to be slightly higher in August than in June, which can be attributed to a higher number of intermorphic queens contributing to offspring production earlier in the season. In both reproductive periods relatedness asymmetry between adult workers and female and male brood was close to 3 : 1. Consequently, the observed population-wide investment ratio of 3 : 1 of alate reproductives in June conforms to the worker optimum (Fisher, 1930; Trivers and Hare, 1976). Yet, as the relatedness asymmetries from the perspective of reproductive females were not calculated and multiple queens per nest reproduce, it is possible that the observed investment ratio could also be in the evolutionary interest of the queens.

The investment sex ratio in *H. opacior* should be influenced by local factors only for the within nest-mating, low dispersing morphs raised in late summer. Apterous reproductives of a single nest intensely compete with each other for mates (males, LMC (Alexander and Sherman, 1977; Hamilton, 1967) and for resources (females, LRC (Clark, 1978). In contrast to the classical model developed by Hamilton (1967) for species with wingless males, in *H. opacior* apterous queens also show strongly reduced dispersal abilities. Their winglessness promotes LRC, as the reduced dispersal capability of ergatoid males facilitates LMC. The population-wide investment ratio of apterous reproductives in August was 1.60 : 1, despite similar within-nest relatedness values in June and August. This ratio, although overall still female-biased, is clearly lower that is more male-biased than sex allocation in June and may suggest a preponderance of LRC. This is furthermore supported by a lower absolute production of intermorphic queens compared to alate queens and a population-wide male-biased numerical sex ratio in late summer. The relatively high investment in apterous males can be explained by an even stronger reduction of the value of apterous queens through LRC or less intense competition among related males, possibly through male dispersal to neighbouring nests on foot. Indeed relatively low relatedness values suggest that males sometimes migrate to adjacent nest chambers through the extended tunnel system. However, despite a similar biology, the highly female-biased investment ratio among apterous reproductives of *T. albipes* and *C. obscurior* (Cremer and Heinze, 2002; Tsuji and Yamauchi, 1996) strongly indicates that LMC can considerably reduce the colony value of apterous ant males.

In ant species that reproduce by budding, local resource competition among nestmate females can occur over two important commodities. First, intermorphic queens compete for workers, which are essential for successful dependent nest foundation, and second, newly established daughter nests compete for resources such as high-quality nest sites and food. In such a situation, accompanying workers are parts 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). Intense competition among neighbouring daughter nests should still lead to an overall male-biased investment, even if workers are counted as part of the female investment. Yet, albeit we did not investigate worker production in detail, including workers into the calculation of the investment ratio in August would make it even more female-biased, approaching the value observed for alates in June. This indicates a somewhat balanced tug-of-war between the local forces acting on the reproductive values for queens and males.

In addition to the population-wide patterns in the sex allocation, we found a strong bimodal distribution of investment ratios on the colony level (split sex ratios) in August. Furthermore during the production of apterous reproductives, larger colonies with an increased sexual investment allocated relatively more resources into queens. These findings can be explained by the resource limitation hypothesis (Nonacs, 1986a; Nonacs, 1986b), which suggests that larger colonies with a higher food provision rate should invest in the costly production of queens, while smaller nests with a lower and less secure food supply should favour the production of males. Finally, our findings that many colonies invest only in reproductives of a single sex are inconsistent with the constant male- or constant female hypothesis (Frank, 1987).

## Conclusions

The here documented 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 highly unusual clear temporal separation of the production and the mating season of the two sets of reproductive morphs provides the unique possibility to investigate how seasonal changes influence nest foundation success and how dispersal and competition affect optimal investment strategies. Our data indicate that the timing of the alate production is governed by the North American Monsoon and the approaching winter, while a well-balanced tug-of-war between local forces acting on the reproductive values for queens and males influences allocation patterns in the apterous season.

## Acknowledgements

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# Chapter II

# Polymorphic microsatellite loci in the ponerine ant *Hypoponera opacior* (Hymenoptera, Formicidae)

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# Abstract

The ant genus *Hypoponera*, with its high diversity of alternative reproductive tactics, is of particular interest in studies on sexual selection. In the species *Hypoponera opacior*, winged and wingless males and queens co-occur and molecular markers are essential to study the reproductive success of these sexual tactics. Primers were developed for five polymorphic microsatellite loci isolated from *H. opacior*. Their variability was tested on 34 colonies from a population in the United States. Nine to 21 alleles per locus were found with observed heterozygosities between 0.1 and 0.7. A significantly positive $F_{IS}$ value suggests inbreeding in this ant with predominant intracolonial matings.

In most ant species, sexual selection is less intense because nuptial flights do not allow males to monopolize females. The ant genus *Hypoponera*, however, contains a large diversity of reproductive strategies and tactics both in males and queens (Foitzik et al., 2002; Le Masne, 1956; Yamauchi et al., 1996; Yamauchi et al., 2001). In the Neotropical *Hypoponera opacior*, winged reproductives copulate during nuptial flights in early summer, while in August wingless males guard females and mate with them within their mother nest. These reproductive strategies are associated with differences in colony organisation: dealate queens head mostly monogynous, monodomous colonies, while colonies with wingless sexuals commonly contain several queens and occupy multiple nests. The latter social organization resembles that of unicolonial, invasive ants and indeed some *Hypoponera* species are cosmopolitical tramps (Giraud et al., 2002; Holway et al., 1998). High resolution molecular markers are necessary to measure the reproductive success of the various sexual tactics to shed light on the evolution and maintenance of alternative reproductive strategies. Here, we characterize five polymorphic loci for *H. opacior*, which will further allow to analyse the impact of inbreeding and reproductive behaviours on the fine-scale genetic structure of colonies and populations.

These soil dwelling ants were collected in the Chiricahua Mountains near the South Western Research Station (SWRS), Portal, AZ, USA. About 260 individuals were ground in liquid nitrogen and the tissue was digested with RNAse and Proteinase K (MBI Fermentas) at 56 °C over night. The DNA was extracted with a standard Phenol/Chloroform protocol and digested with Tsp 509 I, which was later removed by a second Phenol/Chloroform step. Two adaptors (Tsp AD short and Tsp AD long (Tenzer et al., 1999)) were ligated to the DNA, resulting in blunt-ended fragments. Ultrafree-4 spinning columns (Millipore) were used for purification and the ligation products were amplified by using the adaptor Tsp AD short as primer. The 25 µL reaction mixture for each of the 32 polymerase chain reactions (PCR) contained 0.5 µL ligation product, 2.5 µL 10 x Taq polymerase buffer (-$MgCl_2$), 2.5 mM $MgCl_2$, 1.0 mM dNTPs (MBI Fermentas), a final concentration of 1 µM Tsp AD short and 1.25 units of Taq DNA polymerase (Promega). For the PCR a T1 Thermocycler (Biometra Whatman) was used and the following program was applied: 72 °C for 5 min to synthesize the nick between the linker and the genomic DNA, followed by 20 cycles at 93 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. The purified amplification product was enriched with repeat motives by hybridising them with biotinylated oligonucleotides of either $(CA)_{13}$ or $(GA)_{13}$ that had been linked to streptavidin-coated magnetic beads (Dynabeads M-280m Streptavidin;

Dynal) (Tenzer et al., 1999). Unhybridized fragments were removed in four washing steps. Another PCR was performed directly with 1 µL of bead solution under the same conditions but without the initial extension step.

The DNA enriched with repeat motives was ligated into the plasmid pcR2.1 and transformed into Top 10F' cells (TA Cloning Kit; Invitrogen). Positive clones were dot-plotted on nylon membranes (Hybond-$N^+$ membrane; Amersham Life Science) and probed with fluorescein-11-dUTP labelled oligonucleotides $(CA)_{13}$ and $(GA)_{13}$ (Gene Images 3'-oligolabelling and Gene Images CDP-Star detection module; Amersham Life Science).

We detected 96 complementary Plasmid inserts, 82 of which were sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and M13 forward and reverse primers (MWG) on an ABI Prism 310 Genetic Analyser (Applied Biosystems). Primer pairs were designed for 13 of the 55 clones, which possessed repeat motives and these were tested on DNA from individuals of eight different colonies, which was extracted with PureGeneKit (Promega). Gradient PCR was carried out in 20 µl reactions with approx. 10 ng DNA (1.0 µL), 2.0 µL 10 x Taq Polymerase buffer (-$MgCl_2$), 2.5 mM $MgCl_2$, 0.2 mM each dNTP (MBI Fermentas), 0.5 µM each primer, 4 µL Enhancer Solution P (peQlab) and 0.4 units of Taq DNA Polymerase (Promega). The following PCR-program was used: denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1.15 min, 55 °C for 45 s and 72 °C for 45 s, then 72 °C for 7 min.

For each of the nine microsatellite loci where we obtained an amplification product, the forward primer was labelled with an ABI fluorescent dye at the 5'-end (either HEX, TET or FAM; MWG Biotech). All reactions were performed in a T-GRADIENT Thermocycler (Biometra), the fragments were detected on the ABI and analysed using GENESCAN (Applied Biosystems). Five of the nine loci were polymorphic for *H. opacior* and the number of alleles ranged between nine and 21 for 106 individuals from 34 nests (Table 1). All ant nests were collected from a single population in the Chircahua Mountains, Arizona and thus, we could not explicitly test for population substructure. The program RELATEDNESS calculates F-statistic by taking the nest structure into consideration, that is the program uses only nests as independent units, not individuals.

A significant $F_{IS}$ value was calculated for each of the five loci with the program RELATEDNESS 4.2 (Goodnight and Queller, 1994) with a mean of 0.44 (± SE 0.04; 95 %CI = 0.34-0.54) over the five loci indicating true inbreeding in *H. opacior*. Consequently all loci show significant deviations from Hardy-Weinberg equilibrium (Raymond and Rousset, 1995) (Table 1). Linkage disequilibrium was calculated with the software

GENEPOP (Raymond and Rousset, 1995) and all loci were found to be independent with the exception of the ones amplified with the primer pair HoP 26 – HoP 58 ($P < 0.01$). The five primer pairs were also tested in two tramp species of the genus, *Hypoponera schauinslandi* and *Hypoponera* sp., and all of them except HoP 26 yielded an amplification product. However, variability was demonstrated only at a single loci so far in the limited sample ($n = 1$ colony for each species). These powerful genetic markers will thus prove to be an important step forward in the analysis of the evolutionary basis of the fascinating reproductive strategies in *Hypoponera* ants.

**Table 1:** Primer sequences and characterization of five microsatellite loci in the ant *Hypoponera opacior*.

| Locus | Primers (5' → 3') | Repeat motif | $T_a$ (°C) | *n* | $N_A$ | Size of mca (bp) | Frequency of mca | $H_O$ | P-HWE | $H_E$ | Accession n. (EMBL) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| HoP 26 | F :*TCGCGTTAAGTTCCGTTAAGC<br>R : TCGCGCGAAGCGTCTAACTCG | $(CA)_{16}$<br>CG<br>CACG<br>$(CA)_2$TA<br>$(CA)_8$ | 55 | 99 | 9 | 180 | 0.389 | 0.310 | 0.0001 | 0.767 | AJ745075 |
| HoP 54 | F :*TCCGCACGTGGCAAATAGC<br>R : AGCCTGTAGTCCAACTTATCG | GTGC<br>$(GT)_4$<br>GC$(GT)_{11}$<br>GC$(GT)_2$ | 55 | 103 | 16 | 118 | 0.208 | 0.700 | 0.0001 | 0.885 | AJ745077 |
| HoP 58 | F :*AATTACCGTGATTAGC<br>R : AAGGATCGCCGTTACG | $(AC)_{16}$ | 55 | 87 | 10 | 146 | 0.470 | 0.100 | 0.0001 | 0.634 | AJ745076 |
| HoP 60 | F:*GATAACGGGACGTGATCTAGC<br>R: GCAAATCTAAACAGCGAACG | $(CA)_{26}$CG<br>$(CA)_6$CG<br>$(CACG)_2$<br>$(CA)_2$ | 55 | 100 | 21 | 172 | 0.159 | 0.581 | 0.0001 | 0.898 | AJ745078 |
| HoP 64 | F :*CGATTTACAAACGATAATGC<br>R : ATACAAGGTGACTCCACTCG | $(TG)_{12}$<br>GG $(TG)_8$<br>GGTG | 55 | 100 | 12 | 139 | 0.261 | 0.616 | 0.0001 | 0.849 | AJ745079 |

$T_a$ annealing temperature; *n* sample size; $N_A$, number of alleles; $H_O$, observed heterozygosity; $H_E$, expected heterozygosity; *mca*, most common allele; * Forward (F) primer was FAM, TET or HEX-labelled; P-HWE P-value for Hardy-Weinberg equilibrium with α-value after Bonferroni correction of 0.01 (Raymond and Rousset, 1995).

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# Chapter III

# Macro- and microgeographic genetic structure in an ant with alternative reproductive tactics in males and females

Markus H. Rüger and Susanne Foitzik

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# Abstract

In ants alternative reproductive tactics of winged and wingless reproductives often represent complementary dispersal strategies. The two sexual morphs each in males and females of the ant *Hypoponera opacior* mate during different seasons and allow colonizing and rapidly populating habitat islands. Here, we used microsatellite markers to investigate the genetic structure of this species on different levels. On a macrogeographic scale we found considerable structure between and within various mountain ranges in Southeastern Arizona, USA. However, generally genetic and geographic distances did not correspond. Only subpopulations from within the Chiricahua Mountains were genetically more similar than those from different mountain ranges. This lack of an association between geography and genetic structure can be explained by the analysed sites being relics from a large ancestral population. Post-pleistocene climate changes led to desertification of lowland sites, so that *H. opacior* populations retreated to more humid habitats at higher elevation and consequently populations became isolated. On a local scale, we show multicolonial nest structure, also evident from behavioural recognition trials, which revealed consistent aggression between non-nestmates. Yet, polygyny and dependant nest foundation also led to detectable population viscosity. Frequent sibmatings between wingless reproductives caused a high inbreeding coefficient of 0.45. Wingless males, expected to avoid inbreeding to reduce diploid male load, actually either selectively mated with close relatives or showed random mating. Finally, we could not detect diploid males in this highly inbred species, suggesting elimination of diploid male brood or a sex determination mechanism other then complementary sex determination.

# Introduction

In contrast to solitary animals, where behavioural actions are predominantly under direct selection, cooperative behaviours in social species are often best explained by kin selection (Hamilton, 1964). Hence in social animals, analyses of genetic relationships within and between groups are important to gain a deeper understanding of the evolution and maintenance of behavioural strategies. Multiple reproductive tactics or strategies in solitary species often represent alternatives selected by frequency- or state dependant selection (Gross, 1996). In contrast, alternative tactics in social insect species can have a complementary function, for example winged morphs are responsible for long distance dispersal, while wingless reproductives allow the fast exploitation of local resources (Heinze and Keller, 2000).

The tiny *Hypoponera* ants exhibit, in contrast to the vast majority of ants, a great variety of alternative reproductive strategies and tactics in both males and females. Some species produce winged (alate) reproductives, which are typical for ants and which mate on nuptial flights, while in other *Hypoponera* species obligate wingless (apterous) males mate with females in or close to the mother colony (Foitzik et al., 2002; Le Masne, 1956; Yamauchi et al., 1996; Yamauchi et al., 2001). Our focal species *H. opacior* represents an interesting intermediate form and produces both types of reproductives during two discrete mating seasons of the year (Foitzik et al., 2002; Rüger and Foitzik, submitted).

After a mating and dispersal flight in July, inseminated alate *H. opacior* queens shed their wings and found new colonies independently. During a second mating season in late August - early September apterous males mate with apterous (intermorphic) queens within or close to the mother nest. These matings, which have been described as mate guarding, are quite unusual as apterous males copulate with intermorphic queens for hours, while the latter are still partially enclosed in their cocoons (Foitzik et al., 2002). Both apterous males and females can only disperse on foot and thus sib-matings are presumably rather the rule than the exception. Inseminated intermorphic queens either stay in the mother nest or start new nests dependently with the help of workers, most likely in close vicinity to the mother nest (Rüger and Foitzik, submitted). The genetic colony and population structure of an ant species with such an unusual and flexible reproductive mode has not been analysed before. In this study, we investigate the genetic structure of *H.*

*opacior* on different geographic scales, from castes and colonies over patches to subpopulations (demes). We thus add to a better understanding on the impact of alternative reproductive tactics onto nest organisation, migration and mate choice in ants.

In particular, we expect the genetic structure of *H. opacior* populations to be strongly influenced by the frequency and magnitude of long distance dispersal events by winged reproductives. If dense *H. opacior* nest aggregations are the result of only a few or even a single colonisation event by an alate queen, a very low genetic variability is expected on a microgeographic scale. The few alleles introduced by founder queens might also get lost in time through extensive inbreeding of apterous reproductives. Hence in addition, the local structure should also be affected by the behavioural strategies of wingless colony members. Our genetic analysis should therefore elucidate how often and how far apterous males and females disperse through the subterranean tunnels, which connect *H. opacior* nests. The evolutionary independence of nests in dense aggregations with often more than 25 nests per 100 $m^2$ in *H. opacior* (Foitzik et al., 2002) can be low through a loss of nestmate recognition abilities of workers, which allow the adoption of non-nestmates and thus promote genetic mixing. In extreme cases this could result in a unicolonial population structure, which was frequently observed in invasive ant species (Drescher et al., 2007; Giraud et al., 2002; Goodisman et al., 2007; Jaquiéry et al., 2005; Thomas et al., 2006). Albeit many *Hypoponera* ants are tramp species, which recently established new populations abroad (Delabie and Blard, 2002; McGlynn, 1999; Seifert, 2004), *H. opacior* is native to Southeastern Arizona and presumably populated this area for a long time (Hunt and Snelling, 1975; MacKay and Vinson, 1989).

The Chiricahua Mountains in Southeastern Arizona, our main study area, belong to the so called “Sky Islands”, mountain ranges at the contact zone of the Rocky Mountains and the Sierra Madre, isolated by long stretches of desert habitat (Heald, 1951). Climate changes since the Pleistocene let to the retreat of oak-juniper and pine forests to the more humid sites along the slopes and on-top of these peaks, which can reach more than 3000 m in elevation (Marshall, 1957). *H. opacior* chiefly settles in oak-juniper forests at intermediate elevations, and hence its habitat is highly fragmented. The investigation of genetic differentiation in this special geographic setting is of exceptional interest. Our macrogeographic population genetic analyses may thus provide novel insights into migration rates of the tiny alate reproductives between different slopes of the same and in-between mountain ranges, which are often separated by more than 50 km of desert scrub or grass habitat.

In many animals, females and sometimes also males actively choose their mating partner to increase their reproductive success (Kokko et al., 2006). Ants, in contrast, commonly mate during nuptial flights (Hölldobler and Wilson, 1990), in which due to intense scramble competition active choice appears to be barely possible. Yet, apterous males of *H. opacior* mate in the safety of the nest and guard females in copula for several hours (Foitzik et al., 2002). Males not in copula constantly antennate developing females and thus might actively choose their mating partners. However, most of the available virgin females in the nest are close relatives, so that males should actively search for less related females to avoid inbreeding. In this study we used highly variable genetic markers to determine whether apterous *H. opacior* males show inbreeding avoidance.

Inbreeding in haplodiploid Hymenoptera with their typical complementary sex determination can have a very detrimental impact on colony fitness, which is 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; Stouthammer et al., 1992). The honey bee sex locus was recently sequenced and was found to be under diversifying selection, as expected to avoid the costly production of diploid males (Beye et al., 2003; Hasselmann and Beye, 2004). Especially during colony foundation, when workers are important, diploid males are a large burden to the colony and often lead to an early colony death. Only very rarely direct fitness costs of diploid males are postponed, when they manage to mate and father triploid offspring, which, however, are always sterile (Agoze et al., 1994). Thus, to evade the fitness costs associated with the production of diploid males, most Hymenopterans strongly avoid inbreeding through population-wide mating swarms and long distance flights of both sexes e.g. in *Apis* and *Melipona* bees (Michener, 1974) or by temporal separation of female and male dispersal (Hölldobler and Bartz, 1985; Hölldobler and Wilson, 1990).

However, in Hymenopterans with within-nest matings such as many *Hypoponera* or *Cardiocondyla* species (Foitzik et al., 2002; Schrempf et al., 2006; Schrempf et al., 2005; Yamauchi et al., 1996; Yamauchi et al., 2001), inbreeding is often the rule, so that an alternative sex determination system has to be used or diploid males have to be eliminated at a very early stage, as in honey bees (Santomauro et al., 2004). Our genetic analysis will also allow us to estimate the frequency of diploid males.

In summary, the aims of our microsatellite study are fourfold. First, in a population genetic project we investigated structure and gene flow between subpopulations both within and between different mountain ranges in Southeastern Arizona. Second, we analysed intranest relatedness, colony and population structure of *H. opacior* on a microgeographic scale, using both genetic methods and nestmate recognition experiments. Third, we analysed whether apterous males mate assortatively to avoid inbreeding. Finally, we calculated inbreeding coefficients and investigated the occurrence and frequency of diploid males.

## Material & Methods

### *Samples and microsatellite analysis*

*Hypoponera opacior* colonies were collected in Southeastern Arizona (USA) in 1998 and between 2003 and 2005. We genotyped a total of 855 individuals from 376 nests.

Samples were preserved in 100 % ethanol and frozen at –20 °C until DNA extraction. Ants were washed in $ddH_2O$ 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 investigate general patterns of genetic diversity, a subsample with only diploid individuals from the Chiricahua Mountains were used. Deviations from Hardy-Weinberg equilibrium, observed and expected heterozygosities and the inbreeding coefficient $F_{IS}$ at each locus were calculated with the program GENEPOP version 3.4 (Raymond and Rousset, 1995) or FSTAT Version 2.9.3 (Goudet, 2001). Furthermore, unidirectional and symmetrically relatedness values between castes were calculated with the program RELATEDNESS 5.0.8 (Goodnight and Queller, 1994; Queller and Goodnight, 1989). For these calculations we weighed colonies equally and 95 % confidence intervals were estimated by jackknifing over nests (or in cases with sample sizes smaller then the number of loci, over loci). The frequency of null alleles was estimated using the equation: $r = (H_E –$

$H_O$) / (1 + $H_E$) (Brookfield, 1996). A general absence of linkage disequilibrium between these five microsatellites was formerly shown in *H. opacior*, with the single exception of the loci HoP 26 – HoP 58, which behave not entirely independent (Rüger et al., 2005).

### *Macrogeographic structure*

We sampled six geographically separated subpopulations from the Chiricahua Mountains. In addition, we collected *H. opacior* nests from one subpopulation each in three adjacent mountain ranges; the Santa Catalina-, the Pinaleno- and the Huachuca Mountains (Figure 1). For population genetic analysis we genotyped 268 workers, a dealate and an intermorphic queen. We sampled on average 30 nests per deme with a range from 27 to 34. Only a single individual per nest was genotyped to investigate the genetic differentiation between demes. 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. $F_{ST}$-values were calculated with the program Microsatellite Analyser (MSA) (Dieringer and Schlötterer, 2003).

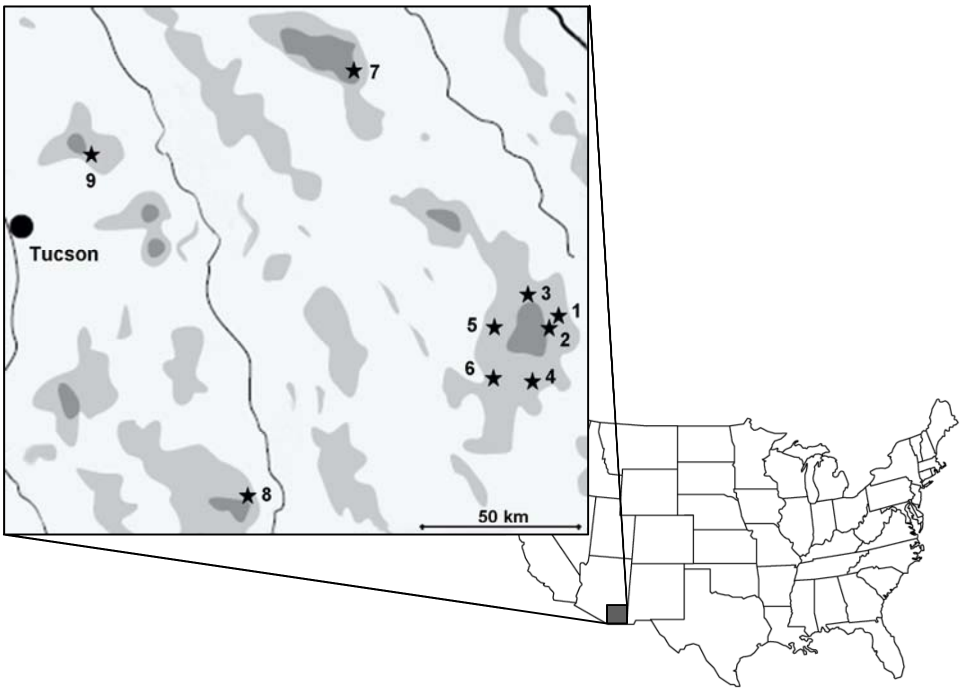

**Figure 1:** Collecting sites of *Hypoponera opacior* in Southeastern Arizona; (USA) 1 = Cave Creek (Chiricahua Mts, N 31° 52.867′, W 109° 11.682′); 2 = F-Plot close to SWRS (Chiricahua Mts, N 31° 52.000', W 109° 12.609'); 3 = Pinery Canyon (Chiricahua Mts, N 31° 57.089', W 109°18.595'); 4 = Price Canyon (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').

### *Microgeographic structure and behavioural observations*

The microgeographic genetic structure of *H. opacior* was investigated on a 6.0 m x 16.5 m area (F-plot) with a high nest density, which was located on the eastern slope of the Chiricahua Mountains (Figure 1; close to subpopulation 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 cm x 10 cm 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. With a subsample of 14 nests we conducted aggression tests in mid-August 2004. 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, and so far enough workers were present in a nest, each worker was used only once. 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 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. Matrices comparisons were conducted with the Mantel test (program: XLSTAT) (Mantel, 1967; Mantel and Valand, 1970).

### *Assortative mating and diploid males*

Inbreeding in Hymenopteran species with the typical complementary sex determination entails tremendous costs in the production of sterile diploid males. Therefore, we expected apterous males to avoid inbreeding by preferentially mating with less related virgin queens. In August 2004 and 2005, *H. opacior* nests, which were maintained in the laboratory at the Southwestern Research Station, were checked regularly for ongoing copulations. Copulations between apterous reproductives are infrequent but long-lasting, as mate guarding is typically for apterous males (Foitzik et al., 2002). To investigate mate choice, we removed mating pairs and stored them in 100 % ethanol. As a control, we removed in each case a randomly chosen intermorphic queen and an apterous male from the same nest and preserved them in 100 % ethanol. The caste of the female was determined under the stereo microscope, because apterous males sometimes accidentally try to copulate with sterile workers. For this analysis we genotyped 152 individuals at our five microsatellite loci. We only genotyped reproductives and assessed their unidirectional relatedness from male to female in both groups (mating pairs and nestmate controls) with the program RELATEDNESS 5.0.8 (Goodnight and Queller, 1994; Queller and Goodnight, 1989). The unidirectional relatedness values per nest were statistically compared with the t-test for dependant samples. Due to laboratory maintenance, apterous males, which emerged from the cocoon in the laboratory, were unable to disperse to other nests and were thus restricted to mate with intermorphic queens from the same nest.

As pointed out above, *H. opacior* might suffer severe costs from the production of diploid males caused by regular sib-mating between apterous reproductives. We attempted to estimate these costs by analysing the frequency of diploid males. For this purpose we genotyped 93 apterous and 18 alate males at all five microsatellite markers.

## Results

***General patterns of genetic diversity***

Our five microsatellite markers were highly variable for *H. opacior* with between 14 – 36 alleles per locus (Table 1). Observed heterozygosities per locus were generally high, yet lower than expected given the high number of alleles. Consequently, inbreeding coefficients ($F_{IS}$) were consistently positive, and overall loci strong inbreeding was detected ($F_{IS} = 0.447$, 95 % CI: 0.33 – 0.60). This finding is consistent with the intra-nest matings of the apterous reproductives. Furthermore, deviations from Hardy-Weinberg equilibrium were significant ($p < 0.00001$, for each of the five loci).

An estimation of null alleles, which could also cause heterozygote deficiencies, was significantly higher than the actually missing amplification products in our samples for each of the five loci (Chi square test for each locus separately: $p < 0.00001$, respectively). Thus null alleles can not explain the high frequency of homozygotes; rather regular inbreeding appears to be the cause.

**Table 1:** Number of alleles ($N_A$), observed and expected heterozygosities ($H_O$, $H_E$) and the inbreeding coefficient ($F_{IS}$) after (Weir and Cockerham, 1984) for all five loci. Calculations were based on a data set of 630 diploid *H. opacior* individuals from 283 nests from the Chiricahua Mountains (AZ, USA).

| Locus | $N_A$ | $H_O$ | $H_E$ | $F_{IS}$ |
|---|---|---|---|---|
| HoP 26 | 24 | 0.462 | 0.848 | 0.456 |
| HoP 54 | 33 | 0.584 | 0.866 | 0.326 |
| HoP 58 | 14 | 0.188 | 0.810 | 0.768 |
| HoP 60 | 36 | 0.562 | 0.933 | 0.398 |
| HoP 64 | 26 | 0.614 | 0.864 | 0.289 |
| Over all loci | | | | 0.447 ± SE 0.08<br>(95 % CI: 0.33 – 0.60) |

All unidirectional- and symmetrically relatedness values between the different castes in *H. opacior* nests are given in Table 2. The relatively low relatedness values for Hymenopteran colonies of 0.20 can be explained by regular polygyny, multiple mating and polydomy in nests headed by intermorphic queens. Relatedness coefficients were generally higher in nests collected in August than in June and these differences were significant for queens and workers (Table 2).

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

| June | | | | August | | | | |
|---|---|---|---|---|---|---|---|---|
| $r_{xy}$ | N | r ± SE | CI 95 % | $r_{xy}$ | N | r ± SE | CI 95 % | P |
| Worker → alate queen | 9 | 0.299 ± 0.091 | ± 0.210 | Worker → intermorphic queen | 39 | 0.393 ± 0.943 | ± 0.087 | 0.272 |
| Alate queen → worker | 9 | 0.297 ± 0.078 | ± 0.180 | Intermorphic queen → worker | 39 | 0.367 ± 0.055 | ± 0.112 | 0.560 |
| Worker → alate male | 9 | 0.196 ± 0.082 | ± 0.189 | Worker → apterous male | 18 | 0.247 ± 0.047 | ± 0.100 | 0.338 |
| Alate male → worker | 9 | 0.278 ± 0.086 | ± 0.198 | Apterous male → worker | 18 | 0.416 ± 0.824 | ± 0.174 | 0.662 |
| Alate queen → alate male | 10 | 0.194 ± 0.059 | ± 0.147 | Intermorphic queen → apterous male | 30 | 0.332 ± 0.029 | ± 0.059 | 0.069 |
| Alate male → alate queen | 10 | 0.238 ± 0.093 | ± 0.211 | Apterous male → intermorphic queen | 30 | 0.452 ± 0.038 | ± 0.078 | 0.082 |
| Worker – worker | 8 | 0.205 ± 0.080 | ± 0.188 | Worker – worker | 57 | 0.416 ± 0.035 | ± 0.070 | **0.036** |
| Alate queen – alate queen | 9 | 0.244 ± 0.101 | ± 0.233 | Intermorphic queen – intermorphic queen | 40 | 0.489 ± 0.047 | ± 0.095 | **0.013** |

### *Macrogeographic scale*

Population genetic analysis revealed significant structure in *H. opacior* from Southeastern Arizona and as a rule restricted gene flow between the different subpopulations (Table 3). The estimated $F_{ST}$-value over all loci and subpopulations was 0.071 (95 % CI: 0.057 – 0.085). Over all nine study sites, geographic distance was significantly and positively associated with genetic differentiation (Figure 2; Mantel test: $r = 0.648$, $p < 0.0003$). However, this association was entirely due to subpopulations from the Chiricahua Mountains being genetically more similar.

**Table 3:** Genetic diversity at microsatellite markers of nine *H. opacior* subpopulations from four different mountain ranges in Southeastern Arizona.

| | HoP 26 | HoP 54 | HoP 58 | HoP 60 | HoP 64 |
|---|---|---|---|---|---|
| Chiricahua Mts. (Subpopulation 1; n = 30) | | | | | |
| $N_A$ | 10 | 18 | 10 | 21 | 14 |
| $N_P$ | 0 | 2 | 0 | 2 | 2 |
| $A_R$ | 8.62 | 16.19 | 8.99 | 17.30 | 11.54 |
| Ho | 0.467 | 0.586 | 0.200 | 0.567 | 0.567 |
| $H_E$ | 0.838 | 0.946 | 0.863 | 0.940 | 0.845 |
| Chiricahua Mts. (Subpopulation 2; n = 27) | | | | | |
| $N_A$ | 10 | 17 | 6 | 17 | 11 |
| $N_P$ | 2 | 1 | 0 | 0 | 0 |
| $A_R$ | 9.44 | 14.33 | 5.98 | 15.09 | 9.81 |
| Ho | 0.519 | 0.556 | 0.111 | 0.704 | 0.296 |
| $H_E$ | 0.884 | 0.908 | 0.813 | 0.933 | 0.832 |
| Chiricahua Mts. (Subpopulation 3; n = 61) | | | | | |
| $N_A$ | 7 | 14 | 6 | 19 | 9 |
| $N_P$ | 1 | 0 | 0 | 0 | 0 |
| $A_R$ | 5.95 | 12.01 | 5.42 | 15.76 | 8.53 |
| Ho | 0.258 | 0.484 | 0.032 | 0.710 | 0.548 |
| $H_E$ | 0.628 | 0.906 | 0.512 | 0.939 | 0.774 |
| Chiricahua Mts. (Subpopulation 4; n = 30) | | | | | |
| $N_A$ | 11 | 19 | 10 | 18 | 14 |
| $N_P$ | 2 | 1 | 0 | 2 | 0 |
| $A_R$ | 8.78 | 16.47 | 9.07 | 15.54 | 11.03 |
| Ho | 0.467 | 0.633 | 0.310 | 0.750 | 0.793 |
| $H_E$ | 0.819 | 0.947 | 0.869 | 0.934 | 0.791 |
| Chiricahua Mts. (Subpopulation 5; n = 29) | | | | | |
| $N_A$ | 9 | 11 | 10 | 19 | 6 |
| $N_P$ | 0 | 0 | 0 | 1 | 0 |
| $A_R$ | 7.75 | 9.95 | 8.61 | 15.32 | 5.70 |
| Ho | 0.286 | 0.577 | 0.276 | 0.448 | 0.552 |
| $H_E$ | 0.740 | 0.873 | 0.605 | 0.919 | 0.742 |

| | | | | | |
|---|---|---|---|---|---|
| Chiricahua Mts. (Subpopulation 6; n = 30) | | | | | |
| $N_A$ | 12 | 13 | 8 | 20 | 11 |
| $N_P$ | 1 | 0 | 0 | 0 | 2 |
| $A_R$ | 9.76 | 13.00 | 7.70 | 16.12 | 9.59 |
| Ho | 0.400 | 0.722 | 0.172 | 0.690 | 0.733 |
| $H_E$ | 0.829 | 0.900 | 0.927 | 0.930 | 0.830 |
| Pinaleno Mts (Subpopulation 7; n = 28) | | | | | |
| $N_A$ | 11 | 7 | 7 | 9 | 5 |
| $N_P$ | 1 | 0 | 1 | 0 | 0 |
| $A_R$ | 9.86 | 6.15 | 5.79 | 8.42 | 4.60 |
| Ho | 0.357 | 0.500 | 0.214 | 0.357 | 0.370 |
| $H_E$ | 0.837 | 0.634 | 0.493 | 0.808 | 0.458 |
| Huachuca Mts (Subpopulation 8; n = 34) | | | | | |
| $N_A$ | 11 | 14 | 7 | 13 | 11 |
| $N_P$ | 2 | 0 | 0 | 0 | 1 |
| $A_R$ | 8.85 | 11.69 | 6.10 | 9.33 | 8.96 |
| Ho | 0.618 | 0.576 | 0.152 | 0.485 | 0.500 |
| $H_E$ | 0.803 | 0.878 | 0.603 | 0.825 | 0.625 |
| Santa Catalina Mts (Subpopulation 9; n = 31) | | | | | |
| $N_A$ | 9 | 13 | 5 | 13 | 12 |
| $N_P$ | 0 | 0 | 0 | 0 | 1 |
| $A_R$ | 8.37 | 11.62 | 4.46 | 10.70 | 9.66 |
| Ho | 0.387 | 0.567 | 0.129 | 0.484 | 0.600 |
| $H_E$ | 0.864 | 0.898 | 0.294 | 0.864 | 0.763 |
| Total (Subpopulation 1-9; n = 270) | | | | | |
| $N_A$ | 24 | 27 | 15 | 32 | 26 |

$N_A$: total number of alleles; $N_P$: number of private alleles; $A_R$: allelic richness; $H_O$: observed heterozygosity; $H_E$: expected heterozygosity; n: number of analysed ant colonies.

**Table 4:** Results of Mantel tests (r- and p-value) on the relationship between the internest distance, symmetrical relatedness and aggression (number of bites during the behavioural tests). Significant p-values are given in bolt. Workers from thirteen larger nests from a 99 m² Chiricahua study plot were used.

| Groups | R | p |
|---|---|---|
| Distance – relatedness | - 0.270 | **0.014** |
| Distances – aggression | 0.215 | 0.057 |
| Relatedness – aggression | - 0.137 | 0.229 |

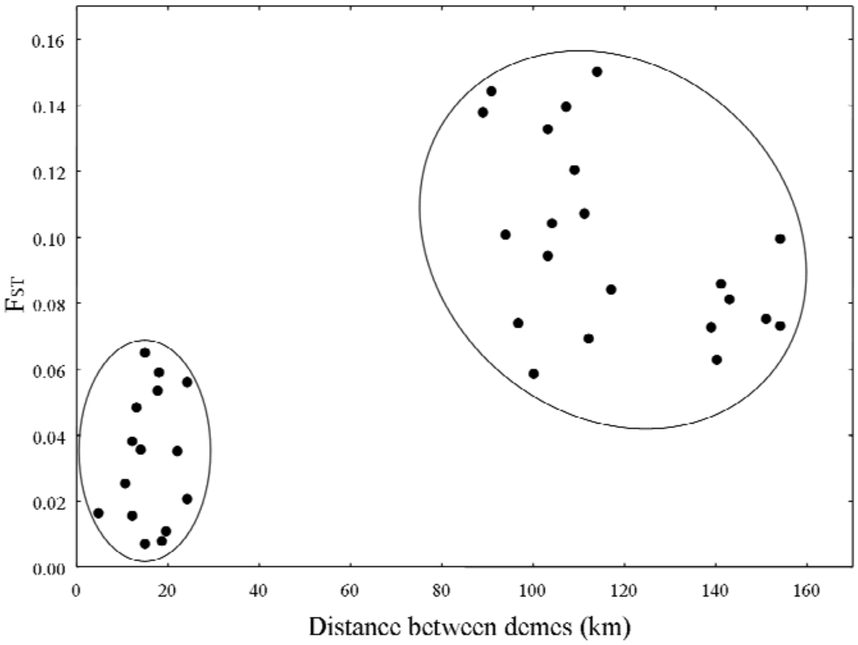

**Figure 2:** Isolation-by-distance in *Hypoponera opacior* from Southeastern Arizona. Positive association between geographical distance (km) and multilocus microsatellite estimates of pairwise differentiation between demes ($F_{ST}$; Mantel test: $r = 0.648$, $p < 0.0003$). Lower oval comprises pairwise comparisons between subpopulations within Chiricahua Mts. and the upper oval those between subpopulations from different mountain ranges.

In a second analysis, we concentrated on structure within the Chiricahua Mountains. Pairwise $F_{ST}$-values between the six demes showed a mean of 0.033 and ranged between 0.007 and 0.065. Only three of all possible comparisons were not significantly different from zero. The $F_{ST}$-values between Chiricahua Mountain subpopulations were significantly lower than those between subpopulations from different mountain ranges (t-test for independent samples: $n_{1,2} = 15, 21$, $t = 7.59$, $p < 0.00001$). Our study sites within the Chiricahua Mountains had a mean distance of 15.9 km (range: 4.6 km to 24.1 km), yet linear distance was not associated with genetic differentiation (Mantel test: $r = 0.079$, $p = 0.774$).

To investigate population genetic structure between the four different mountain ranges in more detail, we merged the genetic data from the different Chiricahua Mountains subpopulations into a single sample. Consequently, we found a mean pairwise $F_{ST}$-value of 0.099 between these four mountain ranges (range: 0.059 to 0.151) at a mean distance of 117.7 km (range: 89.0 km to 154.0 km) between these subpopulations. Surprisingly, in this data set, geographic distance and genetic differentiation were negatively correlated (Mantel test: $r = -0.886$, $p = 0.025$), indicating more distant sites to be genetically more similar. This negative association could not be detected, when we included a single, randomly chosen deme from the Chiricahua Mts. (for all six Mantel tests: $p > 0.05$).

### *Microgeographic scale and behavioural observations*

The microgeographic structure of *H. opacior* was investigated in a 99 m² plot from the eastern slope of the Chiricahua Mountains (Figure 1; close to subpopulation 2: F-plot). In total, we found 40 *H. opacior* nest chambers, i.e. nest density was high with 0.40 nests / m². We genotyped 102 workers from 30 nests (mean number of individuals per nest = 3.4, range: 1 to 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 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 Mts. Linear distance and pairwise symmetrical relatedness values were not associated (Mantel test: $r = -0.045$, $p = 0.351$).

To analyse behavioural colony boundaries, we conducted simple aggression tests for which we used a subsample of nests (N = 14). These nests were larger and contained at least ten workers. 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 nest mate 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 distance and symmetrical relatedness values between these larger nests (Table 4). Further, aggression (N of bites) slightly increased with distance between the nests. Finally, aggression and relatedness between nests were not associated.

### *Assortative mating and diploid males*

In order to elucidate inbreeding avoidance by apterous males we compared relatedness values between copulating pairs and non-mating controls from 13 nests in August 2004 and 2005, respectively. Relatedness values did not deviate from a normal distribution (Kolmogorov-Smirnov test: $p > 0.20$) and the results are given in Table 5. Interestingly, in

August 2004 males were copulating with females, which were significantly more closely related to them than randomly chosen males and intermorphic queens from the same nests (control). Yet, in the August 2005 data set, relatedness between mating pairs and controls did not differ. Overall, males obviously do not select mates according to their relatedness (Table 5).

**Table 5:** Mate choice in *H. opacior*. Comparison of the relatedness values between mating pairs of wingless reproductives (mating) and non-mating controls (control) from the same nest. Significant p-level for the t-test for dependant samples is given in bolt.

| | **Mating**<br>Male → queen<br>r ± 95 % CI, n | **Control**<br>Male → queen<br>r ± 95 % CI, n | T-test for dependant samples |
|---|---|---|---|
| August 2004 | 0.601 ± 0.194, 13 | 0.402 ± 0.194, 13 | T = 3.410, **p = 0.005** |
| August 2005 | 0.363 ± 0.205, 13 | 0.408 ± 0.166, 13 | T = - 0.559, p = 0.586 |
| August 04 & 05 | 0.480 ± 0.138, 26 | 0.405 ± 0.119, 26 | T = 1.179, p = 0.250 |

Finally, our genotyping did not uncover a single diploid male among 111 *H. opacior* males investigated. All males were shown to be hemizygous at all of these highly variable genetic markers, indicating an absence or a very low frequency of adult diploid males in *H. opacior*.

## Discussion

This first genetic study on the population and colony structure of *Hypoponera* ants with their fascinating reproductive strategies revealed strong geographic structuring and high levels of inbreeding. Genetic structuring on a microgeographic scale was influenced by the reproductive tactic of apterous reproductives in *H. opacior* with their restricted dispersal abilities, intra-nest matings and regular polygyny and polydomy. Nevertheless, relatedness analysis demonstrate that nests have a distinct genetic composition different from that of surrounding nests and that, on a behavioural level, workers treat nest mates with less aggression than workers from neighbouring nests. In this aspect, *H. opacior*, which is native to Southeastern Arizona, differs from many tramp and invasive ant species with their typical unicolonial colony structure (Drescher et al., 2007; Giraud et al., 2002; Goodisman et al., 2007; Jaquiéry et al., 2005; Thomas et al., 2006). Furthermore, our intranest relatedness estimates varied between the seasons. Higher relatedness values in late summer indicates a higher number of queens contributing to the offspring emerging in June, which is supported by demographic analysis (Rüger and Foitzik, submitted). Our genetic analysis support observations that apterous reproductives are highly related. Moreover, males do not appear to select less related mating partners. Indeed our data from 2004 suggests that they might actively choose more closely related intermorphic queens for mating. Albeit our behavioural observations and genetic data demonstrated regular inbreeding, we uncovered no evidence for the production of diploid males. This indicates that *H. opacior* colonies either remove diploid male eggs or larvae at an early stage or more likely that *Hypoponera* ants use a sex determination mechanism other than the typical Hymenopteran complementary sex determination system (Cook, 1993).

Structuring (mean $F_{ST}$ of 0.10) was very pronounced between the different mountain ranges given the relatively short distances of around 100 km between sites. Comparable or lower genetic differentiation was shown for a number of ant species between subpopulations, which are separated by several hundred up to a thousand kilometres (Brandt et al., 2007; Sanetra and Crozier, 2003). Moreover, the $F_{ST}$-values between the six Chiricahua sites indicate moderate genetic differentiation (Hartl and Clark, 1997) between subpopulations, which were from 4 to 24 km apart. Indeed, significant restriction in gene flow was found in twelve of 15 possible pairings of Chiricahua subpopulations. Albeit we found such strong structuring, genetic differentiation in general did not increase with distance. Only subpopulations from within the Chiricahua Mountains were genetically more similar than demes from other mountain ranges. Yet, even within the mountain range of the Chiricahuas, linear distance could not explain genetic differentiation. This is supposedly due to the strong physical structuring of the area, with steep mountain slopes and unfavourable habitats both on the hot desert plains and on the cold boreal fir forests on the mountain tops. Air-line distance does not take into account physical migration barriers or the regular direction of the wind. *H. opacior* alates participate in mating flights in July (Rüger and Foitzik, submitted), during the North American Monsoon, when winds predominantly blow from a westerly direction in Southeastern Arizona (Chakraborty and

Krishnamurti, 2003). Yet, wind direction could not explain fixation indices between the four mountain ranges (data not shown). Hence passive transport of winged males or females is not responsible for the observed macrogeographic genetic structure.

Surprisingly air-line distance was negatively correlated to genetic distance between mountain ranges, when all subpopulations from the Chiricahuas were lumped into one. Analyses based on just a single, randomly chosen Chiricahuan subpopulation instead revealed the complete absence of isolation-by-distance. Taken together with the unexpectedly high allelic diversity in all studied subpopulations, extant populations of *H. opacior* in the "Sky Island region" potentially represent refuge populations from a very large ancestral population. Indeed, several climate changes since the Pleistocene strongly reduced the area with adequate habitat conditions for *H. opacior* in the Southwestern United States. During the late Wisconsinan (22,000 to 11,000 years before present), when the climate in this area was meseic and cooler, woodland communities spread across present desert sites at lower elevations, serving as a link between mountain ranges (Thompson and Anderson, 2000; Van Devender and Spaulding, 1979). Yet, about 11,000 years ago the climate became warmer and dryer. Desertification of the lowlands apparently forced species such as *H. opacior*, which are adapted to more humid conditions, to retreat to mountain slopes. Genetic data also support such a scenario for populations of the ridge-nosed rattlesnake *Crotalus willardi* from the Sky Island region (Holycross and Douglas, 2007). Our study species, *H. opacior*, has its centre of distribution in tropical Central America and the southern parts of the United States are the most northern parts of its range. For species at the ecological boundaries of their distribution, minor climate changes can have dramatic consequences. An alternative hypothesis to explain the current distribution of *H. opacior* in Southeastern Arizona would be that subpopulations populations were established through long distance dispersal over extended stretches of uninhabitable desert. Yet, this would have resulted in extreme genetic bottlenecks and a strong association between distance and genetic differentiation, assumptions, which can not be supported by our genetic data.

On a microgeographic scale, we found significant relatedness among nest mates and, on a behavioural level, recognition and aggression towards non-nestmates. Hence *H. opacior* populations in Southeastern Arizona have a multicolonial structure, which strongly differ from supercolonial species as the invasive Argentine ant *Linepithema humile* with its two unicolonial supercolonies, which cover a stretch of 6000 km along the shore of southwestern Europe. Workers of each supercolony are peacefully accepted in every nest throughout its range, but aggression is extremely high between both colonies (Giraud et al., 2002; Jaquiéry et al., 2005). Despite the general multicolonial nest structure in *H. opacior*, we found relatedness between nests to decrease with internest distance for larger nests. An increased relatedness among neighbouring nests, also known as population viscosity, is generally associated with low dispersal capabilities of founding queens (Hamilton, 1964; Pamilo, 1998). Population viscosity, the result of philopatry in queens or dependant founding of new colonies is typical for polygynous (several queens per nest) ant species (Crozier et al., 1984; Giraud et al., 2000; Pamilo, 1983). Correspondingly, the low, yet detectable viscosity at the fine scale in *H. opacior* can be explained by polydomy, polygyny and dependant colony foundation of apterous queens on foot, which are accompanied by workers (Foitzik et al., 2002; Rüger and Foitzik, submitted).

Clearly the main function of alate reproductives is long distance dispersal and discovery of new, unoccupied sites, while local exploitation appears to be more efficient by apterous reproductives. This dual reproductive strategy in *H. opacior*, with outcrossing alates in June and inbreeding apterous reproductives in August, 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 typically 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, which might be advantageous for colonizing new habitats, while selfing (inbreeding) conserve favourable gene combinations for the local habitat.

Our study further demonstrates a strong footprint of regular inbreeding in *H. opacior*. This was expected by our behavioural observations of habitual within-nest mating (Foitzik et al., 2002), but is unusual for Hymenopterans with single locus complementary sex determination, where the production of sterile, diploid males should incur high fitness costs (Cook, 1993; Crozier and Pamilo, 1996). Indeed, our analysis of mate choice by apterous males indicated that males either actively choose closely related mating partners or show random mating in respect to relatedness. Additional studies will demonstrate whether *H. opacior* males indeed under certain circumstances preferentially mate with close relatives as our 2004 data suggest. Active inbreeding was recently shown in a cichlid fish, where mating partners might benefit from a higher relatedness because it enhances cooperation

(Thunken et al., 2007) and is quite common in plants, where selfing prohibits the beak-up of locally adapted gene combinations (Goodwillie et al., 2005).

In respect to our analysis of relatedness between mating partners in *H. opacior* we have to note that our laboratory nests disallowed males to leave the mother nest and disperse. In dense nest aggregations of *H. opacior* nest chambers are often connected through subterranean tunnels (Foitzik et al., 2002). Apterous males, albeit equipped with a weakly sclerotised cuticule, can potentially migrate to neighbouring nests, where they could find less related mating partners. Nevertheless, our genetic data unambiguously demonstrate high rates of inbreeding. Indeed, the $F_{IS}$-values in *H. opacior* were only slightly lower than the highest values ever reported in ants, which were found in the ant *Cardiocondyla batesii* 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 relatively high allele numbers, which points towards multiple, or even frequent colonisation events of suitable habitats by alate queens. Although nests headed by a dealate queen were relatively rare (about 5 % of all nests), all colony types contributed to the production of alate sexuals, which emerge in late June and participate in nuptial flights after the onset of the North American Monsoon in July (Rüger and Foitzik, submitted).

Albeit inbreeding was exceedingly high, we did not detect adult diploid males. This might indicate a sex determination system in *Hypoponera* ants other than single locus complementary sex determination common for Hymenopterans. Indeed, the production of diploid males has been reported for more than 40 species of social Hymenoptera, and supported in these species complementary sex determination (van Wilgenburg et al., 2006). An in-depth investigation of the sex determination mechanism in *H. opacior* will be difficult as it demands laboratory breeding and has to be the focus of future studies.

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# Chapter IV

# Larval cannibalism and worker-induced separation of larvae in *Hypoponera* ants: a case of conflict over caste determination?

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# Abstract

Reproductive altruism and cooperative brood care are key characteristics of eusocial insects and reasons for their ecological success. Yet, Hymenopteran societies are also the stage for a multitude of intracolonial conflicts. Recently, a conflict between adult and larval colony members over caste fate was described and evidence for overt conflict was uncovered in several bee species. In theory, diploid larvae of many Hymenopteran species should experience strong fitness benefits, if they would be able to change their developmental pathway towards the queen caste. However, larval self-determination potential is low in most advanced eusocial Hymenopterans, because workers often control larval food intake and queen-worker caste dimorphisms are generally high. In the ant genus *Hypoponera*, larvae actively feed on food provided by workers and here we show extremely low queen-worker size differences in these ants: the lowest in *H. opacior,* where fertile wingless (intermorphic) queens weigh on average only 13 % more than workers. Thus, slightly better nutrition during development might change the fate of a *Hypoponera* larva from a completely sterile worker to a fertile queen. One possibility to obtain extra food for *Hypoponera* larvae with their well-developed mandibles would be to cannibalise adjacent larvae. Indeed, we observed frequently larval cannibalism in natural nests. Yet, adult workers apparently try to prohibit larval cannibalism by carefully separating larvae in the nest. Larvae, which were experimentally brought into close contact, were rapidly set apart. Workers further sorted larvae according to size and responded swiftly to decreasing food levels, by increasing interlarval distance. Still, an experimental manipulation of the larval cannibalism rate in *H. schauinslandi* failed to provide conclusive evidence for the link between larval cannibalism and caste development. Hence, further experiments are needed to determine whether the widespread larval cannibalism in *Hypoponera* and the untypical brood distribution can be explained by an overt caste conflict.

## Introduction

The tremendous success of eusocial Hymenopterans is based on cooperation between behaviourally and often also morphologically distinct female castes (Hölldobler and Wilson, 1990). Queens, the reproductive caste, generally gain direct fitness, while workers only obtain indirect fitness benefits from their altruistic behaviour. In the vast majority of Hymenopteran species, female morphological castes are determined through environmental factors during larval development (Wilson, 1971; Wheeler, 1986; Wheeler, 1994). Only very rarely, for example in hybrid zones of seed harvester ants, genetic predisposition prevails (Winter and Buschinger, 1986; Fraser et al., 2000; Julian et al., 2002; Helms Cahan and Keller, 2003; Volny and Gordon, 2002; Heinze and Buschinger, 1989). Caste determination leads to different developmental pathways of diploid larvae. Recent studies on eusocial bees clearly demonstrate differential gene expression in queen and worker larvae (Cnaani et al., 2000; Corona et al., 1999; Evans and Wheeler, 1999). Also in ants, different genes are expressed in adult ant queens and workers (Gräff et al., 2007), as could be expected seen the differences in morphology, behaviour and life expectancy. *Lasius niger* queens were found to show an overexpression of genes involved in somatic maintenance and reproductive division of labour.

In most ant species with environmental caste determination, totipotent diploid larvae that receive an above threshold quality or quantity of food develop into queens, while less well-nourished individuals will become workers (Wilson, 1971; Wheeler, 1986; Wheeler, 1994). Most ant larvae are unable to actively feed, but rely entirely on food and water provided via trophallaxis by adult nestmate workers. Hence, the primary decision whether an individual will develop into a worker or a queen is thus generally made by adult workers, which control larval provisioning (Hunt and Nalepa, 1994). However, ant larvae are able to actively signal their needs to workers through a hunger cue as observed in the fire ant *Solenopsis invicta* or via begging behaviour as in *Gnamptogenys striatula* (Cassill and Tschinkel, 1995; Cassill and Tschinkel, 1999; Kaptein et al., 2005). In the latter species acquiring extra food through begging might allow *G. striatula* larvae to manipulate their future reproductive options. Similarly, *Hypoponera* larvae might have more control over their food intake as they have well-developed mandibles and actively feed on prey delivered by workers. The tiny soil-dwelling *Hypoponera* ants are well-known for their diversity of morphological queen and male castes, both within and between species (Foitzik et al., 2002; Yamauchi et al., 1996; Yamauchi et al., 2001). In *H. opacior*, our main study species, we could recently demonstrate that alate queens and males develop from overwintered larvae and conduct mating flights in early summer (Rüger and Foitzik, submitted). In August / September, *H. opacior* intermorphic queens and wingless (ergatoid) males mate within the mother nest. They are raised from eggs laid in spring and early summer. The completely sterile workers emerge in both reproductive seasons. Hence, there are three potential developmental pathways for diploid larvae, and the question arises whether *Hypoponera* larvae try to control their caste fate.

Despite the high level of cooperation in eusocial insect societies, divergent genetic interests between colony members can lead to aggressive interactions and even the killing of nest mates (Bourke and Franks, 1995; Crozier and Pamilo, 1996). Intracolonial conflicts between adults over reproduction and sex allocation were intensely studied during the last three decades, and just recently a conflict between adult and larval colony members on caste determination was brought to our attention, based on kin selection (Nonacs and Tobin, 1992; Bourke and Ratnieks, 1999). The conflict over caste determination arises because diploid larvae that develop into queens instead of workers, can obtain higher fitness by own reproduction (Bourke and Ratnieks, 1999; Ratnieks, 2001; Wenseleers et al., 2003). Conversely, colony productivity increases when a larger proportion of the diploid brood develops into workers instead of fertile queens, by which the inclusive fitness of adult workers will be higher by indirect fitness. The conflict over caste fate was thought to be especially intense, when workers and reproductives are reared simultaneously, when larvae have a high self-determination potential due to low queen-worker size dimorphism or when larvae have control over food intake (Bourke and Franks, 1999). Open conflict over caste fate was recently described in *Melipona* bees, where queens are worker-sized and develop in identical, similarly provisioned cells as workers. In *M. beecheii* roughly 5 - 25 % of the diploid larvae in natural colonies develop into queens and a large fraction of these superfluous queens are killed upon emergence by worker sisters (Ratnieks and Wenseleers, 2005; Moo-Valle et al., 2001; Wenseleers et al., 2004). In *Schwarziana quadripunctata*, another stingless bee, normal queens are larger than workers and are reared in special, larger cells on the comb periphery. Yet, in this species a second worker-sized queen caste evolved. These dwarf queens are reared in worker cells and end up heading more than one fifth of all colonies, albeit they do not have the full reproductive potential of normal-sized queens (Wenseleers et al., 2005). Instead of trying to develop into a small queen on low food provisioning, there are more ways to influence the caste fate. Hymenopteran larvae can take a more active role by influencing their food regime. In some stingless bees of the genera *Frieseomelitta* and *Leurotrigona* larvae can obtain more food during larval development by biting through cell walls and feeding on the provision mass of neighbouring cells (Ribeiro et al., 2006; Faustino et al., 2002). Theoretically, ant larvae could enhance their provisioning by feeding on nest mate larvae. In *Hypoponera* active cannibalism between larvae was observed. By their high head mobility, well-developed mandibles and active feeding behaviour of *Hypoponera* larvae may allow them to selfishly influence their caste development towards intermorphic queens which are only slightly larger than their complete sterile workers sisters. It is expected according their genetic interest that adult workers try to prohibit cannibalism among nest mate larvae and the production of superfluous intermorphic queens by securely separating larvae and controlling their food intake. In this study, we quantified caste differences and the occurrence of larval cannibalism in three *Hypoponera* species, documented the natural distribution of larvae in ant nests and the influence of workers on their distribution. Furthermore, we analysed experimentally the impact of food levels on larval distribution and finally on caste development.

## Material & Methods

### *Study system, ant collecting and rearing*

Three *Hypoponera* species were used in this study. Colonies of the Neotropical ant *H. opacior* were collected in the Chiricahua Mountains AZ, USA close to the “Southwestern Research Station” between 2003 and 2005. This native ant exhibits two distinct alternative reproductive tactics with both alate and apterous males and females (Foitzik et al., 2002). Colonies from the tramp ant *H. schauinslandi* (Emery) (formerly *H. bondroiti*, revised by (Seifert, 2004)) were decedents of a laboratory stock originally derived from ants found in the soil of a box of African rose chafers, which was sent from the “Aqua-Zoo Düsseldorf” (Germany) to a colleague of ours. This Tropical-cosmopolitan species occurs in buildings with stable heating conditions throughout Europe (Seifert, 2004) and exhibits alate and intermorphic queens. *H. schauinslandi* males are always wingless (ergatoid males), but two different morphs, “majors” and “minors”, were described (Yamauchi et al., 1996). The undescribed *H.* sp., colonies of which were collected at the "Tierpark Berlin Friedrichsfelde" (Germany) in 2003, produced during three year laboratory maintenance only apterous females and "minor" males. Although this species is morphologically very similar to *H. schauinslandi* it is genetically clearly separated based on sequence data from the mtDNA gene Cytochrome Oxidase I and II (Sequence divergence of 7.2 %, unpublished data). All three species live subterranean. *H. opacior* modifies pre-existing cavities in soil, favourably under large stones, while *H. schauinslandi* and *H.* sp. prefer to nest under and in rotten wood (Yamauchi et al., 1996; Foitzik et al., 2002).

Ant colonies were maintained in the laboratory in artificial nests in plastic boxes (200 mm x 100 mm x 58 mm). Two round nest chambers (20 mm diameter, 1 mm deep) were created in moisturized plaster and covered with a microscope slide. The bottom of the chamber was stained with black watercolour to achieve a better contrast to the whitish larvae. Ant nests were kept in a climatic chamber at 24 °C constant and a 12/12 h day and night rhythm or at room temperature. All colonies were fed ad libitum with living, wingless *Drosophila* sp. and cookie crumbs three times a week.

### *Castes dry weight*

Individuals of all castes from *H. opacior* and *H. schauinslandi* were killed by freezing at -20 °C. To measure differential investment in alate and apterous morphs, the samples were dried at 60 °C for 48 h and weighed subsequently with a micro-scale (Satorius).

### *Occurrence of cannibalism*

To analyse the occurrence of cannibalism between larvae in a standardized way we allowed close contact between two ant larvae in the absence of workers for six hours in two different positions: two larvae were positioned side-by-side on their two dorsal pairs of fleshy tubercles, so called "sticky doorknobs" (parallel position) or one larva was placed on the ventral side of the other (on-top position). The latter resembles the position of food, which is usually placed by workers on larva's ventral side in front of their head. As only the on-top position resulted in repeated cannibalism events, all further experiments were carried out by placing larvae in this position. In a second part of the experiment, we were interested in the influence of larval development on the frequency of cannibalism. Larvae were grouped into three different size classes: small (< 0.7 mm), medium (0.7-1.3 mm) and large (> 1.3 mm). The frequency of cannibalism was subsequently observed between larvae of the same size class. We decided to conduct all cannibalism experiments over six hours, to give larvae enough time for the attack, while remaining in good condition in the absence of food and care normally provided by workers. During the experiments larval pairs were regularly checked for the occurrence of cannibalism and their position. If a larva moved and was not anymore in the desired position, the position was restored. Finally, the pairs of larvae were controlled under a dissecting stereo microscope. We noted the occurrence of cannibalism when one larva was clearly feeding on the other and the victim larva was shrivelled and did not show the typical turgor of healthy larvae.

### *Larval distribution*

To investigate the distribution of *Hypoponera* larvae in undisturbed artificial ant nests, we took digital images of eleven *H. opacior* nests, which contained between 20 and 129 larvae and 14 *H. schauinslandi* nests with 8 to 26 larvae. In order to obtain precise measurements of the length and width of a larva and the distance between its mandibles and its nearest neighbour and the length and width of the neighbouring larva, distances were measured with "AnalySIS" V 2.11 (Soft-Imaging Software GmbH). Measurements were obtained with high accuracy; mean and standard error of ten repeated measurements of the length of the same *H. schauinslandi* larva was 1.28 mm ± 0.01 mm.

To analyse whether workers purposely cause the unusual larval distribution, we experimentally placed all larvae on a large brood pile. For these trials, we used artificially composed nests of *H. opacior* (N = 8) with five workers and 14 larvae and *H. schauinslandi* (N = 8) with eight workers and 20 larvae, each. The larvae were positioned on a pile in the centre of the nest chamber, before workers were reintroduced. As soon as the first worker discovered a larva, timing started. Digital images were taken after 0, 5, 10, 15, 30, 60, 120 and 1440 min and the distances between each larvae and its nearest neighbour were measured on the screen or as described above.

If cannibalistic behaviour of larvae is dependant on food levels, workers could react by varying inter-larval distances. To investigate whether workers react adaptively on differences in the larval provisioning rate, ten artificially composed *H. opacior* and ten *H. schauinslandi* nests were assigned to either of two treatments for four consecutive days. Half of the nests from each species in the "low food level group" (low) was fed with two

*Drosophila* sp. flies on day two and four, whereas the “high food level group” (high) received half a fly three times a day. Each nest contained 20 larvae and four workers, respectively. Nests were digitally photographed before the onset of the experiment, and at the end of each of the four days. The distances between each larvae and its nearest neighbour were measured with the “AnalySIS” system. We analysed the effects of the parameters “number of larvae per nest”, “point in time” and “low / high” on the distance to the nearest neighbouring larva. Therefore, we conducted a general linear model for each parameter separately and for all possible combinations. Non-significant predictors were removed sequentially until only significant ones remained.

### *Impact of cannibalism on caste fate*

Finally, we investigated in *H. schauinslandi* whether larvae, which were allowed to cannibalise other larvae, exhibit a higher probability to develop into reproductives in comparison to their non-cannibalistic sister larvae. By feeding on conspecifics larvae cannibalistic larvae received additional food, which under the expectation of a nutrition dependent caste determination could lead to a higher probability to reach the queen caste. Therefore, we artificially composed 20 nests with one intermorphic queen, ten workers and nine larvae (one small, five medium and three large; same size classes were used as described above). Adult individuals per nest were taken from the same stock nest and were marked with a yellow gloss paint dot, whereas larvae were chosen randomly. Ten nests were treated as the “cannibalistic group” and the other ten as the “control group”. In the “cannibalistic group” all larva from each nest were moved to a separate nest specific box with moistened plaster floor. Every larva was provisioned with a living larva, which was placed on their ventral side in on-top position, as described above. From now on, the lower larva had two hours to cannibalise the upper one. In cases where cannibalisation was not achieved at that time, we artificially perforated the upper larva with a tiny needle and placed it back in the on-top position. After in total six hours, all larvae were placed back into their source nests. The “control group” was similarly treated to induce similar stress through handling, except that they did not receive the possibility to feed on other larvae. Both treatments were repeated for four consecutive days. The demography of all nests was monitored every day, until the last larva had metamorphosed and emerged from its cocoon. All callows were frozen at –20 °C in individually marked Eppendorf caps. They were weighed with a micro-scale (Satorius) and their eye diameter was measured under a dissecting microscope. Finally, we dissected the callows to determine their ovary status.

### *Statistics*

In general, our data were not normally distributed. Consequently, we used non-parametric statistics. Abbreviations are as follows: Mann Whitney U test (MWU test), Spearman rank order correlation (SRC) and Chi square test ($\chi^2$ test). For multiple regression (MR) and general linear model (GLM) analyses, we tried to normalize skewed data with a square root transformation. Further, ANOVA and subsequent LSD post hoc test were applied. All statistical calculations were performed with the program Statistica 6.0 from StatSoft.

## Results

### *Castes dry weight*

Castes of *H. opacior* differed in their dry weight (ANOVA: $F_{(4,55)} = 73.36$, $p < 0.00001$; Figure 1), albeit the weight ranges of the three female castes overlapped to a certain extent. Alate queens, the largest female caste, weighted about 21 % more than intermorphic queens. These intermorphic queens in turn were about 13 % heavier than workers. Weight differences in our measurements might slightly underestimate differentiation between the two *H. opacior* queen castes, as most intermorphic queens were inseminated and had started ovary development, while alate queens were predominantly virgins at collection.

The dry weights of the five *H. schauinslandi* castes were significantly different from each other (ANOVA: $F_{(4,40)} = 59.22$, $p < 0.00001$; Figure 1). However, in this species we found no weight differences between dealate (alate queens, which shed their wings) and intermorphic queens (Fisher LSD post hoc test, $p < 0.25$). Dealate queens were 48 % and intermorphic queens 56 % heavier then workers. Alate males in *H. opacior* and major males in *H. schauinslandi*, respectively, were heavier then their ergatoid brother-castes (Figure 1). In *H. schauinslandi* queens of both castes, which were included in our measurements, had fully developed ovaries.

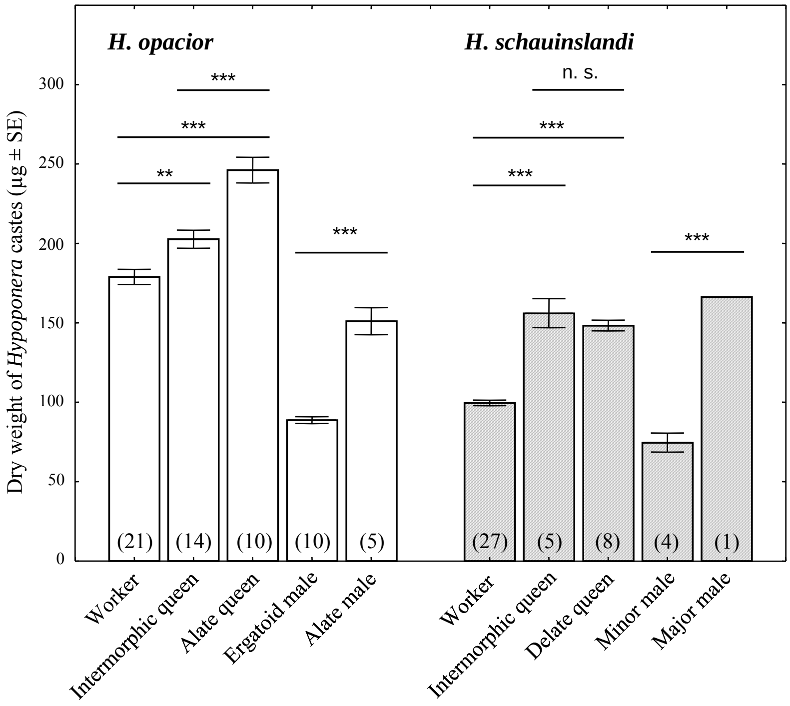


**Figure 1:** Dry weights (mean ± SE) of *H. opacior* and *H. schauinslandi* castes are given in µg. They are significantly different within both species (ANOVA: *H. o.*: $F_{(4,55)} = 73.36$, $p = 0.00001$; *H. s.*: $F_{(4, 40)} = 59.22$, $p < 0.00001$). Asterisks indicate the p-value of Fisher's LSD post hoc test, n. s. = not significant, ** = $p < 0.005$, *** = $p < 0.0001$.

### *Cannibalism frequencies in* Hypoponera *larvae*

Cannibalism was never observed when two larvae were placed side-by-side. In contrast, cannibalism regularly occurred in all three *Hypoponera* species, when two larvae were placed in the on-top position. Over all experiments, the proportion of cannibalism in *H. opacior* was 7.6 % and in *H. schauinslandi* 4.2 %. Cannibalism rates were not different from each other ($\chi^2 = 1.27$, df = 1, p = 0.26, n = 251; Table 1). Cannibalism was with 42.9 % exceptionally common in the undescribed *H.* sp. ($\chi^2$ tests: *H. o.* - *H.* sp., $p < 0.0001$; *H. s.* – *H.* sp., $p < 0.00001$). The frequency of cannibalism did not depend on larval size in *H. opacior* and *H. schauinslandi* ($\chi^2$ tests: $p > 0.05$). In every case of cannibalism the lower larva, which was positioned in a regular feeding position, attacked the upper larvae.

**Table 1:** Results of two trial series on the occurrence of cannibalism in *H. opacior*, *H. schauinslandi* and *H.* sp.. In the first trial series larval size was not taken into account, while in the second, larvae were grouped according to size: small (< 0.7 mm), medium (0.7-1.3 mm) and large (> 1.3 mm).

| Species | Trial series | Larval class | On-top trials | Cannibalism | |
|---|---|---|---|---|---|
| | | | N | N | % |
| ***H. opacior*** | 1 | | 25 | 6 | 24.0 |
| | 2 | Small | 58 | 1 | 1.7 |
| | | Medium | 33 | 2 | 6.1 |
| | | Large | 16 | 1 | 6.3 |
| | Σ | | 132 | 10 | 7.6 |
| ***H. schauinslandi*** | 1 | | 58 | 2 | 3.5 |
| | 2 | Small | 20 | 2 | 10.0 |
| | | Medium | 20 | 0 | 0.0 |
| | | Large | 21 | 1 | 4.8 |
| | Σ | | 119 | 5 | 4.2 |
| ***H.* sp.** | 1 | | 14 | 6 | 42.9 |

### *Distribution of brood in* Hypoponera *ant nests*

In all three *Hypoponera* species, larvae were clearly not positioned on a brood pile. Contrary to eggs and cocoons, they were noticeably separated from each other in the nest chamber. All eggs were placed on a pile and had direct contact to other eggs. Cocoons in natural nests of *Hypoponera* were also grouped together in three-dimensional chambers in the soil or in cavities in rotten wood (pers. obs.). Our measurements demonstrated a strong association between larval width and length (SRC: *H. opacior*: $p < 0.00001$; *H. schauinslandi*: $p < 0.00001$). Here, we present only the results for larval width, as width is probably more relevant for workers, which are responsible for larval sorting, as they grab larvae laterally for transport and may assess their size by their weight and/or the mandible opening angel. Larval distribution was not random, but larvae were sorted according to width and inter-larval distances were positively correlated with larval width. In *H. opacior* and *H. schauinslandi* the mean distance between larval mandibles and the body surface of its nearest neighbour was 1.09 mm ± SE 0.02 mm (range: 0.11 – 6.39 mm) and 1.43 mm ± SE 0.08 mm (range: 0.13 – 6.03 mm), respectively. A typical larval distribution in an artificial *H. opacior* nest is shown in Figure 2. In each of the two species we found a significantly positive correlation between larval width and the width of its nearest neighbour overall nests (SRC: *H. opacior*: $r_s = 0.268$, $p < 0.00001$, $n = 755$; *H. schauinslandi*: $r_s = 0.501$, $p < 0.00001$, $n = 224$, respectively). However, we also noted strong differences between nests, ranging from highly significant positive correlations over slight positive tendencies to no correlations at all. Likewise, larval width and distance to its nearest neighbour were positively associated (SRC: *H. opacior*: $r_s = 0.171$, $p < 0.00001$, $n = 755$; *H. schauinslandi*: $r_s = 0.308$, $p < 0.00001$, $n = 224$). Again, between-nest variation was high.

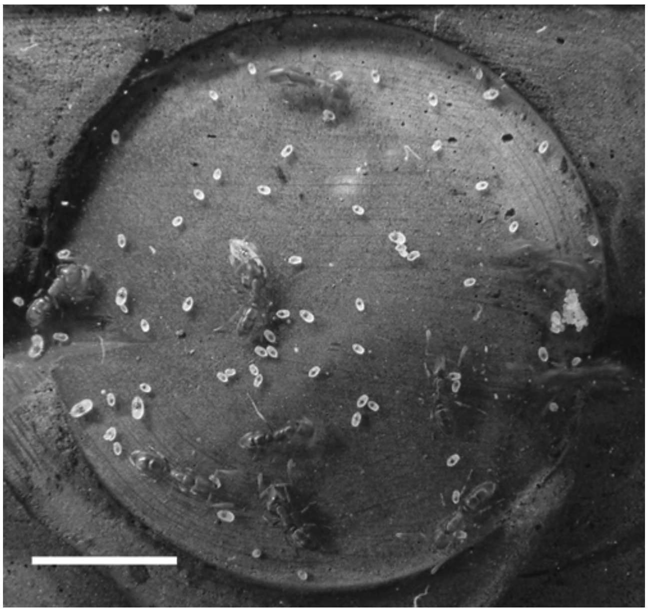


**Figure 2:** Typical larval distribution pattern in an undisturbed *H. opacior* laboratory nest. Workers redistribute the larvae on the entire nest surface to provide a minimum space between neighbours. Different to larvae, eggs are placed on a pile (above the right nest entrance). The scale bar is equivalent to 5 mm.

To investigate larval distribution and sorting in more detail, a multiple regression analysis was conducted to investigate the influences of three predictors: width of the first larva, number of larvae per nest and width of the second larva on the distance to the nearest neighbouring larvae. It turned out, that both the overall effect and the width of the first larva were significantly positive in both species. The number of larvae per nest was in both cases negatively associated, but only in *H. opacior* significantly so. In detail, we found in *H. opacior* a significant positive overall effect ($F_{(3,751)} = 30.065$, $R^2 = 0.107$, $p < 0.00001$), a significant positive influence of the width of the first larvae ($\beta = 0.131$, $p = 0.0004$), a significant negative influence of the number of larvae per nest ($\beta = -0.273$, $p < 0.00001$) and an insignificant value for the width of the second larva. For *H. schauinslandi* the overall effect was again significant positive ($F_{(3,220)} = 10.419$, $R^2 = 0.124$, $p < 0.00001$). The width of the first larvae was significant positive ($\beta = 0.156$, $p = 0.041$), the influence of the number of larvae per nest was not significant, but again negatively correlated ($\beta = -0.690$, $p = 0.309$) and the width of the neighbouring larvae was with $\beta = 0.211$ and $p = 0.006$ significant positive, too. Due to a high correlation between larval length and width, we obtained similar results for larval length (SRC: $r_s = 0.834$, $p < 0.00001$).

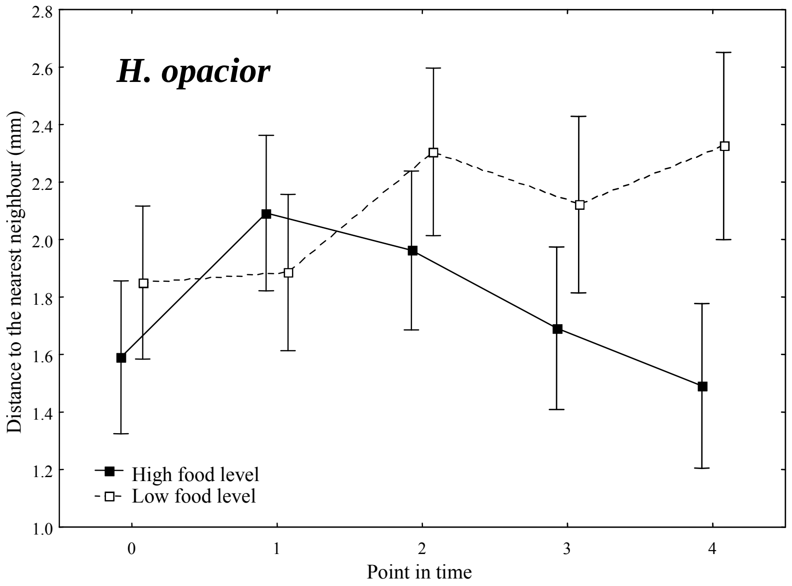
The r-values for spatial relationships were calculated after (Clark and Evans, 1954), where zero indicates a complete aggregation, one random distribution and 2.15 complete over dispersion. R for the larval distribution varied in *H. opacior* between 0.69 and 1.05 and in *H. schauinslandi* between 0.48 and 1.03.

Our experimental manipulation of larval distribution, where larvae were positioned on an artificial brood pile at the beginning of the experiment, resulted in a rapid redistribution of larvae by ant workers in all trials. Distance to the nearest neighbour increased rapidly with time within the next few hours (SRC over all colonies: *H. opacior* $r_s = 0.481$, $p < 0.00001$; *H. schauinslandi* $r_s = 0.425$, $p < 0.00001$; Table 2).

**Table 2:** Workers of *H. opacior* and *H. schauinslandi* actively redistribute larvae that were artificially positioned on a brood pile. The $r_s$ and p-values of the Spearman rank order correlations between time and distance of each larva to its nearest neighbour are given for each nest and over all nests, respectively.

| | *Hypoponera opacior* | | *Hypoponera schauinslandi* | |
|---|---|---|---|---|
| Nest # | $r_s$ | P | $r_s$ | P |
| 1 | 0.407 | <0.0001 | 0.386 | <0.00001 |
| 2 | 0.441 | <0.00001 | 0.554 | <0.00001 |
| 3 | 0.336 | <0.001 | 0.360 | <0.00001 |
| 4 | 0.532 | <0.00001 | 0.605 | <0.00001 |
| 5 | 0.302 | <0.003 | 0.484 | <0.00001 |
| 6 | 0.691 | <0.00001 | 0.379 | <0.00001 |
| 7 | 0.450 | <0.0001 | 0.424 | <0.00001 |
| 8 | 0.631 | <0.00001 | 0.362 | <0.00001 |
| Total | 0.481 | <0.00001 | 0.425 | <0.00001 |

Different food levels affected the distances between larvae and its nearest neighbour in *H. opacior* (GLM: $F_{(1,847)} = 51.6$, $p < 0.00001$; Figure 3) and distances between neighbouring larvae were smaller in the well-nourished group (MWU test: U = 78406.0, p = 0.001). In addition, the interaction between the food level treatment and the number of larvae per nest influenced inter-larval distances (GLM: $F_{(1,847)} = 46.5$, $p < 0.00001$). In contrast, in *H. schauinslandi* only time, the number of larvae per nest and the interaction between both factors were significant (GLM: $F_{(1,972)} = 11.4$, $p = 0.0008$; $F_{(1,972)} = 5.0$, $p = 0.03$; $F_{(1,972)} = 11.6$, $p = 0.0007$). During the experiment, the number of larvae per nest decreased. This was especially pronounced in *H. opacior* with a reduction of 33.3 % in the low food group and 13.5 % in the high food group. Larval dwindling was negligible in *H. schauinslandi* with only 4.0 % and 0.1 %, in the two food treatments respectively. Differences within the low and the high group were significant (MWU test: U = 29330.0, p < 0.00001; U = 36314.0, p = 0.00001).



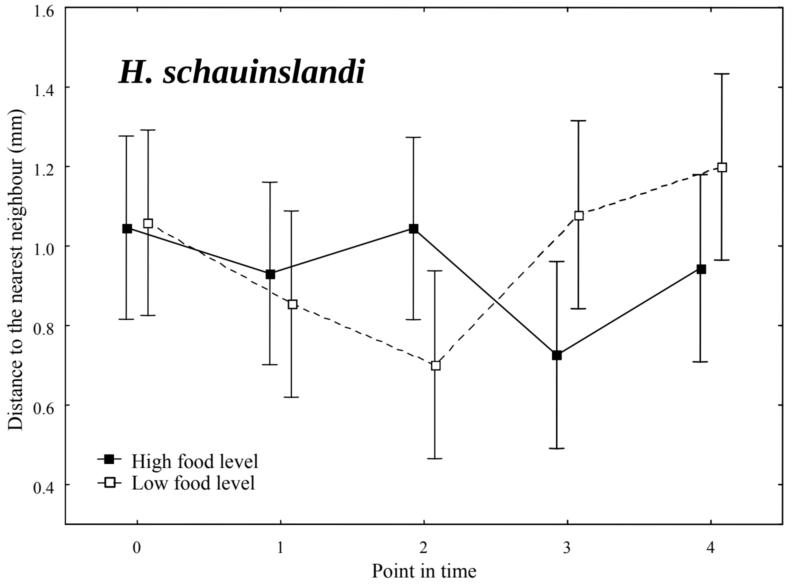


**Figure 3:** Distances to the nearest neighbour of ten *H. opacior* and *H. schauinslandi* nests, respectively, are given as means and 95 % CI during two different food level treatments on four consecutive days (0 = start situation, 1 – 4 = situation on the evening of each day). High food level colonies received food ad libitum and low food level colonies were kept under regular feeding conditions.

### *Experimentally induced cannibalism and caste fate*

More than seven percent of the larvae in the "cannibalistic group" cannibalised the offered larvae within the first two hours, yet over 75 % fed on the artificially perforated larvae during the following four hours. After 45 days (17.01. – 02.03.2006) all experimentally treated *H. schauinslandi* larva had emerged. We obtained 39 callows from 90 larvae in the "cannibalistic group" and 41 callows form 90 larvae in the "control group". Thus, a surprisingly low number of larvae managed to develop into adulthood. We did not find any differences between the callows in the "cannibalistic group" and in the "control group". Neither the weight (mean ± SE: 181 ± 6 µg, 178 ± 6 µg) nor the eye diameter (mean ± SE: 21.1 ± 0.76 µm, 20.5 ± 0.53 µm) were different (MWU test: U = 758, p = 0.69; U = 734, p = 0.53, $n_{1,\,2}$ = 39, 41). Correspondingly, all dissected callows contained no ovaries and were consequently workers. The duration of the pupal stage, between pupation and eclosion was with 25.1 ± 0.48 and 24.2 ± 0.53 (mean ± SE) days also not significantly different.

## Discussion

An open conflict on caste determination is expected in the small societies of *Hypoponera* ants, because of the high potential of the larvae to influence their own caste fate by active feeding behaviour, i.e. cannibalism, and the existence of intermorphic queens. Indeed, we found very low size dimorphisms between workers and reproductive females. The dry weight of intermorphic queens was only 13 % larger than that of sterile workers in *H. opacior*, and in *H. schauinslandi* both intermorphic and winged queens were about 50 % heavier than workers. These caste differences are very low compared to other ant subfamilies. In the myrmicine *Pheidole desertorum* gynes are more then three times heavier than major and 26 times heavier than minor workers (Helms, 1995). Similarly, in *Temnothorax nylanderi* virgin queens reach the four and a half-fold weight of workers (Foitzik and Heinze, 2000), and in the formicine *Lasius flavus* (Formicinae) virgin queens were 14.4 times heavier than workers (Steinmeyer, pers. comm.). Albeit dry weight differences vary considerably between taxa, most ant species exhibit strong queen-worker size dimorphisms. The exceptions are some ponerine ants with relatively low caste differences (Peeters, 1997).

The occurrence of cannibalism between sibling larvae in the context of a selfishly increased food intake was situation-dependent. No cannibalism was observed between two *Hypoponera* larvae positioned side-by-side. Possibly numerous spire-like tubercles and hairs that cover almost the entire body surface of *Hypoponera* larvae can serve as protection against cannibalism by fellow larvae (Wheeler, 1910). Yet, the frequent cannibalism observed in all three species in the on-top position is consistent with the finding that ponerine larva can be a menace for their sister larvae (Wheeler and Wheeler, 1979). Frequencies of eight and four percent in *H. opacior* and *H. schauinslandi* within six hours appear to be low just at first glance. But many larvae would be victimised during their larval development time of about two months in *H. opacior* and three weeks in *H. schauinslandi* (unpubl. data), when positioned on a regular brood pile with physical contact.

We found no variation in cannibalism rates between different larval developmental stages, although this may have been expected, due to a potentially higher benefit of extra food in critical developmental periods. Developmental pathways are fixed after so-called decision points and subsequently larvae are unable to further influence their caste fate (Wheeler, 2002; Hölldobler and Wilson, 1990). Yet, larvae in each of the three size categories were able and did pierce the integument of fellow larvae without the help of workers. Adult Hymenopterans cannibalise brood during periods of food shortage (Wilson, 1971). However, in our experiments *Hypoponera* ant colonies were fed adequately to sustain colony growth. Cannibalism rates varied between species and might be no general feature of this genus, as no cannibalism was reported from an Australian *Hypoponera* species, which also lacked intermorphic queens (Peeters and Hölldobler, 1992).

Larvae, which managed to develop into female reproductives can reproduce and gain direct instead of only indirect fitness benefits. Especially in *H. opacior* it appears highly beneficial to become an intermorphic queen as her intranidal mating and dependant foundation secures a high chance of successful nest establishment (Rüger and Foitzik submitted). Indeed, dissections of 64 young intermorphic queens from 16 *H. opacior* nests showed that all but one were inseminated and had developing eggs in their activated ovaries (unpubl. data). This is different from the bee *Schwarziana quadripunctata* where dwarf queens show a reduced fecundity and both dwarf and normal queens conduct risky mating flights (Wenseleers et al., 2005). Similarly in the ant species *H. schauinslandi* intermorphic queens, albeit of similar weight, laid fewer eggs and raised fewer larvae under controlled conditions than dealate queens. However, this effect levelled of during colony development (unpubl. data), so that it could be equally favourable for a diploid larvae to develop into either of the two reproductive morphs.

Whereas female larvae should behave selfishly and try to maximise their food intake to develop into reproductives, sterile adult workers, whose behaviour is selected mainly on the colony level, should have opposing interests (Bourke and Ratnieks, 1999). We observed that adult workers thoroughly separate larvae in the nest, supposedly to prevent larval cannibalism and consequently an overproduction of reproductive females. Indeed, we found that larvae in undisturbed laboratory nests of *H. opacior* and *H. schauinslandi* almost always have a minimum distance to its nearest neighbour. Additionally, larval size is positively correlated with the distance to the nearest neighbour and larvae are sorted by size. Workers do not exhibit this distinct sorting behaviour towards eggs and cocoons. Furthermore, they regularly checked egg piles for emerging first instars, which were immediately removed and redistributed in the nest. Rapid separation and redistribution of larvae from an artificially created pile started as soon as the larvae were discovered by workers. Distances between nearest neighbouring larvae increased swiftly with time in all trials with *H. opacior* and *H. schauinslandi*. The fast and sensible reaction of ant workers constantly adjusting larval spacing, suggests an adaptive nature of this behaviour. In some genera of stingless bees such as *Frieseomelitta* and *Leurotrigona* a selfish strategy of developing larvae was observed, which can be compared to larval cannibalism in *Hypoponera*. Instead of consuming nestmate larvae, they bite through the cell wall and feed on the provision mass of the neighbouring cell to bias their developmental pathway to become a queen. Similar to the spacing of larvae in *Hypoponera* nests by ant workers, bee workers normally try to prevent direct contact between cells by building small wax bridges. Only as an emergency queen rearing mechanism, workers build cells directly adjacent to others (Faustino et al., 2002; Ribeiro et al., 2006). In addition to the observed regular spacing patterns, *H. opacior* workers reacted to varying food levels. Distances between neighbouring larvae in the low food level group increased over time, indicating that hungry larvae are more prone to cannibalise their nest mates.

Sorting of larvae was also observed in the myrmicine ant *Temnothorax unifasciatus*, where workers arrange brood by size in two-dimensional concentric annuli (Franks and Sendova Franks, 1992). In contrast to *Hypoponera* larvae, *Temnothorax* larvae are fed by trophallaxis, and larval sorting was thought to ease systematic brood care through workers. Active cannibalism between these myrmicine larvae with their poorly developed mandibles is improbable, while cannibalism between *Hypoponera* larvae appears to impose sufficient costs to explain larval spacing. Hence brood sorting may have arisen independently in these unrelated genera as their brood care systems vary greatly.

We would have preferred to conduct our final experiment with several *Hypoponera* species, however at this point we had merely *H. schauinslandi* colonies available. Despite a slightly increased weight and eye diameter in eclosing individuals from the cannibalistic group, intermorphic or alate queens were produced in neither of the two treatments. The small, artificially created colonies were still in their growth phase and hence invested only in workers (Hölldobler and Wilson, 1990). Yet, if caste determination in *H. schauinslandi* is solely nutrition dependent, a diet supplemented through cannibalistic consumption of nestmate larvae should have changed the caste fate of developing larvae. Hence irrefutable evidence that larval cannibalism in *Hypoponera* ants should be explained by the selfish attempt of developing larvae to become queens is as yet missing. Nevertheless minor queen-worker caste differences, frequent larval cannibalism and the distinct brood sorting behaviour of workers all point to an overt conflict over caste determination in *Hypoponera*.

*Hypoponera* workers are completely sterile and thus all males are sons of the queen. For such a situation, under larval self-control of caste fate and the assumptions of Hardy-Weinberg with monogynous, monandrous colonies, 20 % of all totipotent female larvae should develop into queens (Ratnieks, 2001). The conditions in *Hypoponera* with their frequently polygynous colonies headed by intermorphic queens diverge from the assumptions of this model. This might lower the benefit for female larvae to develop into intermorphic queens, whereas the high success rate of dependant nest foundation might favour queen development. Whereas in *Melipona* bees, colonies pay costs due to selfish queen overproduction and killing (Koedam et al., 1995), *Hypoponera* colonies suffer through brood destruction. Yet in both cases selfish behaviour of larvae lead to a reduced inclusive fitness of adult colony members. All three *Hypoponera* species reproduce at least partially by budding, which reduces the size of the mother colony in the same way as the swarm founding behaviour of bees. But contrary to stingless bees, *Hypoponera* intermorphic queens and workers disperse on foot. Consequently, local resource competition (Clark, 1978; Pamilo, 1991) should reduce the relative fitness benefit of queens.

The equation $z^* = (1- R_f) / (1 + R_m)$ from (Wenseleers et al., 2003) originally developed for swarm-founding social Hymenoptera allows an even more precise calculation of the optimal proportion of diploid larvae developing into intermorphic queens ($z^*$) in *H. opacior*. We obtained life-for-life relatedness values to females ($R_f = 0.39$) and males ($R_m = 0.12$) from microsatellite data (Rüger and Foitzik, sumitted) and calculated accordingly $z^* = 0.55$. *H. opacior* has two reproductive periods per year (Rüger and Foitzik, submitted) and intermorphic queens eclose almost exclusively during late August. Consequently conflict over caste fate is expected to be strongest during the summer brood.

However, excess production of intermorphic queens in *Hypoponera*, especially in *H. opacior*, is not easy to detect. Typically, average nest sizes in *H. opacior* with fifteen workers and 1.5 intermorphic queens (Rüger and Foitzik, submitted) indicate, that the worker force, which accompanies an intermorphic queen, during budding is very small. In contrast, *Apis mellifera* colonies produce 15 – 25 queens and 150,000 workers per swarming season (Seeley, 1985; Winston, 1987). Repeated budding events in *H. opacior* through an overproduction of intermorphic queens might be the cause for the tiny nest size observed in the field. Clearly, additional work is needed here. We suggest focusing on the self-determination potential of female larvae in *H. opacior* before workers and reproductives are produced in August and on their female caste allocation at the time of eclosion.

Finally the context of caste fate conflict might provide an explanation for the exceptional larval morphology in *Hypoponera* and a few other ponerine genera (Wheeler, 1900; Wheeler and Wheeler, 1952; Taylor, 1967). A pair of fleshy tubercles on each of the 4th and 5th abdominal somites (Wheeler and Wheeler, 1971), the so called "doorknobs" (Peeters and Hölldobler, 1992), are used by workers to position larvae on the floor, wall or ceiling of the nest chambers, similar to the anchor-tipped hairs described from *Tetramorium* larvae (Wheeler and Wheeler, 1976). Various, yet not completely convincing ideas on the adaptive function of the "sticky doorknobs" in *Hypoponera* larvae were given. They could improve head and neck movement during feeding (Wheeler, 1900), or serve to keep the body surface from the humid substrate (Peeters and Hölldobler, 1992). Our study suggest that sticky doorknobs allows *Hypoponera* workers to securely separate larvae, in contrast to some other ponerine ants without these fleshy tubercles, where larvae are rather mobile. Clearly a higher mobility of *Hypoponera* larvae with their cannibalistic tendencies would cause the early death of many larvae. Hence, sticky doorknobs prevent larval movement and frequent cannibalism between sister larvae - for the good of the colony. If true, larvae might exhibit at the same developmental stage behavioural features (cannibalism) that are shaped by "selfish" individual selection, while selection on the colony level forced them to develop sticky doorknobs, which prohibit their cannibalistic tendencies together with larval sorting behaviour of the workers.

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# General Discussion

The seemingly unspectacular genus *Hypoponera* with its tiny ants, which belong to the primitive subfamily Ponerinae, shows probably the highest diversity of alternative reproductive strategies and tactics in both sexes within the family Formicidae. This peculiarity makes *Hypoponera* an ideal model genus for studies on the evolution of alternative tactics in male and female reproduction. However, to gain a deeper understanding which social and ecological conditions shape the reproductive behaviour and morphology, it is necessary to gather detailed knowledge on the behaviour, life history, ecology and sociogenetics of the focal species. In my dissertation I approached this important and complex evolutionary question in a Neotropical *Hypoponera* species, *H. opacior*. As mentioned in the introduction, scientific information on the large ant genus *Hypoponera*, with more than 100 species, is only slowly accumulating. My study was prompted by preceding work on *H. opacior*, which demonstrated two morphological different reproductive phenotypes in each sex (Foitzik et al., 2002). Winged (alate) and wingless (apterous) males and queens were reported from a population in Southeastern Arizona. Both queen morphs are associated with a certain social structure of the colonies they head. Once an alate queen has mated on the wing, she sheds her wings (now called dealate queen) and independently found a new colony (haplometrotic) mainly in areas with a low nest density. In contrast, apterous (intermorphic) queens are generally mated by apterous (ergatoid) brothers within the mother nest. New nests tend to be founded dependently by budding of young intermorphic queens (pleometrotic) with a part of the work force. This leads to areas with a very high nest density (Foitzik et al., 2002).

### *The seasonality of reproduction*

Despite this general picture, nothing was known on the timing of alate reproduction and the proximate causes influencing the production of the different reproductive morphs. Is the latter mediated by genetic, social or environmental factors? And how is the population structure of *H. opacior* affected by the dispersal abilities of alate and apterous morphs? Within many species, individuals exhibit diverse phenotypes despite an absence of genotypic variation, a phenomenon described by the term phenotypic plasticity. Evolution can either act stabilizing on the plasticity of the phenotype or can exploit its plasticity. As pointed out by Nijhout, (2003), “phenotypic plasticity is the primitive character state for most if not all traits” and therefore their development depend on variable and manifold internal and external influences. Reaction norms describe a continuous distribution of phenotypic morphs, while discrete phenotypes are called polyphenisms. In insects, developmental processes are generally mediated by various regulating mechanisms of hormone secretion, which in turn are influenced by a number of environmental factors (e.g. nutrition, temperature) (Nijhout, 2003). Other than in most non-social insects, where offspring receive almost no further attention after egg laying, larval development in (eu)social insects, like in ants, is additionally governed by workers, which tend and feed larvae until pupation.

My long-term study, including six field stays during the years 2003 to 2005 demonstrated that *H. opacior* in Southeastern Arizona possesses a bivoltine lifecycle with two offspring generations per year (CHAPTER 1). These two generations clearly differed from each other, albeit in both workers and reproductives were produced. The first generation emerged in late June and reproductives were predominately alates. Whereas in the second generation, which emerged in late August, reproductives were exclusively apterous.

Interestingly, the emergence of alate reproductives is strongly correlated with the onset of the North American Monsoon, a weather phenomenon, which constantly causes the by far highest precipitation rates in this area in the month of July (see CHAPTER 1, Figure 3). This leads to a higher air humidity, which provides favourable conditions for *H. opacior* mating flights and in addition moistens the soil. Overall, it results in a prospering nature, e.g. blooming and growing of plants and likewise in a richer fauna, providing a wealth of nutrition for the food chain. This improved provisioning may also support the second generation in the year, because it allows rapid larval growth. High food availability may explain the short developmental time of apterous compared to alate reproductives. Additionally, hibernation may trigger larval developmental pathways towards alate morphs, as it is the case in other ant species (Brian, 1955; Brian, 1975). Yet, *H. opacior* is a Neotropical species and hibernation may only play a role in the most northward parts of its range. In the ant *Cardiocondyla obscurior* alate males are produced under adverse conditions, however ergatoid males are constantly produced throughout the year (Cremer and Heinze, 2003). Contrary, alate reproductives of the seed-harvesting ant *Chelaner* sp. appeared after a period of sufficient rain, whereas brachypterous (short winged) reproductives were produced after drought, however this study is only based on two nests (Briese, 1983).

Polyphenisms such as the winged and wingless morphs of *H. opacior* have been described as adaptations to reliable and predictable variations in the environment (Nijhout, 2003). Interestingly, for polyphenisms is the inducing environment not the same as the selective environment. Whether the annual reproductive cycle in *H. opacior* from Southeastern Arizona is directly triggered by or adapted to the North American Monsoon remains to be investigated. This could be accomplished by a comparative field study, for example in the northern parts of Arizona or adjacent areas of Utah, Colorado or New Mexico, in which *H. opacior* has its most northward distribution. Contrary to Southeastern Arizona these areas experience no monsoon and show a relatively constant precipitation rate throughout the year. Of interest would also be a comparison with populations from Southern California, which experience the typical Mediterranean winter rains.

The production of dispersing and non-dispersing polyphenisms in separate seasons allows an optimal use of their complementary functions. Alates ensure genetic recombination by outbreeding and promote colonisation of new favourable habitat patches by dispersal on the wing. In contrast, apterous reproductives avoid all risky endeavours such as mating flights and solitary founding by within-nest matings and dependant founding by budding. Thereby they retain an existing genetic composition, which might provide advantages in the actual habitat patch, and allow its quick colonisation and exploitation. Such dual strategies may be evolutionarily favoured under reliable variations in ecology and were described in several animal (e.g. *H. nubatama* (Yamauchi et al., 2001) and *Technomyrmex albipes* (Tsuji et al., 1991; Yamauchi et al., 1991) and plant species (*Viola pubescens* (Culley, 2002)).

In a large number of ant species two queen morphs occur. Yet, morph differences are not necessarily as pronounced as in *H. opacior*, often the second morph is just an isometric reduced version of the regular alate queen, but typically, nests headed by these microgynes or macrogynes differ in their social organisation (Hölldobler and Wilson, 1977; Stille, 1996). This is the case in *Myrmica ruginodis* and in *Temnothorax rugatulus*, where microgynes abound in nests with several queens, while outbreeding macrogynes found new colonies independently and occur mainly in monogynous colonies (Elmes, 1991; Rüppell et al., 1998). By contrast, in *Solenopsis geminata* both queen morphs disperse, but still macrogynes found independently, while microgynes become queens by infiltration or adoption in established colonies (McInnes and Tschinkel, 1995). Microgynes somewhat resemble wingless queens in *H. opacior* as they show matings close to or within the mother nest and often are only able to found new nests with the help of workers. In most ant species with macrogynous queens, their nests show a higher relatedness and are monogynous. Different to the situation in *H. opacior*, macrogynes and microgynes are produced in the same reproductive period (yet, *S. geminata* produced macrogynes from May to September and microgynes from September to November (McInnes and Tschinkel, 1995)) and these alternative reproductive tactics are only found in queens, not in males.

My study confirms earlier findings by Foitzik et al. (2002), that queen morphs in *H. opacior* are associated with social nest organisation. Monogyny, monodomy and independent colony founding were again found for alate queens, which participate in nuptial flights, while intermorphic queens occurred in polygynous and polydomous nest aggregations. These fundamental differences have also been found between monogynous and polygynous ant species (Hölldobler and Wilson, 1977).

It is a general pattern in ants that alates are larger than apterous reproductives, implying that they require more nutrition during their development, that is, they are more costly to produce. Furthermore, these size differences of gynes give a good indication of their mode of colony founding. Gynes with high energy reserves found independently, while dependently founding queens do not rely on those (Keller and Passera, 1989). Although, size dimorphism between female reproductives in *Hypoponera* is by far not as pronounced as in other ant species, alate reproductives of both sexes of *H. opacior* and *H. schauinslandi* are still larger and heavier than apterous ones. Beside the costs, the differential reproductive potential of the two queen morphs is of evolutionary interest. For insects in general it has been suggested, that wingless individuals are more fecund than the respective winged form, since resources, which are usually used for the development of the flight apparatus can now be reallocated to reproduction (Groeters and Dingle, 1989; Roff, 1984; Roff, 1986; Roff and Fairbairn, 1991). This is not the case for both queen morphs of *H. opacior*, *H. schauinslandi* and *H.* sp., which have an identical ovariole number (3 + 3) and additionally, *H. schauinslandi* produced equal numbers of adult worker offspring (M.H. Rüger, unpubl.). Apart from their fertility, investment in intermorphic queens in *H. opacior* has a higher likelihood to pay off, as colonies headed by dealate queens are rare. Nevertheless, colonies invest regularly in alate reproductives to ensure outbreeding and dispersal. The proportional investment in the winged and wingless morphs is assumed to be a trade-off between the advantages of dispersal and the associated costs. The temporal and special habitat structure is often also important as habitat stability my promote winglessness and vice versa (Harrison, 1980; Heinze and Tsuji, 1995; Roff, 1986). A detailed analysis by Roff (1994) supported his hypothesis that flightlessness in insects is favoured in stable woodland sites and at high altitude or latitude (Roff, 1990). The first two points go along with the habitat preference of *H. opacior* in Southeastern Arizona, which nest in mountainous oak-juniper forests.

Sequence analysis of about 1450 base pairs of the mitochondrial Cytochrome Oxidase I and II region of dealate and intermorphic queens demonstrated that the alternative reproductive tactics in *H. opacior* are indeed exhibited within a single species (M.H. Rüger, unpubl.). Furthermore, I could document participation to sexual production in both seasons of all nest types irrespective of whether they were headed by an alate or one to several apterous queens. This consequently excludes a genetic morph determination and strongly supports the role of the environment on morph determination. Hence, the species *H. opacior* expresses a conditional strategy with two alternative tactics per sex (Gross, 1996).

### *Sex allocation*

The entirely different dispersal patterns of the reproductive morphs in *H. opacior* provide the rare opportunity to compare the impact of local factors on the sex allocation. While alates disperse, barely dispersing apterous reproductives compete with each other for mates and resources. Intermorphic queens may compete locally for rare nest sites, limited food or a restricted number of workers required for dependent founding (local resource competition, LRC) (Clark, 1978), while ergatoid males compete for mating partners (locale mate competition, LMC) (Alexander and Sherman, 1977; Hamilton, 1967). Theory predicts that LRC biases sex allocation towards males, whereas LMC acts in the opposite direction. Queen influence on sex allocation in *H. opacior* is probably weak, since over 80 % of reproductive offspring producing nests were queenless. *H. opacior* workers are sterile and have no ovaries. Consequently they lack the opportunity to directly bias the primary sex ratio by producing males. However, workers can increase their fitness biasing the sex ratio towards the more related sex. Indeed, microsatellite analyses demonstrate that workers are about three times more closely related to queens than to males. Yet, this relatedness asymmetry (relatedness of workers towards reproductive females divided by the relatedness of workers towards males) does not differ between June and August, which otherwise may have affected investment ratios. As found, relatedness values suggest a 3 : 1 investment in queens, if workers are able to bias sex allocation in their interest.

I found strong differences in sex allocation ratios between the two reproductive periods. In June, sexual investment was biased towards alate queens, while in August it was split into nests investing predominantly in intermorphic queens and others invested strongly in ergatoid males. On the population level, the proportional investment in queens was more male-biased in August then in June, albeit general inbreeding suggested local male competition in the former season. My data thus indicate that local selection pressures and competition among intermorphic queens are more pronounced than local mate competition. In contrast to the expectation under local mate competition, ant nest did not generally produce low ergatoid males numbers, but either invested only in just a single sex or strongly in males. The high number of single sex-brood suggests that either ergatoid males or intermorphic queens disperse on foot to mate. Indeed, ergatoid males in the laboratory were observed outside the nest chamber after eclosion of all pupae (pers. obs.). In the myrmicine ant *C. elegans*, which typically mates within-nests, foreign ergatoid males are not attacked by workers and dispersal of young queens and ergatoid males may lower negative effects of inbreeding as suggested by a recent genetic study (Lenoir et al., 2007).

### *Population structure and sociogenetics*

Behavioural field observations require typically huge endeavours, and for cryptic species like *H. opacior* they are sometimes unfeasible. However, indirect inferences on dispersal abilities, here for alate and apterous sexuals, respectively, can be drawn by thorough sampling and mapping in the field and subsequent genetic analysis. State of the art genetic markers are microsatellites (also called simple sequence repeat (SSR) or simple sequence length polymorphism (SSLP)). These are heritable small nuclear repeats, which are usually not under selection. I tested several primer pairs for microsatellite loci from other ponerine ants e.g. *Diacamma ceylonense* (Gopinath et al., 2001), *Gnamptogenys striatula* (Giraud et al., 1999) and *Platythyrea punctata* (Schilder et al., 1999). Unfortunately, neither of them was suitable for *H. opacior*, because they were either not polymorphic or did not yield amplification products. This may suggest that these ant genera are too distantly related to *H. opacior*. Consequently, I developed five highly polymorphic microsatellites for *H. opacior* (CHAPTER 2, (Rüger et al., 2005)).

The level of gene flow and consequently the dispersal abilities of individuals, or in the case of ants of reproductives, is reflected in the genetic composition of subpopulations (demes). An absence of structuring between demes can be traced back to high dispersal abilities and panmictic matings. The stronger the differentiation between the demes, the weaker the dispersal abilities of reproductives. In a genetic study, I investigated differentiation between nine different demes of *H. opacior* from four different adjacent mountain ranges (Sky Islands) of Southeastern Arizona (CHAPTER 3). The analyses included one deme each from the Huachuca Mts, the Santa Catalina Mts, the Pinaleno Mts and six demes from within the Chiricahua Mts.

In general, gene flow between all demes was restricted. Isolation by distance was significantly positive over all study sites, but was not present within the Chiricahua Mts and when single demes of the four Sky Islands were compared. This is due to a less pronounced substructuring between demes from within the Chiricahua Mts. This shows clearly, that dispersal between the Sky Islands is strongly restricted and that even within the Chiricahua Mts alate dispersal capabilities are by far not sufficient to maintain panmixia. The substructuring in *H. opacior* is compared to other ant species, and with respect to the relatively short spatial distance between the demes of this study, clearly pronounced (Brandt et al., 2007; Clémencet et al., 2005; Goropashnaya et al., 2004; Lenoir et al., 2007; Sanetra and Crozier, 2003). A potential explanation can be found in the climatic history of the Sky Island region. During the Pleistocene, the area was more humid and habitats favoured by *H. opacior* were not restricted to the mountainous regions as today, but instead covered also the flats, which in the present are inhospitable dry desert lands (Thompson and Anderson, 2000; Van Devender and Spaulding, 1979).

Microgeographic analysis revealed some evidence for population viscosity, i.e. an increased genetic similarity between neighbouring nests, which is generally found in species with highly restricted dispersal abilities. Internest relatedness and nest distance on a 99 $m^2$ plot were not associated for the entire sample, but in a smaller subsample of the larger nests. The latter comparison allowed also contrasting genetic and behavioural structure as I used these larger colonies to investigate behavioural colony boundaries. Aggression tests showed well-developed nest mate recognition, and a slight increase in aggression with internest distance. Overall, in addition to the finding of significant relatedness among nestmates, these results suggest a multicolonial nest structure and low population viscosity. Information from nuclear markers such as microsatellites also reflects dispersal abilities of males. Ergatoid males of *H. opacior* contrary to winged males in other ant species with non-dispersing queens do not facilitate long-range dispersal (Crozier et al., 1984; Sanetra and Crozier, 2003) and consequently, my finding partly diverge from an expected clear population viscosity. Additionally, I found high allele diversity within this plot what may be due to occasional independent nest foundations by dealate queens, however no such nests were sampled. As already mentioned above, it may be possible that apterous males or females disperse farther than previously assumed, either during the mating or the nest founding period.

*H. opacior* ergatoid males were expected to avoid inbreeding to reduce costs associated with the production of diploid males. Diploid males in Hymenoptera are generally sterile and occur when diploid individuals are homozygous at the sex locus (Cook, 1993). A genetic comparison between mating apterous reproductives and a control in August 2004 showed that males preferentially copulated with more closely related intermorphic queens. Yet, the repetition of this experiment in the consecutive year indicated random mating. Clearly, *H. opacior* males do not avoid inbreeding. Evolutionary it may make sense for ergatoid males to go for closer relatives, as this reinforces the effect of the dual strategy in *H. opacior*. In the absence of costs of inbreeding, ergatoid males would hence accumulate alleles, which were already successful in a local habitat patch. However, a generalisation is questionable as this active inbreeding was only found in one year. In addition, ergatoid males might be unable to accurately measure relatedness differences between potential mating partners as recognition is normally based on cuticular hydrocarbon profiles (Singer, 1998). Ergatoid males mate with young intermorphic queens, while these are still partially enclosed in the cocoon. Young ants during the first days after emergence and young slave making ant queens are known to be odourless (chemical insignificance) and do not exhibit a pronounced cuticular hydrocarbon signature (Lenoir et al., 2001). The same is true for cocooned pupae (Lenoir et al., 2001; Witte, pers. com.). Contrary, males of a Bethylidae species can recognize female cocoons by their unique hydrocarbon profile (Howard, 1992). Inbreeding may be not very costly in *H. opacior* as I could not detect diploid males by genotyping, despite regular brother-sister matings.

### *Conflict over caste determination*

Clonal societies may represent the only social situation, where inner-nest conflicts are expected to be entirely absent. But despite a very high inbreeding coefficient ($F_{IS} = 0.44$) *H. opacior* is far from being clonal. Relatedness between nestmates varied between 0.2 and

0.5. Hence, conflicts between nest-mates may still occur. Only queen-worker conflict over direct reproduction can be ruled out, as *Hypoponera* workers lack ovaries and are invariably sterile. However, this circumstance may emphasise larval selfishness as becoming a queen is their only option to gain direct fitness (Bourke and Ratnieks, 1999; Ratnieks, 2001; Wenseleers et al., 2003).

My weight analyse show that size differences in *H. opacior* and *H. schauinslandi* between workers and both queen morphs are small compared to other ant species. Moreover, it was documented that larvae are able to actively cannibalise other larvae in natural nests as well as during experiments. Assumed that caste determination in *Hypoponera* is nutrition dependent as suggested for other Formicidae (Wheeler, 1986; Wheeler, 1994), an increased food intake by cannibalism may lift worker destined larvae over a threshold and open the developmental pathway towards the queen morph. This two findings, small queen-worker size difference and larvae, which are in control of their food intake, meet the requirements by Bourke and Ratnieks (1999) and open the possibility for an overt conflict over caste determination between larvae and adult workers. It is possible that larval distribution maintained by workers, which prohibits physical contact between larvae, counteract larval cannibalistic tendencies. Workers separated larvae carefully and sorted larvae by size in both species and even extended the distances between starving *H. opacior* larvae (CHAPTER 4).

The presence of an overt caste conflict is nicely described for meliponine bees. In *Melipona beecheii* all larvae develop in identical, mass provisioned and sealed cells, and consequently queens, who have the same body mass as workers are by far overproduced. However, a large fraction of these virgin queens is killed by workers shortly after emergence (Moo-Valle et al., 2001; Ratnieks and Wenseleers, 2005; Wenseleers et al., 2004). Another stingless bee, *Schwarziana quadripunctata*, even produces in addition to “normal” queens, dwarf (worker-sized) queens, which are also able to head a colony on their own. But again, workers aggressively discriminated against dwarf queens (Wenseleers et al., 2005).

Unfortunately, the final experiment with *H. schauinslandi*, which allowed additional food intake by cannibalism in one group of larvae in comparison to a control group, failed to link larval cannibalism and caste development. Further experiments are necessary to elucidate if observed patterns are indeed explained by an overt conflict over caste determination in *Hypoponera*. It may be most promising to focus on *H. opacior*, where worker-queen size differences are smallest and the larval dispersal behaviour of workers was most pronounced. In this respect the period of larval growth from late spring to early summer when apterous sexual larvae develop may be especially interesting.

Alternatively, larval sorting may be seen in the context of brood care as it is described by Franks and Sendova Franks (1992) for *Temnothorax unifasciatus* and cannibalism may occur just accidentally as larvae feed on every item in their reach.

### *Further directions*

As mentioned before, a promising approach would be to investigate how the annual reproductive cycle in *H. opacior* is shaped by annual variation in precipitation and temperature. This would include comparing the timing of reproduction between populations from various climate zones. Sex allocation studies indicate that local resource competition is rather important and follow-up studies could analyse how queens compete for workers and how sterile worker decide which queen to follow. In addition the potential conflict over caste fate needs more attention, as pointed out above. Another logical and promising step is the establishment of a solid *Hypoponera* phylogeny in combination with the alternative reproductive strategies of the already investigated *Hypoponera* species to shed light on their evolution.

# Summary

Evolution ultimately leads to diversity, both within and between species and sexes. Reproductive strategies within a single sex are sometimes expressed as discrete alternative phenotypes, so called polyphenisms. Ants of the genus *Hypoponera* almost certainly exhibit the highest diversity of alternative reproductive strategies and tactics within the family Formicidae.

This long-term study revealed a bivoltine lifecycle (two reproductive periods per year) in a population of the ponerine ant species *Hypoponera opacior* from Southeastern Arizona. During the first reproductive period in late June, predominantly winged (alate) reproductives were produced, presumably from hibernated larvae. Contrary, in the second reproductive period in late August, exclusively wingless (apterous) reproductives emerged after a comparatively short developmental time. Sterile workers were produced during both reproductive periods. The emergence of alates was strongly correlated with the onset of the North American Monsoon, which cause the highest precipitation rates in this region. An increased humidity may be advantageous for the mating flight and dispersal of alates, as well as for colony founding. Alate production early in season is probably required as only dealate queens, who successfully raise the first workers before winter, will survive hibernation. Both male and female alternative phenotypes are not genetically determined in *H. opacior*. Demographic analyses revealed that nests headed by both dealate (dewinged) and apterous (intermorphic) queens invested in both phenotypes in the characteristic periods. This indicates that the observed phenotypes of queens and males are alternative tactics of a conditional strategy, which is environmentally mediated.

The two distinct queen morphs show apparent differences in mating behaviour and dispersal and head nests with a fundamentally different social structure. While alate reproductives exhibit an outbreeding mating behaviour, where males and females mate on the wing and thus ensure dispersal concurrently, apterous reproductives show an inbreeding mating behaviour, where copulations typically occur between siblings within the mother nest (intranidally). Interestingly apterous males show mate guarding behaviour and stay in copula with apterous queens, which are still partially enclosed in the cocoon. Following the dispersal flight, single dealate queens found new colonies independently, resulting in monogyny and unidomy of nests headed by this queen morph. In contrast,

intermorphic queens disperse on foot accompanied by workers and thus found new nests dependently. This mating and dispersal behaviour of intermorphic queens, lead to a polygynous, polydomous yet also multicolonial nest structure.

The tiny apterous reproductives have only low dispersal abilities, especially when compared to alates. Sex allocation in the wingless sexuals should therefore be strongly influenced by local selection pressures as males and females compete with relatives of the same sex for resources. Multi-year comparison in sex investment ratios between alate and apterous reproductives documented a male-biased allocation in apterous reproductives when compared to the alates. This high investment in males indicated that local mate competition is less intense than local resource competition among nestmate queens. Intermorphic queens compete mainly for workers, which go along with them during nest foundations and greatly increase the founding success of intermorphic queens.

A genetic approach on the basis of newly developed highly polymorphic microsatellites detected a strong substructuring between subpopulations of different mountain ranges of the Sky Islands and even between subpopulations in one of them, the Chiricahua Mts. However, isolation by distance was only present over all subpopulations and was due to a comparatively lower substructuring within the Chiricahua Mts. This pattern may be regarded as the outcome of post Pleistocene climatic changes, where reduced precipitation rates restricted a once widely distributed *H. opacior* population to the more favourable habitats at higher elevations. On the microgeographic scale only low population viscosity was detected. A subsample of larger nests, with which also aggression tests between workers were conducted, showed a correlation between inter-nest relatedness and distance. A decrease in relatedness between nests with distance is expected for founding queens with low dispersal capabilities. Albeit aggression decreased with distance, the behavioural data indicate strong nestmate recognition, suggesting a multicolonial population structure on a microgeographic level.

Ergatoid males, which mate with nestmate intermorphic queens within their mother colony, were expected to avoid inbreeding, assuming kin recognition abilities. A comparison of the relatedness between copulating pairs and nestmate controls, revealed the contrary in 2004. Males significantly selected more closely related partners, while random mating was detected the year after. Inbreeding avoidance by ergatoid males may be not that important as despite a high inbreeding coefficient no sterile diploid males were detected by microsatellite analysis.

Finally, behavioural observations showed that all larval stages actively cannibalise nestmate larvae. Further, larvae in natural nests and experiments were deliberately separated from one another by workers to avoid physical contact. Moreover, queen-worker size differences are small compared to other ant genera. These findings were interpreted and discussed in terms of the kin selected conflict over caste determination. However, a final experiment could not verify a conclusive connection between an enhanced nutrition by cannibalism and development towards the queen morph. Alternatively it is possible that the unusual larval sorting behaviour by workers could be regarded in the context of brood care and larval provisioning.

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