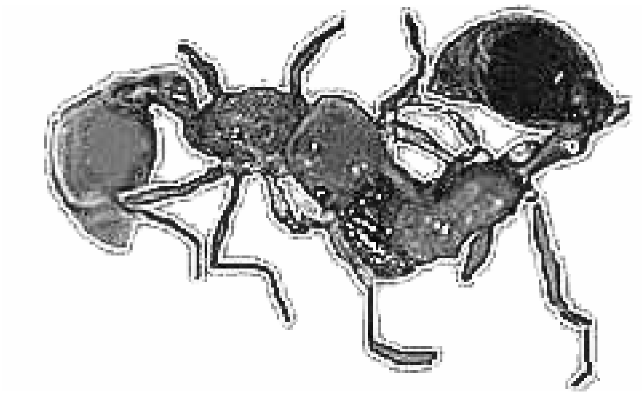
# Alternative reproductive tactics and their consequences in the ant genus *Cardiocondyla*



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# Alternative reproductive tactics and their consequences in the ant genus *Cardiocondyla*

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# GENERAL INTRODUCTION

“One of the greatest problems facing Darwin´s (1859) theory of evolution by natural selection concerned conspicuous male traits, (….). These and other extravagant male characters would seem to reduce survival, and so should be opposed by ordinary natural selection” (Andersson, 1994).

To solve the problem, Darwin (1871) developed his theory of sexual selection as a special case of natural selection, of which a key aspect is the competition between males over access to females, which can increase the variance in the reproductive success of individual males and thus the opportunity for sexual selection. As a consequence, alternative reproductive behaviours of males can evolve, for example, small, weak or young males that have low competitive abilities do better using completely different tactics. Frequently used alternatives are "sneaking" matings without paying the cost of fighting, or, mimicking females to avoid being attacked by territorial males (Andersson, 1994; Neff, 2001; Shuster and Wade, 1991a; Shuster and Wade, 2003).

The expression of alternative reproductive tactics is known from a variety of taxa, including mammals, fish, birds and arthropods (for reviews see Alcock, 1998; Andersson, 1994; Austad, 1984; Brockmann, 2001; Dominey, 1984; Gadgil, 1972; Neff, 2001). They are often accompanied by morphological correlated traits such as the development of weapons (e.g. mandibles in the coleopteran *Dendrobias mandibularis,* Goldsmith, 1987, forelegs in the thrips *Hoplothrips pedicularis*, Crespi, 1986, and cerci in the earwig *Forficula auricularia*, Eberhard and Gutierrez, 1991; see also below), and can be either genetically or environmentally determined.

In case of genetic polymorphism, the fitness of the different phenotypes is expected to be on average equal, otherwise, the most successful genotype would spread and replace the others (Dominey, 1984; Gadgil, 1972; Gross, 1996). The different strategies can be maintained for example due to environmental heterogeneity, accompanied by the occupation of different niches (Dominey, 1984). Most often, however, they are maintained by negative frequency dependent selection (Gadgil, 1972; Gross, 1996; Maynard Smith, 1982). Thus, male fitness depends on the frequency of rival male types, and each morph has a fitness advantage when rare (Alonzo and Warner, 2000; Henson and Warner, 1997). However, such alternative strategies are rare in nature but do exist e.g. in fish (Zimmerer and Kallman, 1989), a bird (Lank et al., 1995), lizards (Sinervo and Lively, 1996; Zamudio and Sinervo, 2000) and the marine isopod *Paracerceis sculpta.* In the latter, three phenotypes correlate with three alleles at a single autosomal locus. Big fighter males occur alongside of intermediate-sized males which mimic females and also, alongside of small males which "sneak" matings (Shuster and Wade, 1991b).

Conversely, alternative tactics within a conditional strategy are frequent in nature (Gross, 1996). Under the conditional strategy, individuals are genetically monomorphic. Theory predicts that the “decision", which tactic is expressed, is dependent on the status of the individual and will result in higher fitness for the individual (Gross, 1996). Individuals naturally differ in their status, e.g. because of environmental influences or because they differ in their developmental stages. The fitness of the alternative tactics are different, and are maintained by status-dependent selection (with or without frequency-dependent selection). A switch-point in status exists at which the fitness from the alternatives are equal (intermediate status; Repka and Gross, 1995). For example, individuals with a status above the switch-point will adopt a territorial tactic, whereas individuals with a status below the switch-point will adopt a sneaking tactic. By doing so each individual can maximise its fitness according to its status, even if this is assumed to be only the "best of a bad lot (job)" for sneaking individuals (Brockmann, 2001; Eberhard, 1982; Gross, 1996). However, as stated by Lee (2005), it is important to be aware that fitness may vary greatly within tactics and overlap between tactics.

In many cases (especially in vertebrates), the individuals switch between behavioural tactics according to their age or size class, for example in many young, small anuran males, which express satellite behaviour on the territories of older, larger males (e.g. Emlen, 1976; Sullivan, 1982; for a review on age specific behaviour see Caro and Bateson, 1986) or in several fish, in which up to four distinct tactics can be observed simultaneously according to different size classes (e.g. Taborsky et al., 1987). Conversely, in case discontinuous phenotypes are expressed as is the case in many insects, the morph is determined at a given time during development and tactics are irreversible and adopted for the whole lifetime. Then, for example, only individuals above the switch-point will express weapons, while individuals below the switch-point will go without weapons and/or develop wings to mate far away from "territorial" males. Examples are territorial and dispersing males in the ground nesting bee, *Perdita portalis,* and fighting and sneaking males in the dung beetle *Onthophagus taurus* as well as in the mite *Sancassania berlesei* (Danforth, 1991; Moczek and Emlen, 1999; Tomkins et al., 2004). It is important to notice that the switch-point is adjusted to ecology and demography, as e.g. to predation, sex ratio or density (Gross, 1996; Tomkins and Brown, 2004; Tomkins et al., 2004). For example in the mite *Caloglyphus berlesei*, individual choice of tactic is dependent on density as well as body size (Radwan, 1993).

In theory, a mixed strategy is also conceivable (Gross, 1996; Maynard Smith, 1982). It is suggested that genetic monomorphic individuals can use any tactic and that the choice of the tactic is purely probabilistic. All tactics have equal fitness and are maintained by frequency dependent selection. However, no support for the existence of a mixed strategy has been found so far.

Different types of alternative reproductive tactics - driven by natural selection - can be found mainly in insects, especially in the female sex, as an adaptation to different ecological conditions (Crespi, 1988; Harrison, 1980; Roff, 1986).

Most often, the polymorphism concerns dispersal, which is sometimes reflected phenotypically by a wing polymorphism (Roff, 1986; Roff and Fairbairn, 1991; for review of dispersal polymorphism see Zera and Denno, 1997). However, the development of wings does not necessarily mean that an individual is able to fly, as wing muscle reduction can occur together with long wings (see, e.g. long winged, flightless water striders; Kaitala, 1988). As the production and maintenance of a flight apparatus (especially flight muscles) is costly, natural selection can, under certain conditions, for example habitat stability, result in the loss of flight capability in favour of reproduction (Roff, 1990; Roff, 1994). In the cricket *Gryllus firmus*, a long winged morph coexists with an obligatory flightless short winged morph. In the latter, ovarian growth is greater during the first weeks of adulthood (Zera and Brink, 2000; Zera et al., 1997). Frequently, the production of long winged individuals is density dependent (Denno et al., 1991; Dixon, 1985) and in some species, the tactics expressed change regularly between generations due to environmental fluctuations, for example in waterfleas (Lynch, 1980).

In ants, dispersal polymorphism of females is often correlated with differences in colony founding strategies and linked with this, differences in queen size and colony social status: queens which disperse usually found colonies in an independent way (e.g. without the help of workers). After the mating flight, they shed their wings and use the voluminous flight muscles and fat reserves as an energy source for raising the first brood. Those colonies typically remain monogynous (one single reproductive queen). Conversely, queens which do not disperse are usually smaller and sometimes lack wings, as they seek adoption into established colonies after mating or even mate inside of the colony and consequently do not require significant energy resources (Bourke and Franks, 1995; Heinze and Keller, 2000; Heinze and Tsuji, 1995; Hölldobler and Wilson, 1990; Passera and Keller, 1990; Rüppell and Heinze, 1999; Stille, 1996). In such colonies, several queens reproduce together (polygyny). Polygyny and dependent colony founding has probably evolved due to a high risk of dispersal for solitary founding queens and/or high population densities close to saturation (Heinze and Tsuji, 1995).

This, and stable, uniform habitats (e.g. deserts), similar to non-social insects, can promote winglessness of queens (Heinze and Tsuji, 1995).

Differences in queen morphology can be found not only between monogynous and polygynous species, but also within species. In many cases, a pronounced polymorphism of two distinct queen classes can be seen, adapted to different dispersal and founding strategies (Heinze and Keller, 2000; Heinze and Tsuji, 1995; Rüppell and Heinze, 1999). Dimorphism can be either of size (e.g. in *Solenopsis*, McInnes and Tschinkel, 1995), or wing development (e.g. in *Plectroctena*, Villet, 1991), or even a combination of both (e.g. in *Lepthothorax*; for review see Heinze and Keller, 2000; Heinze and Tsuji, 1995).

Similarly, determination can be genetic or environmentally. In the ant *Harpagoxenus sublaevis* and *Leptothorax* sp A., a genetic basis for the queen morph has been demonstrated (Buschinger, 1978; Heinze, 1989; Winter and Buschinger, 1986). In *Technomyrmex albipes*, winged queens are replaced by wingless queens during the colony life cycle that suggests an environmental determination of queen morph (Yamauchi et al., 1991).

Alternative tactics in ant males are rare as mating is usually a short event, generally occurring in large swarms (Wilson, 1971), where males do not have the possibility to monopolize females. Thus, competition between males is usually low (Boomsma et al., 2005). However, in some species of the genera *Hypoponera* (Foitzik et al., 2002; Yamauchi et al., 1996), *Technomyr*mex (Yamauchi et al., 1991), *Formicoxenus* (Loiselle and Francoeur, 1988) and *Cardiocondyla* (Heinze, 1999; Heinze et al., 1999; Heinze et al., 1998), wingless, ergatomorphic males can be found beside winged males and is probably associated with the loss of between colony dispersal and pairing inside of the nest (Hölldobler and Wilson, 1990). Accompanied with this, competition between males is re-established, and in several species of *Cardiocondyla* and in *Hypoponera punctatissima* (Hamilton, 1979), ergatoid males fight each other to monopolize the females.

The study genus *Cardiocondyla* reveals both male and female polymorphism. *Cardiocondyla* belongs to the subfamily *Myrmicinae* and to date 48 species have been described. However, due to their small size, many *Cardiocondyla* species have probably been overlooked, and many more species are expected to be discovered (Seifert, 2003). Some have high invasive potential and thus belong to cosmopolitan tramp species (e.g. *C. obscurior, C. mauritanica, C. wroughtonii, C. emeryi, and C. minutior*). They are widely distributed around the tropics and subtropics, probably often passively distributed via human commerce (Seifert, 2003). Only a few workers together with some brood are able to establish a new, reproductive colony (Heinze et al., in press), which is typically polygynous. Other, non invasive species are restricted to Palearctic deserts, semideserts or dry steps (*C. ulijanini, C. elegans, C. batesii, C. nigra, C. sahlbergi, C. bicoronata*), and those of which queen number is known are monogynous (*C. elegans, C. batesii, C. nigra, C. ulijanini;* Seifert, 2003).

The species used for the investigations and experiments in this work were mainly *C. obscurior* and *C. batesii.* In addition, *C. minutior* and *C. nigra* were included in one project. Both tramp species (*C. obscurior* and *C. minutior)* were collected in Bahia, Brazil. *C. obscurior* lives in young coconut pods or in rolled lemon leaves and are therefore easy to collect (see Figure 1). *C. minutior* are, as *C. batesii* and *C. nigra*, soil-dwelling ants, their nests contain usually only a single very small nest entrance, of which a small duct leads to chambers in various depths up to 1.50 meters (Seifert, 2003, pers. observation). Consequently the detection of the nest entrance and the collection of a whole colony is difficult. *C. batesii* and *C. nigra* were collected in the surroundings of Granada, Spain and in the southern part of Cyprus, respectively (see Figure 1; further details to species characteristics are given below).

**Figure 1**. Nest sites of colonies of *C. obscurior*: coconut pods and a lemon tree leaf (left), and a typical habitat of *C. batesii* in Spain (right).

In all species of *Cardiocondyla*, ergatoid wingless males can be found instead or in addition to the normal typical winged males (e.g., all of the above mentioned tramp-species are male dimorphic except *C. mauritanica*, whereas in the monogynous species only ergatoid males exist). Phylogenetic data suggest that the ergatoid male morph evolved only once early in this genus (in addition to the "ancestral" winged males), but that the winged male morph has been lost independently several times (e.g., due to low probability to mate after dispersal; Boomsma et al., 2005; Heinze et al., 2005).

Ergatoid males are adapted to colonial life, they lack wings and ocelli, have reduced eyes and pigmentation, and their spermatogenesis continuous throughout their life. They are often aggressive and adaptations to fighting such as sabre-shaped mandibles (Figure 2) and the production of a propaganda substance can be seen in some species (*C. obscurior, C. wroughtonii,* ”*C. argentea*–group”; Heinze et al., 1998; Heinze et al., 1993; Heinze et al., 2005; Stuart et al., 1987; Yamauchi and Kawase, 1992; Yamauchi and Kinomura, 1993) . The males hold their competitors with their mandibles (Figure 2) and smear the rival male with the propaganda substance, which leads to aggression by the workers. In other species, males do not develop special "weapons", but are able to kill pupae or young callows of rival males with their worker-like mandibles (*C. minutior, C. emeryi, C. mauritanica;* Heinze et al., 1998; Heinze et al., 1993; Yamauchi and Kinomura, 1993). Consequently, ergatoid males occur either alone or with very few other ergatoid males in the colony (exception see below).

Contrary, the winged males are peaceful and adapted to dispersal by the expression of wings, large eyes and ocelli (Figure 2). However, winged males also mate inside of the nest before leaving the colony. To prevent being killed by their ergatoid rivals, it has been shown that winged males of *C. obscurior* mimic females chemically during the first days of their life (Cremer et al., 2002). Furthermore, recent studies have revealed that the male morph in *C. obscurior* is not determined genetically, but that "stress" conditions (e.g. drastic reduction of temperature or splitting of colonies into small subunits) can induce the production of winged males (Cremer and Heinze, 2003). The mechanism as well as the timing of morph determination were unknown at the beginning of the study and consequently constitute a central question of this work.

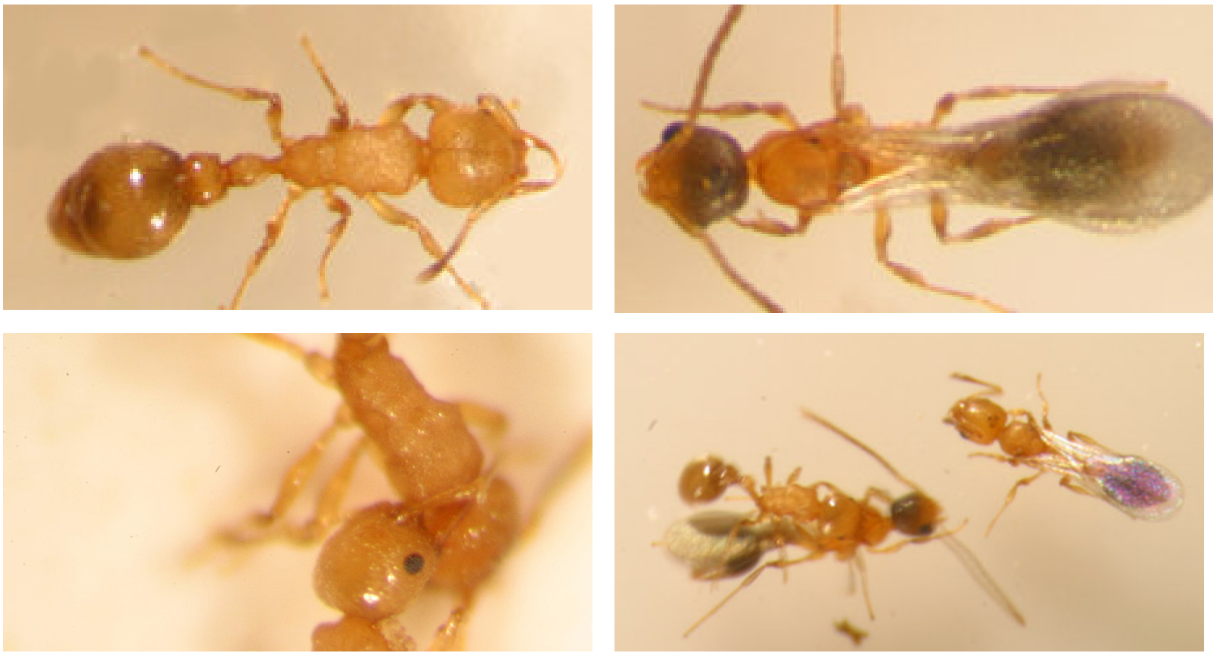


**Figure 2**. Ergatoid (top left) and winged male (top right) of *C. obscurior*, a fight between two ergatoid males (one male grasps his rival with its large mandibles; bottom left), and an ergatoid male, which tries to mate with a winged male instead of the virgin queen due to female mimicry of the winged male (bottom right).

Brood exchange experiments, behavioural observations and treatment with methoprene, a juvenile hormone analogue, revealed a significant role of juvenile hormone both for morph and caste determination in *C. obscurior*. Furthermore, workers, not the larvae themselves, react to changing environmental conditions and determine the morph of the male larvae at the end of the second (of three) instar by treating larvae differently (Chapter 1).

As the influence of workers on male morphs became obvious, it was especially important to compare the reproductive potential of the two male morphs, because, in the case that the “interests” of selfish males and the colony differ, there is a potential source of conflict, and individuals may try to manipulate each other. In order to investigate this, a fitness comparison of the different male morphs was conducted as a basis for future modelling (Chapter 2). Results suggest that males indeed should prefer to be winged, but that the colonies instead produce "cheaper" ergatoid males as "a living, long- term sperm-supply".

In monogynous *Cardiocondyla* species, as e.g. *C. batesii* and *C. nigra*, the winged morph has been lost and ergatoid males are peaceful and do not try to monopolize the females (Figure 3). The loss of aggressive behaviour is probably an adaptation to monogyny: accompanied with the single-queen colonies is a seasonal production of many females, so that one single male would not be sufficient to inseminate all females. Furthermore, all these species are probably inbred (see below) and thus competing males are highly related. Phylogenetic data revealed that monogyny is the derived state in the genus and evolved only once (Heinze et al., 2005). In at least some species from this monogynous clade, polymorphism in the female sex can be found: short winged queens occur alongside of "normal", long winged queens. To investigate how queens deal with the transition from polygyny to monogyny and whether the different queen morphs represent alternative reproductive tactics, behavioural and morphological investigations were conducted (Chapter 3).

**Figure 3**. Two ergatoid males together with a young queen (left) and a close up of a young queen and an ergatoid male of *C. batesii* (right).

It is obvious, that intranidal mating and, accompanied with this, the expression of alternative reproductive tactics and the territorial male morph in particular have extensive consequences for colonies of *Cardiocondyla* in various ways.

For example, sex ratio in *Cardiocondyla* has been shown to be adjusted to local mate competition (Cremer and Heinze, 2002) instead of relatedness asymmetry (as it was shown for many other ant species, Bourke and Franks, 1995), because related ergatoid males compete for females locally (within the colony). The theory of local mate competition predicts that only enough males should be produced to inseminate all females, and thus, sex ratio is strongly female-biased. Similar sex ratio adjustment to local mate competition is mainly known from non-social fig-wasps and parasitoids (Fellowes et al., 1999; Hamilton, 1967; Herre, 1987; Luck et al., 2001; West and Herre, 1998), but also from other ant species where mating inside of the nest is the rule (e.g. *Myrmoxenus kraussei,* Bourke and Franks, 1995; Winter and Buschinger, 1983).

As another consequence, inbreeding is expected to occur in *Cardiocondyla* in various degrees, depending on the number and relatedness of reproducing queens in the colonies (monogynous or polygynous colonies), the frequency of outbreeding (e.g., with alien winged males in male dimorphic species) and whether inbreeding avoidance between sexuals occurs or not. However, no concrete data has been made available to date. To investigate colony genetic relatedness and the extend of inbreeding, a population and colony genetic analysis was conducted in *C. batesii* (Chapter 4). It became clear that more than 80% of all matings in the obligate monogynous species are among siblings, and a similar number was also obtained for *C. nigra* (Schrempf and Heinze, unpubl.). Even in the case that more females reproduce together in one colony, relatedness and inbreeding in colonies is expected to be high, and in fact, preliminary data in the facultative polygynous and male dimorphic species *C. obscurior* have revealed a similar inbreeding coefficient to that in the above mentioned monogynous species.

The high levels of inbreeding are in turn expected to have consequences for the underlying sex determination system in *Cardiocondyla*. In Hymenoptera, the common mode of sex determination is parthenogenetic arrhentoky. Males develop from unfertilised eggs and are haploid, whereas females develop from fertilized eggs and are diploid (haplo-diploidy). The mechanism of sex determination in many hymenopteran species has been shown to be single-locus complementary sex determination (sl-CSD), where individuals, which are hemizygote at the sex determination locus are male, and individuals heterozygote at the sex determination locus are female. In case of a mating between individuals sharing the same alleles at the sex locus ("matched" mating), diploid males will be produced. Diploid males develop from fertilized eggs, which are homozygous at the sex determination locus. Usually, diploid males are sterile and a burden for the colony, as they do not work (Cook, 1993a; Cook, 1993b; Stouthamer et al., 1992; Whiting, 1943; but see Cowan and Stahlhut, 2004 for viable diploid males). Thus, several authors have suggested that single locus complementary sex determination cannot be the mechanism of sex determination in species, which inbreed regularly, because the probability of a matched mating is 50% in the case of a brother sister mating and thus diploid male production is drastically increased in inbred species (Figure 4).

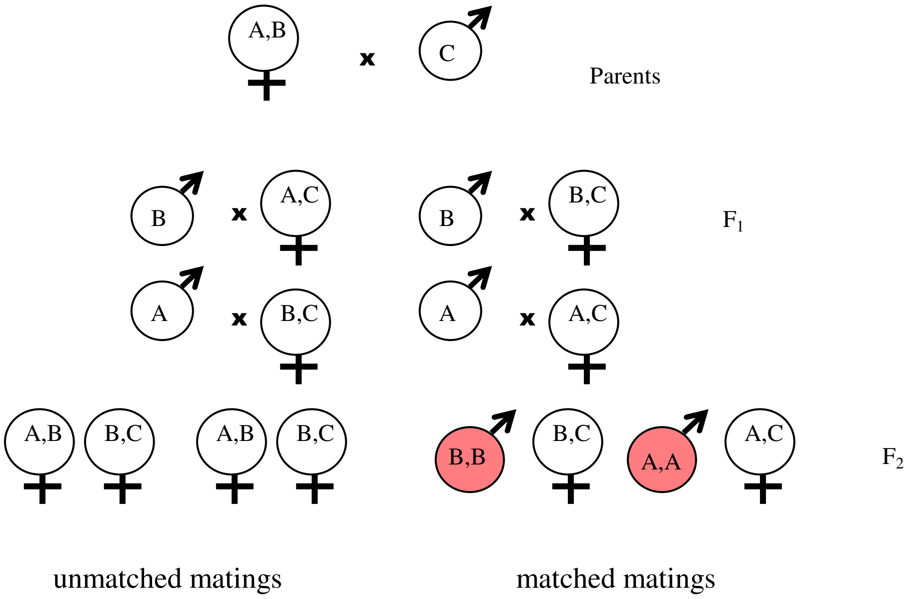


**Figure 4**. A diploid queen mates with a haploid male (parental generation). In case of a mating between a brother and a sister in the next generation ($F_1$) and under the assumption of single locus complementary sex determination, 50% of matings will be unmatched (left, three-allelic), but 50% of the matings will be matched (sharing one allele). 50% offspring of these matings will develop as diploid males (red; F2). In total, 25% of fertilized eggs will be male instead of female.

As the most probable alternative mechanism for inbred species, multiple locus complementary sex determination and genomic imprinting have been suggested (see Cook and Crozier, 1995 for review). Multiple locus complementary sex determination is simply an expansion of sl-CSD, and assumes that more than one locus is responsible for sex determination. Thus, more generations of inbreeding are necessary - dependent on the number of loci and alleles - to result in diploid male production, because all loci have to be homozygous. To date there are no examples of ml-CSD, but few experiments have been conducted so far. Genomic imprinting proposes that one or more loci are differentially imprinted in paternal versus maternal development. Unfertilized embryos contain the “maternal” imprint and result in males. Fertilized embryos contain the “paternal” imprint in addition and result in females

(Beukeboom, 1995; Poiré et al., 1993). Evidence for genomic imprinting was found in the regularly inbreeding wasp *Nasonia vitripennis* (Dobson and Tanouye, 1998).

In Hymenoptera, diploid males have often been reported as having larger body sizes (e.g. Périquet et al., 1993; Ross and Fletcher, 1985a) or wing hair cells (Grosch, 1945). In *Cardiocondyla*, no exceptional males had been found before the beginning of this study and detailed genetic studies have been lacking so far. Thus, Chapter 5 investigates possible mechanisms of sex determination in the ant *C. obscurior.*

Finally, the special life history of *Cardiocondyla* makes it a suitable system for testing the evolution of sexual conflict and cooperation in social insect societies, respectively. In the last years, there has been growing evidence that the reproductive interests of males and females frequently differ and therefore generate sexual conflict (Arnqvist and Rowe, 2002; Chapman et al., 2003; Rice, 2000). The main conflict between males and females arise as a result of female mating behaviour, which can lead to antagonistic traits, whereby males try to control female remating and boost their short term fecundity to gain maximum reproductive success. As a consequence, females have been shown to suffer increased mortality and thus decreased lifetime reproductive success in polygamous systems (e.g. in *Drosophila;* Chapman et al., 1995; Holland and Rice, 1999; Rice, 2000; Wolfner, 1997).

Only in monogamous species is cooperation instead of conflict between partners expected. Social insects are comparable with a monogamous system, as queens usually mate only during a short period of time early in their life and store the sperm afterwards in their spermatheca for the rest of their lives (exception termites, where the king and the queen stay together in the royal chamber). Even in the case that the female mates with more than one male during the short mating period, males can only gain maximum reproductive success if the females live for a long time, as colonies have to grow for a prolonged period of time before sexuals are produced. Thus boosting short term fecundity of females would not benefit male reproductive success. Males can try to hint females from remating, e. g., through the transfer of mating plugs (e.g. in *Bombus,* Baer et al., 2000; in *Solenopsis*, Mikheyev, 2002), but the influence of mating on longevity and female reproductive output is expected to have evolved towards a positive effect for females – the opposite to the effect observed in polygamous mating systems. As it is easy to raise sexuals and afterwards observe matings in the laboratory in *Cardiocondyla obscurior*, we have investigated the effect of mating on female longevity and lifetime reproductive output (Chapter 6).

## Summary: Aims of this thesis

Studies of the last years have provided important insights into male reproductive tactics in *Cardiocondyla obscurior* (Cremer, 2002). Nevertheless, detailed investigations on the proximate mechanism of male morph determination as well as concrete data concerning the reproductive potential of the different male morphs have been lacking. Thus, the chapters one and two deal with alternative reproductive tactics of males in *C. obscurior*.

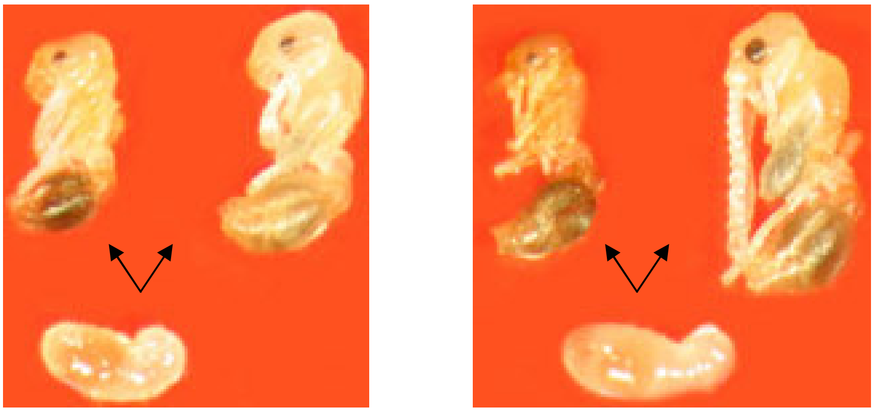
Contrary to male reproductive strategies, studies on female polymorphism in *Cardiocondyla* have been unavailable, despite the fact that the phenomenon has been frequently reported (Heinze et al., 2002; Marikovsky and Yakushkin, 1974; Seifert, 2003). Chapter 3 investigates whether wing polymorphism in females is correlated with alternative dispersal and founding strategies. Moreover, as monogyny is the derived state in *Cardiocondyla*, a comparison of different species clarifies how secondary monogynous queens deal with the transition from polygyny to monogyny.

In the following chapters, the consequences of alternative reproductive tactics on colony and population genetic structure as well as on the mechanism of sex determination have been examined.

Finally, the interactions between ergatoid males and females have been investigated in order to verify a theory of sexual cooperation.

# Proximate mechanisms of male morph determination in the ant *Cardiocondyla obscurior**

Alexandra Schrempf and Jürgen Heinze



Diploid larvae of *Cardiocondyla obscurior* can develop into worker and queen pupae (left), and haploid larvae can develop into ergatoid and winged males (right), respectively

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* Submitted to Evolution and Development

## ABSTRACT

The ant genus *Cardiocondyla* is characterized by an extraordinary male polymorphism, with winged disperser males and wingless, territorial ergatoid males. Winged males are produced only after the colony has experienced stressful environmental conditions, e.g., a drastic temperature decrease. We investigated the proximate basis of male polymorphism and caste dimorphism in *C. obscurior.* The critical stage for both morph and caste determination is the end of the second of three instars. Larval development as well as duration of the pupal stage are extended both in winged males and winged females and winged reproductives need on average 8.8 days longer for the development from egg to adult than wingless ergatoid males and workers. Treatment of first and second instar larvae with methoprene, a juvenile hormone analogue, led to the expression of the winged morph, suggesting an important role of juvenile hormone in both sexes. Though queens are produced year-round in contrast to winged males, the proximate basis of polymorphism seems to be the same in both sexes. Whereas the larvae themselves appear to be insensitive to the environmental changes, behavioural observations revealed that workers react to stress by changing their behaviour towards larvae and in this way trigger them to develop into winged males.

*Keywords*: polymorphism, male morph, caste, juvenile hormone, development, social insects, *Cardiocondyla*

## INTRODUCTION

The differentiation of female social Hymenoptera into wingless, largely or completely sterile workers and initially winged reproductive queens provides one of the most prominent examples of phenotypic plasticity (Hölldobler and Wilson, 1990). Caste polymorphism is essential for an efficient division of labour and thus one of the major causes for the great evolutionary success of bees, wasps, and ants. Male social Hymenoptera do not engage in social activities and their only task is inseminating young female reproductives. Due to their "limited" function and, in most species, the lack of overtly aggressive competition for access to females, males of social Hymenoptera rarely express different morphs.

The ant genus *Cardiocondyla* is one of the few social Hymenoptera with a pronounced male polymorphism (Heinze et al., 1998). Winged males resemble the males typical for other ant species and represent a peaceful disperser morph. In contrast, wingless, "ergatoid" males engage in lethal fighting and attempt to monopolize mating with all female sexuals eclosing in their nests (Kinomura and Yamauchi, 1987). Recent studies revealed an environmental determination of male morph: winged males of *C. obscurior* are produced only under "stressful" conditions, e.g., after a sudden 5°C temperature decrease or food shortage (Cremer and Heinze, 2003). The proximate mechanisms as well as the timing of morph determination are still unknown, but the morphology of gynandromorphs suggested similarities between the determination of male morph and female caste. Gynandromorphs are male on one side of the body and female on the other. In *C. emeryi* they either combined the characteristics of winged males and winged female sexuals or of ergatoid males and workers, but were never winged on only one body side (Heinze and Trenkle, 1997).

Caste differentiation in social Hymenoptera is generally controlled by the endocrine system, in particular juvenile hormone (Hartfelder and Emlen, 2005; Nijhout, 1994; Nijhout, 1999; Nijhout, 2003a). An environmentally induced increase of juvenile hormone titres in female larvae during a certain critical stage induces the development of female sexuals (Hartfelder and Rembold, 1991). Environmental stimuli may act on female larvae directly or indirectly via the behaviour of workers, which may control caste development, e.g., by providing the larvae differentially with food (Wheeler, 1986).

In this study, we investigated the role of juvenile hormone in both caste and male morph differentiation in *C. obscurior* and determined when in larval development differentiation occurs. Furthermore, we investigated whether stressful environmental conditions induce the development of winged males directly or indirectly through worker manipulation.

## MATERIALS AND METHODS

### *Study species, number and duration of larval stages*

Colonies of *C. obscurior* Wheeler, 1929 were collected in Bahia, Brazil in September 2000 and kept in the laboratory at 30°C / 25°C day / night (12hrs / 12hrs) temperature and light cycles. Colonies produce a few female sexuals and ergatoid males throughout the year, but switch to the production of winged males (in addition to a few ergatoid males and female sexuals) approximately six weeks after being fragmented into small parts or subjected to rapidly changed rearing temperature (Cremer and Heinze, 2003). Sexual and worker larvae cannot be distinguished easily from each other, as ergatoid males are of similar size as workers and winged reproductives are only slightly larger than workers. We randomly collected 123 larvae from ten colonies and also investigated 10 prepupae and eight larvae, which were known to represent winged male larvae from stressed colonies with virgin queens. Width of head capsule and length of the total body were measured with an ocular micrometer (magnification 80x). As adult ergatoid males have elongated, sabre-shaped mandibles, we also examined the shape of mandibles in the larvae. To estimate the duration of the larval stages, freshly laid eggs were transferred into orphaned worker colonies and reared by them. Brood development was recorded. *Cardiocondyla* workers do not have ovaries and therefore cannot produce brood.

### *Critical stage of male morph determination*

To determine the critical larval stage, until which environmental changes can influence male morph, we transferred first, second and small third instar larvae (at the beginning of the third instar) from their original colonies (donor colonies) into acceptor colonies, which were either "unstressed" or "stressed" by drastic reduction of worker numbers by splitting. Stressed colonies usually produce winged males (WMP colonies; Cremer and Heinze, 2003), whereas unstressed colonies, which did not experience such a "splitting" event, produce ergatoid males (EMP colonies). All experimental colonies (34 WMP and 36 EMP colonies; 10 donor colonies each and 24 and 26 acceptor colonies, respectively) were kept at normal temperature cycles (30°C / 25°C). We transferred 15 to 30 larvae of each larval stage from each of the WMP and EMP donor colonies into EMP and WMP acceptor colonies, from which the according larval stage had previously been removed. As a control, larvae of EMP colonies were transferred into EMP colonies and the other way round (n = 6 EMP for each larval stage, n = 6 WMP for each stage).

### *The controlling party of male morph determination*

To detect whether male morph was controlled by the larvae themselves or adult members of the colony (queens or workers), we uncoupled the rearing conditions for adults and larvae in the previously determined critical second larval stage in the following way. EMP larvae were reared either by workers together with queens or orphaned workers from WMP (stressed) colonies (n = 6, 15-50 larvae each). Larvae were allowed to stay in their nests and were not moved to ensure that they did not experience any kind of stress. Instead, workers were exchanged in daily intervals to secure that they always "felt stressed". As a control, we transferred workers of EMP colonies to larvae of EMP colonies and workers of WMP colonies to larvae of WMP colonies, respectively (n = 6). If the larvae themselves controlled their development, we would expect that all unstressed larvae developed into ergatoid males. If the stressed workers or queens decided the fate of the morph, winged males would be produced. Furthermore, if queens were important for the switch to winged male morph development, only ergatoid males should occur in queenless colonies.

### *Larval treatment by workers*

We conducted behavioural observations of WMP (n = 18) and EMP (n = 18) colonies to detect whether stressed and unstressed workers treat larvae differently. We observed three second instar larvae from each colony for 30 minutes each. For statistical analysis, observations of the three larvae were pooled and tests were conducted with the means of each colony. Experiments were conducted blindly, i.e., the observer did not know whether the colony represented a WMP or EMP colony.

### *Effect of methoprene on larval development*

Methoprene, a juvenile hormone analogue, was used to verify a possible role of juvenile hormone on male morph and female caste development. Methoprene was diluted in acetone (1µg / µl) and 2µl or 5µl of the solution were applied with a small pipette onto 20 larvae (unstressed conditions) of the three different instars each. As the larvae are very small, we could not prevent that a small amount of the solution was dropping down. Acetone was used as a control. Four replicates were carried out for each stage with the 2 µl treatment. Larvae were reared by workers and morph development was recorded for every single larvae.

# RESULTS

### *Number and duration of larval stages*

*C. obscurior* has three larval stages, which can be clearly separated by head capsule width and mandible shape. Body length cannot be used as an indicator of larval stage, morph, and sex. Only in the last larval instar, winged males can be distinguished from other larvae by reaching a larger size. Moreover, the difference in the length of prepupae indicates that there is a difference between young queen prepupae on one hand and worker and ergatoid male prepupae on the other hand (Figure 1). Mandibles are indistinct and soft in the first larval stage, more marked and white in the second larval stage, and distinct and of brownish colour in the last larval stage. Morph and sex cannot be distinguished by mandible shape.

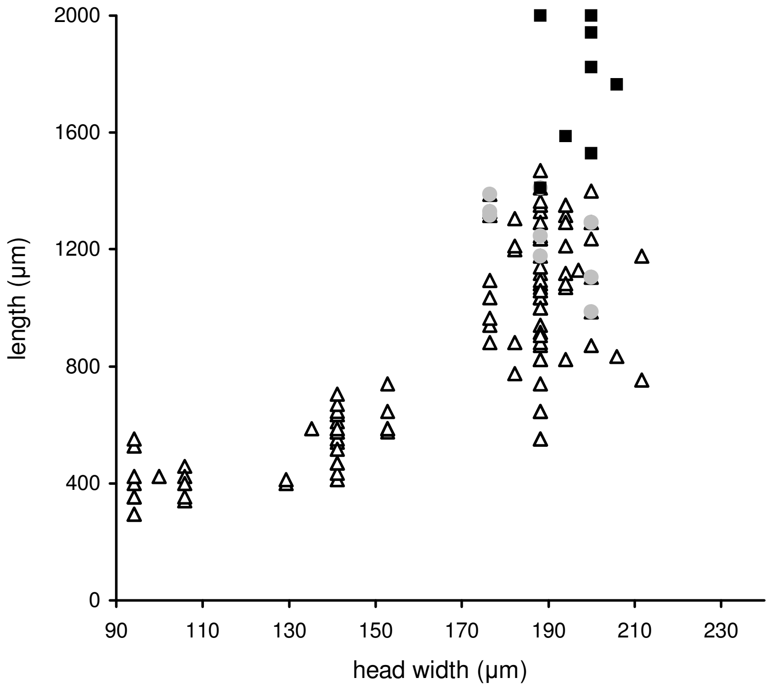

**Figure 1.** Head width and body length of larvae of the ant *Cardiocondyla obscurior*. Three different larval stages can be separated by head capsule width but not body length. Triangles represent larvae taken at random from their colonies; black rectangles represent larvae of winged males and grey circles prepupae.

Both winged male and queen larvae differed significantly in developmental time from worker and ergatoid male larvae. The developmental time from oviposition until the end of the prepupal stage was 16.9 ± S.D. 1.23 days for workers and ergatoid males and 21.9 ± 1.53 days for winged reproductives (n = 32; Mann Whitney U-test: U = 0.0, $p < 0.001$). Furthermore, the pupal stage was longer in winged reproductives (workers and ergatoid males: 4 ± 0.8 days; winged reproductives: 7.8 ± 1.6 days; U = 2.5, p < 0.001; Figure 2).

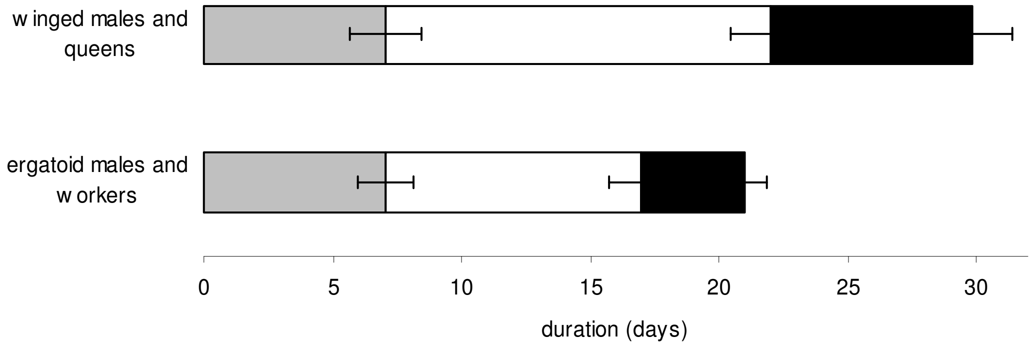

**Figure 2.** Developmental time (days) of male and female larvae of the different morphs reared at 30°C / 25°C temperature cycles. Mean duration and standard deviation of the egg stage (grey), larval stages (white) and pupal stage (black) are given.

### *Critical stage of male morph determination*

Male morph is not already determined during the first larval stage. In all cases, first instar larvae developed into the morph according to the environmental conditions they experienced in the acceptor colony. In the second instar, 43.75% of the male larvae could be "switched" to the opposite morph by transfer into the other environment, whereas others were already "fixed" and a switch was no longer possible. Therefore, male morphology appears to be determined during the second larval stage and probably at the end of this stage. From the early third larval instar on, morph development could no longer be influenced by changing the rearing environment, and all larvae except two developed as expected from the conditions they had experienced before the transfer. In two EMP acceptor colonies one ergatoid male each eclosed together with winged males from third instar WMP larvae (Table 1). However, this is explained by the observation that stressed colonies usually produce a few ergatoid males in addition to a large number of winged males. In all control set-ups larvae developed as expected, thus, they apparently were not stressed by the transfer itself.

### *The controlling party of morph determination*

In all six set-ups, in which male larvae from EMP donor colonies were reared by stressed workers and queens or stressed orphaned workers, first and early second instar larvae developed into winged male pupae, whether queens were present in the acceptor colonies or not. Thus, we conclude that workers control male morph determination. In all control colonies, the expected male morph developed.

**Table 1.** Results of a brood-exchange experiment, in which larvae of different stages from stressed donor colonies (WMP) were reared by workers from unstressed acceptor colonies (EMP) and vice versa (for details see text).

| | **WMP colonies acceptor** | **EMP colonies acceptor** |
|---|---|---|
| **First instar larvae, WMP donor**<br>(n=10; each 15 to 30 larvae) | | 10/10 ergatoid males |
| **First instar larvae, EMP donor**<br>(n=10; each 15 to 30 larvae) | 10/10 winged males | |
| **Second instar larvae, WMP donor**<br>(n=9; each 15 to 30 larvae) | | 5/9 winged and ergatoid males; 4/9 only ergatoid males |
| **Second instar larvae, EMP donor**<br>(n=7; each 15 to 30 larvae) | 4/7 winged and ergatoid males; 3/7 only winged males | |
| **Third instar larvae, WMP donor**<br>(n=7; each 15 to 30 larvae) | | 5/7 only winged males; 2/7 winged and ergatoid males (one single ergatoid male, three and four winged males) |
| **Third instar larvae, EMP donor**<br>(n=7; each 15 to 30 larvae) | 7/7 ergatoid males | |

### *Larval treatment by workers*

There was a significant difference in the way workers treated larvae in EMP and WMP colonies. We recorded antennation, biting (non-aggressive; larvae were treated with the mandibles of the workers, but they were not injured and did not reveal any signs of biting afterwards), carrying, allogrooming and feeding of larvae (by trophallaxis and solid food). Feeding of larvae was too rarely observed for a statistical analysis. Workers in WMP colonies antennated and bit the larvae more frequently than workers in EMP colonies. A similar trend was observed for allogrooming and carrying, however, the difference was not significant (Figure 3; MANOVA; $F = 4.44$, d.f. = 4, $p < 0.01$; Bonferroni-corrected post hoc comparison: antennation: $F=7.25$, $p < 0.01$; biting: $F = 8.98$, $p < 0.005$; carrying: $F = 1.07$, $p = 0.3$; allogrooming: $F = 0.16$, $p = 0.68$).

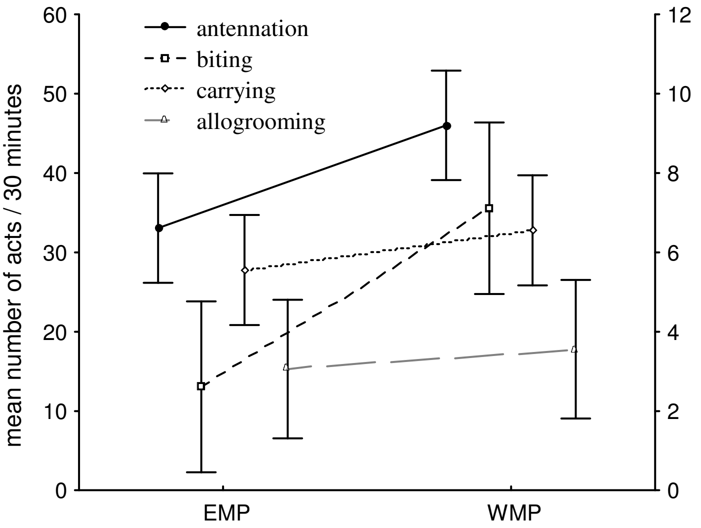

**Figure 3.** Behaviour of workers with regard to larval treatment. In "stressed" colonies, workers contacted larvae more often than in colonies, in which only ergatoid males were produced (number of antennations left y-axis; biting, carrying and allogroming right y-axis).

### *Effect of methoprene on larval development*

All larvae (first, second and third instar) treated with acetone grew and pupated into worker, queen or ergatoid male pupae. The larvae had a longer developmental time compared to untreated larvae, but were similar to larvae treated with acetone / methoprene (duration of larval stages incl. prepupal stage: minimum of 17 days).

None of the third stage larvae treated with 5 µl methoprene pupated, and they all discoloured and died after some days / weeks. Only 19 of 80 (23.75%) third instar larvae treated with 2 µl methoprene pupated. Of these, five large larvae (6.25%) that had already been at the end of this stage when treated developed as worker pupae shortly afterwards. Approximately two weeks later, 13 larvae (16.25%), which had been at the very beginning of the third larval stage when treated, developed into female sexuals. One larva (1.25%) developed into a winged male pupa.

All first and second instar larvae treated with methoprene grew and reached the third larval instar. Of the 200 larvae, 143 (71.5%) pupated, the rest discoloured and died. More larvae died after treatment with 5 µl acetone / methoprene (stage one: 5 / 20, 25%; stage two: 15 / 20, 75%) than after treatment with 2 µl (stage one: 9 / 80, 11.25%; stage two: 28 / 80, 35%). All pupating larvae developed into winged reproductives, independent of the amount of methoprene.

# DISCUSSION

Our experiments documented a number of striking similarities of the male morph and female caste differentiation in the ant *Cardiocondyla obscurior*, including an equally longer developmental time from egg to adult in winged sexuals and a similar impact of topical application of the juvenile hormone analogue methoprene. The extraordinary male polymorphism of this ant genus might therefore have the same proximate basis as queen-worker dimorphism.

Winged individuals in wing-dimorphic species usually have a longer developmental time than wingless morphs (Zera and Denno, 1997), but differentiation into reproductive and sterile individuals seems to be more complex in social insects. For example, developmental time in the ant *Myrmica rubra* is not different between winged queen and wingless worker larvae (Brian, 1965), whereas queen larvae have a longer developmental time in the ant *Tetraponera anthracina* (Terron, 1977). Similarly, developmental time of queen larvae of the bumblebee *Bombus terrestris* is longer than that of worker larvae (Cnaani et al., 2000), though queen larvae of the honeybee *Apis mellifera* develop more rapidly than workers and eclose several days earlier (Nijhout, 1994). Although of these differences, juvenile hormone appears to generally play an important role in both, wing-polymorphic species (e.g. in *Gryllus rubens*, Zera et al., 1989) and during caste differentiation in social insects (Nijhout 1999). Our results regarding the effect of methoprene on the development of male and female larvae of *Cardiocondyla* therefore are in accordance with data from other species.

The fate of male larvae was determined at the end of the second larval stage, and only one early third instar EMP larva could still be induced to develop into a winged adult by direct topical application of methoprene. The developmental programme in females is in agreement with that in males: treatment with methoprene allowed a switch to queen caste until the beginning of the third instar. Many methoprene-treated third instar larvae failed to pupate, presumably since a decline in the level of juvenile hormone is required for the pupal moult (Hartfelder, 2000; Riddiford, 1994).

Changing hormone levels can be triggered by varying environmental conditions, including changes in temperature (as in the butterfly *Bicyclus anynana;* Brakefield and Reitsma, 1991; Kooi and Brakefield, 1999), population density (the mite *Caloglyphus berlesei;* Radwan, 1993), photoperiod (the butterfly *Arashnia levana*; Koch and Brückmann, 1987) and diet quality (the dung beetle *Ontophagus taurus;* Emlen and Nijhout, 1999). In *C. obscurior*, male larvae develop into winged sexuals when their colonies are subjected to a sudden temperature decrease, colony fragmentation, or other drastic changes (Cremer and Heinze, 2003). Male larvae apparently do not react to these changes themselves. Instead, workers respond to stress by starting to treat male larvae differently, e.g., by increasing the frequency of antennations, and in this way determine their developmental pathway. The presence of a queen is not necessary to induce the development of a certain male morph.

Increased antennation frequency might indicate a higher density of workers and lead to the development of winged disperser morphs. Similarly, an alarm pheromone causes increased walking behaviour in aphids and results in increased wing induction (Kunert et al., 2005). However, *C. obscurior* larvae, which developed into winged males, were also more often “bitten” by workers. Such biting might directly affect morph development, because simulated biting induced a switch from queen to worker caste in larvae of *Myrmica* ants (Brian 1973, but see Buschinger, 1993). Alternatively, the increased contact rate of workers and larvae might reflect an increased feeding rate and thus higher provisioning of larvae. Considering that winged male and female sexuals of *Cardiocondyla* are larger than their wingless counterparts and that nutritional status influences juvenile hormone level (e.g., in *Apis mellifera*, Rembold, 1987), food supply might be the most plausible way of morph determination. Whereas in the honeybee, queens are reared in special brood chambers and can be individually provided with “royal jelly”, ant larvae are anonymously placed on a brood pile. To differentially overfeed a few well-defined larvae is therefore not possible, and female larvae might develop into queens, when they were by chance fed more often than other larvae (e.g. Reuter, 1998). Male larvae in *Cardiocondyla* are probably less sensitive to differences in provisioning than female larvae, so that a more extreme shift has to occur to result in the necessary hormone concentration to activate the developmental pathway of a winged sexual. Alternatively, the threshold level of sensitivity to juvenile hormone can be higher in male larvae compared to female larvae (see e.g. Nijhout, 1999). This might explain why colonies normally contain only ergatoid and no winged males, though queens are produced year-round (Heinze and Delabie, in press).

Obviously, phenotypic plasticity is maintained in both sexes during most of larval development, allowing a colony to react quickly to changed environmental conditions. This might be one reason for the successful spread of *C. obscurior* in natural and urban habitats. Colony-level success may be associated with a cost for the individual male. Most ergatoid males are killed by their older rivals before reaching sexual maturity, while winged males usually remain unharmed (Kinomura and Yamauchi, 1987). An individual male larva might therefore selfishly prefer to grow into a winged male with guaranteed survival and a certain chance of mating, while workers under normal conditions probably benefit more from producing only the cheaper ergatoid males, in particular as those, which are killed during male-male fighting, are immediately recycled and fed to the larvae. Male selfishness is prevented by the fact that workers determine the “fate” of their male relatives, but it would nevertheless be of interest to investigate larval begging behaviour. Larvae can actively beg for food (Creemers et al., 2003; Kaptein et al., 2005), and male larvae might try to manipulate workers to enhance their future reproductive options.

## ACKNOWLEDGEMENTS

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# Chapter 2

# Alternative reproductive tactics in males of the ant *Cardiocondyla obscurior**

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and Jürgen Heinze

A winged male, an ergatoid male and a queen of *C. obscurior*

* Manuscript

## ABSTRACT

In the ant species *C. obscurior*, an environmentally induced male polymorphism of winged "normal" males and ergatoid, wingless males can be found. Whereas the latter are aggressive and try to monopolize all females, winged males are peaceful and disperse after several days within the colony. Most environmentally determined polymorphisms can be explained by the status-dependent selection model, which suggests that individuals choose the tactic according to their status, so that they gain the highest possible fitness. In this study, we try to determine the reproductive output of the different male morphs of C. *obscurior* to investigate whether morphs differ in their fitness. This is of special interest in *C. obscurior*, because workers determine into which male morph a larva develops, thus, they have the potential to force male larvae into a certain morph, even in case interests of the individual larva differs. We show that males of both morphs have equal maximum lifetime reproductive success. However, considering the survival probability of males before they start sexual reproduction and the competition between individuals, a male larva should prefer to get winged instead of ergatoid.

*Keywords*: alternative reproductive tactic, status, reproductive success, male morph, social insects, *Cardiocondyla*

## INTRODUCTION

Alternative reproductive tactics within the sexes are widespread in the animal kingdom, and have been explained by three evolutionary stable strategy models: alternative strategies are characterised by a genetic polymorphism, whereas mixed and conditional strategies are determined environmentally. In case of a mixed strategy, alternative phenotypes with equal fitness are maintained through frequency dependent selection, whereas the conditional strategy predicts status-dependent selection (Gadgil, 1972; Gross, 1996; Maynard Smith, 1982). Up to now, no evidence for the existence of a mixed strategy has been found, while the conditional strategy has been shown to apply to most dimorphisms (Gross, 1996). The model of status-dependent selection predicts that the tactic an individual adopts, will depend on the relationship between the status and the fitness payoff associated with each tactic (Gross, 1996). An individual can maximise its fitness by choosing the tactic that yields the highest fitness with regard to its status (e.g. fighting or sneaking), which is often correlated with body size and dependent on the environmental conditions the individual experiences. Average fitness of individuals of different status is unequal, and low status individuals are often meant to do the "best of a bad lot" (Eberhard, 1982). However, this is not always well quantified, and it has also to be considered that the fitness of the morph can be dependent on the prevailing conditions (Roff, 1996).

In the ant species *C. obscurior*, an environmentally determined fighter-flier polymorphism of “normal" winged males and ergatoid (“worker-like”) wingless males is exhibited. The ergatoid males express long, sabre-shaped mandibles, whereas winged males are bigger than ergatoid males and develop large eyes, ocelli and wings. Ergatoid males fight against each other and combats between two males always end in the death of one male, thus, the surviving male can monopolize all virgin females as long as it stays alive and will not be killed by another rival. As sexuals are produced during the whole year and ergatoid male pupae eclose regularly, fights can be observed frequently. Usually, the ergatoid male tries to puncture the cuticle of freshly eclosed rivals with its long mandibles. In case the cuticle of the rival has already hardened (e.g. in big colonies, where males are able to hide in the brood pile during the first hours after eclosion), males are not able to kill the rival on their own and thus smear a substance of their gaster-tip on the body of the opponent (Kinomura and Yamauchi, 1987; Stuart et al., 1987; Yamauchi and Kawase, 1992). This substance leads to worker aggressiveness, and they will kill the male.

Contrary, winged males are peaceful. Although they represent the "disperser" morph, they stay inside the nest during the first days of their life and mate with virgin females. To avoid being killed by ergatoid males, they mimic females chemically and "sneak" copulations (Cremer et al., 2002). Winged males as well as larvae of winged males are bigger and heavier than ergatoid males (adult erg. males: 4.2 x 10 $^{-5}$g, adult winged males: 6.7 x 10 $^{-5}$ g from Cremer and Heinze, 2002; Schrempf and Heinze, subm.). They only have a limited sperm supply, as testes degenerate several days after eclosion and sperm is stored in seminal vesicles. In ergatoid males, spermatogenesis continues during their whole life, so that they have an unlimited sperm stock (Heinze and Hölldobler, 1993; Heinze et al., 1998). This, and the fact that they do monopolize the females in a colony through fighting, was interpreted as indication that ergatoid males have a higher competitive ability than winged males (Anderson et al., 2002). Though, concrete data are rare, and one study by Heinze and colleagues in other *Cardiocondyla* species (1998) revealed that winged males have an unexpected high insemination capability.

The male morph in *Cardiocondyla* is controlled through different worker treatment and not by conditions the larvae experience directly (Schrempf and Heinze, subm.). Usually, only in case environmental conditions turn bad, winged disperser males are produced in the colonies (Cremer and Heinze, 2003). Although it is obvious that males should prefer to get winged under unfavourable conditions, it is not easy to determine what would be best for the males under “usual” conditions – as interests of the colony as a whole and a selfish male may differ. In this study, we collect data regarding the reproductive success of the different males in *C. obscurior* and try to estimate whether morphs differ in their average fitness on basis of a simple equation.

## MATERIALS AND METHODS

### *Study species*

Colonies of *C. obscurior* were collected in Brazil (Bahia) in September 2000, and kept since then in the laboratory in climatic chambers with 30°C/25°C temperature and 12h/12h day/night-cycle. Ergatoid males and young queens are produced year-round. Winged male producing colonies were created by splitting big colonies into smaller subunits (see e.g. Cremer and Heinze, 2003).

### *Reproductive potential*

To estimate the maximum insemination capability of ergatoid and winged males, one day old males (n=6 ergatoid males, n= 5 winged males) were placed together with ten to 20 virgin queens in colonies of approximately 15 workers and some brood. Every day, ten young virgin queens from several large “donor” colonies were added during the whole lifetime of the male, so that each male would have had the possibility to inseminate more than 100 females. Afterwards, all females were dissected to control whether their spermatheca contained sperm. The same set-up was conducted with six winged males at the time they were trying to leave the colony, which can be easily observed as they get extremely active, leave the nest chamber and run around outside.

To get an impression of the course of insemination over time, we prepared the same set-up once again but dissected all females every 24 hours, before adding another ten (n= 2 for each morph).

### *Sperm number and sperm transfer*

Eight virgin, eight to ten day old winged males were dissected on a microscope slide in a drop of Beadle solution (128.3 mM MaCl, 4.7 mM KCl, 2.3 mM $CaCl_2$). Both seminal vesicles were transferred to a new drop of Beadle solution and sperm was released and mixed carefully with the tip of the forceps, to avoid clumping. The position was marked in ink on the bottom of the slide and the solution allowed evaporating. Afterwards, sperm was fixed in 70% ethanol and stained with DAPI (Hoechst). Two different persons counted the sperm number of a quarter or half of a seminal vesicle of each male, using a fluorescence microscope at a magnification of x 200. Afterwards, mean number of total sperm was calculated.

17 mated females were dissected in a drop of Beadle solution 24 hours after one single copulation with the respective (virgin) male morph (mated with an ergatoid male: n=10; mated with a winged male: n=7). After 24 hours, all sperm was expected to have reached the spermatheca. Copulation was observed by eye and video taped, and copulation duration was recorded. The male was separated from the female immediately after the copulation act, as the males frequently mate several times in succession with the same female. The spermathecae of the queens were treated in the same way as the male reproductive organs described above. All sperm was counted by two persons independently.

### *Longevity, survival probability and mean number of competitors*

To determine the mean lifespan of males under natural conditions (e.g. without "reproductive stress" as above), we observed freshly eclosed males in their colonies and removed all additional male pupae. For ergatoid males, this represents the maximum lifespan "without enemies" (under "protected conditions"), however, this is not representative for their real lifespan in the colonies, as they normally fight with eclosing males. Thus, ergatoid males (n=49) were marked in a second experiment by cutting a small part of one tarsus of the freshly eclosed male. Observations in the past revealed that this does not influence fighting ability of males (A. Schrempf, unpubl.). Number and stage of male pupae (e.g. white pupae, brown pupae shortly before eclosion) were recorded four times a week and the number of killed, freshly eclosed males was recorded to estimate the probability of reaching reproductive life.

To investigate how many males compete on average for females in one colony in case of winged male production, we recorded the mean number of males, which were simultaneously as adults in the nest, in 53 winged male producing colonies over a period of four weeks. Frequently, other winged males are in the colony, and more rarely, an ergatoid male develops in addition to the winged male(s). In colonies in which only ergatoid males are produced, one adult male monopolizes all females and does not have to compete for matings.

### *Estimation of the reproductive success of the different male morphs*

The mean reproductive success of the respective male morph $mR_e$ and $mR_w$ can be calculated by $mL_e$ * $mF_e$ and $mL_w$ * $mF_w$, respectively (mL = mean reproductive lifespan of the males in days; mF = mean daily mating frequency of the males), assuming that sperm is unlimited (see below) and females are available. The maximum reproductive success was obtained directly by the experiments conducted as described above (reproductive potential). Theoretically, it could be calculated in the same way as the mean reproductive success (based on maximal values), but in fact, that would lead to an overestimation, as males only can inseminate a certain (lower) amount of females in the practical experiment.

In addition, there are two other significant factors, which can strongly influence male reproductive success in *Cardiocondyla*: mortality rate before reproductive activity or the survival probability S, respectively, and the mean number of males in a colony C (as winged males have to compete for females with other males). Integrating those two factors, mR * S / C (for both the winged and ergatoid male) expresses the "real" reproductive success of the male morphs R under consideration of rival males and the survival probability before any reproduction, on condition that all inseminated queens have the same probability of survival and equal reproductive success, independent of the male morph they mated with. It is important to notice that we only consider the direct fitness of the males.

# RESULTS

### *Reproductive potential*

Ergatoid males inseminated up to 30 virgin queens and winged males up to 23 virgin queens. Winged males inseminated only up to twelve females with a probably already fixed amount of sperm after they left the nest (Table1). Differences between the males are not significant - even in case of treating winged males as one single group (see t-test) - what seems to be mainly due to the high variation between males and not due to limited sample-size (Kolmogorov-Smirnov: n.s.; ANOVA: $n_1 = 6$, $n_2 = 5$, $n_3 = 6$, F=1.41, p=0.27; t-test: $n_1 = 11$; $n_2 = 6$; t=-1.29, p = 0.21; Figure 1). Considering only the "best" male in the group of winged and ergatoid males, the "best" ergatoid male inseminated 30 of 200 possible females in 19 days, the "best" winged male inseminated 23 of 120 possible females in 11 days ($\chi^2$: 0.67; p = 0.41).

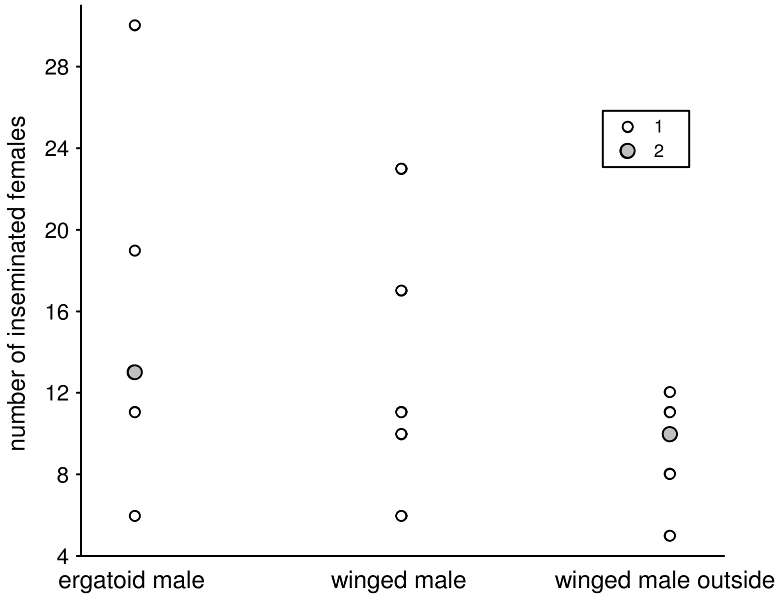

**Figure 1.** Number of inseminated females of the two different male morphs (started at day one of their adult life) and of winged males after they left the nest.

Winged males on average inseminate more females / day, but they have an on average shorter lifespan than ergatoid males. Again, the differences between males are not significant (Kolmogorov-Smirnov: n. s.; $n_1 = 6$, $n_2 = 5$, $n_3 = 6$; ANOVA insemination frequency: F = 2.41, p = 0.13; survival analysis for multiple groups: $\chi^2 = 3.60$, p = 0.17; Table 1). There is a strong tendency that the number of inseminated females is dependent on the longevity of the males

(Spearman-Rank-correlation: n = 17, R = 0.44, p = 0.08; Figure 2). The dissection of females every 24 hours reveals that ergatoid males on average inseminate one queen per day during their whole life (and in case they inseminate more queens this seems independent of their age), whereas winged males on average inseminate two or more females per day during the first days of their life, and less females later on in their life.

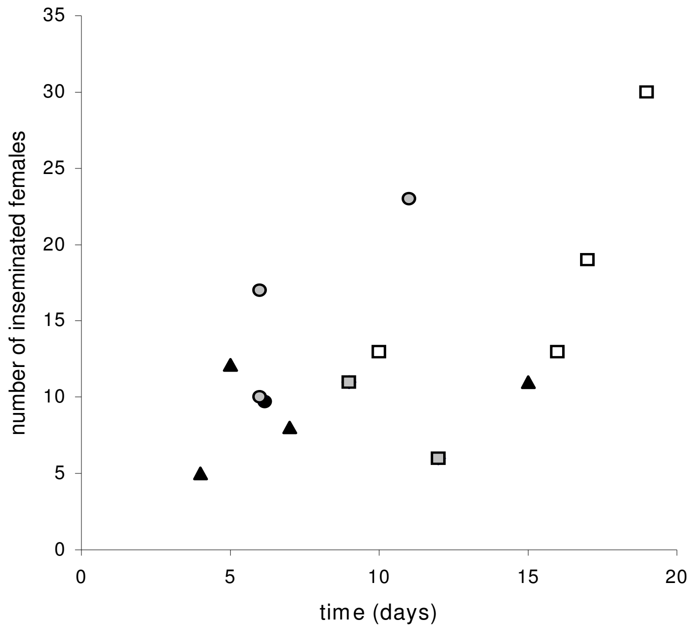

**Figure 2.** Lifespan of ergatoid (white rectangles) and winged males (grey circles) and number of inseminated females under conditions with a surplus of females. Black triangles show the same for winged males from the day on, on which they leave the nest.

### *Sperm number and sperm transfer*

The sperm number in winged males is on average 22 772 ± 5771 (n=8; 3771 sperm cells per count in a quarter of a seminal vesicle, mean difference between the two counts: 247 sperm cells).

All females mated with winged males (n=7) were inseminated, although the sperm amount in the spermathecae differed considerably. However, with the average mean of 953 sperm cells per female, a winged male would be able to inseminate about 24 females.

Three of ten spermathecae of females mated with ergatoid males did not contain any sperm (and were thus not included in the analysis), and as it was the case in females mated with winged males, sperm amount between females differed considerably. There is no difference in the amount of spermatozoa transferred to the females between winged and ergatoid males, although the highest number was transferred by two ergatoid males (Kolmogorov-Smirnov:

n.s.; t-test: $n_1$=7, $n_2$=7, t = 1.09, p = 0.3; Table 1). No correlation of sperm number and copulation duration neither in ergatoid nor in winged males could be detected (Spearman-Rank-correlation: erg. males: n = 10, R = 0.39, p = 0.26; winged males: n= 7, R = 0.22, p = 0.64; Figure 3).

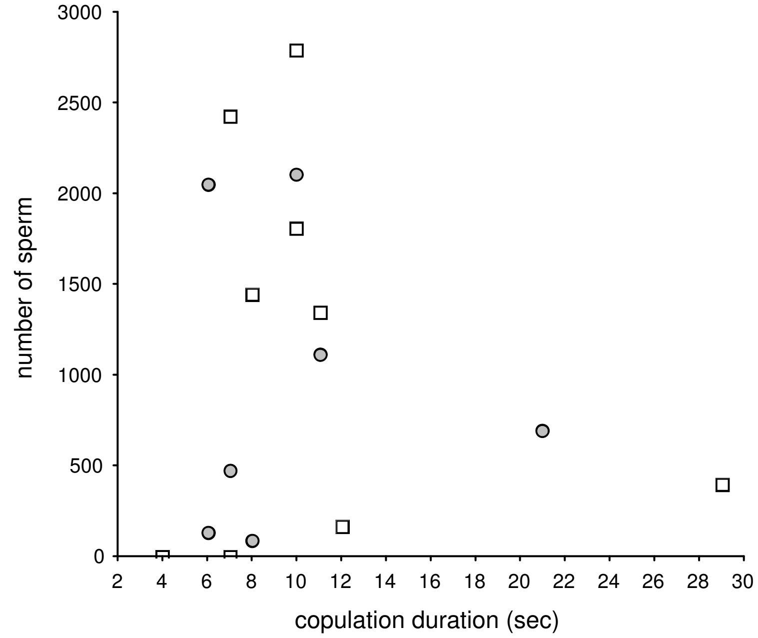

**Figure 3.** Mean sperm number transferred to the spermatheca of a virgin female during one single copulation act of ergatoid (white rectangles) and winged (grey circles) males. Number of sperm in the spermatheca is independent of copulation duration.

### *Longevity, survival probability and mean number of competitors*

Under "natural" conditions the lifespan of both, winged and ergatoid males, is increased in comparison to the lifespan under "reproductive stress" (Table 1, Figure 4). The effect is clearly visible in both male morphs, but not significant (Cox-Mantel Test: ergatoid males: $n_1$= 6, $n_2$=51, U = - 1.42, p = 0.46; winged males: $n_1$= 5, $n_2$=12, U = - 2.21, p = 0.10).

Ergatoid males can reach a higher maximum lifespan than winged males under "protected conditions" (winged males: 12.25 ± 5.7 days; erg. males: 37.2 ± 23.4 days; Cox-Mantel Test: $n_1$ = 12, $n_2$ = 10, U = 5.54, $p < 0.01$), however, under natural conditions (other male pupae in the colony), both morphs live equally long (Cox-Mantel-test: $n_1$ = 12, $n_2$ = 51, U = 3.55, p = 0.16; Table 1, Figure 4). Although 34 of the observed ergatoid males died "by themselves" in the colonies and e.g. not during a fight, their lifespan is reduced compared to the lifespan of males without enemies (maybe because of physiological stress due to previous fights; Cox-Mantel-test: $n_1 = 34$, $n_2 = 10$, $U = -6.51$, $p = 0.02$). In nine cases (17.6%), a freshly eclosed male killed the adult male, and in another eight cases (15.7%), both males were killed from the workers during the fight.

The probability to survive the transition from pupae to adult and thus reach reproductive life is 0.43 ± 0.25 (Table 1). Each male kills on average 1.3 ± 1.6 freshly eclosed males.

On average, 1.45 males are simultaneously together in winged male producing colonies, thus, approximately 45% of winged males have to compete for access to females with either another winged male or an ergatoid male.

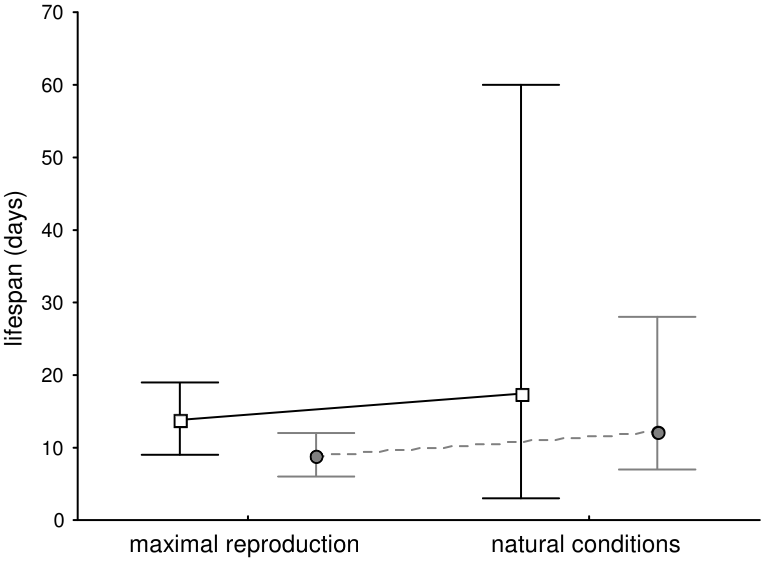

**Figure 4.** Total lifespan of ergatoid (white rectangles) and winged males (grey circles) under maximal reproduction and under natural conditions (mean, min. and max.).

*Estimation of the reproductive success of the different male morphs*

Similar to the maximum reproductive success, the calculated mean lifetime reproductive success of both male morphs is not different ($mR_w = 20.8 \pm 5.13$; $mR_e = 19.14 \pm 5.64$). Even for "sperm limited" winged males, the number of spermatozoa is not a limiting factor, as they are sufficient to inseminate 24 females on average.

However, winged males usually do not experience mortality before or shortly after eclosion, in contrast to ergatoid males. Instead, as winged males are peaceful, they frequently have to “share” females with competitors. In case of winged male production, even ergatoid males have to compete for females, what might decrease their reproductive success as well. For a simple comparison, we assume that ergatoid males do not have to compete for females, and that winged males experience zero mortality. Then, calculating the “real” reproductive success of the different morphs according to $R = mR * S / C$, $R_w > R_e$ (14.3 > 8.23), thus, males should prefer to get winged (Table 1). Even taking the maximum possible reproductive success of the two male morphs as basis for calculation ($n_e = 30$, $n_w = 23$), males have an advantage if they are winged ($R_w > R_e$: 15.8 >12.9).

**Table 1.** Male longevity, insemination capability and sperm transfer during copulations in *Cardiocondyla*. In addition, the mean survival rate as well as the mean number of males in the colonies are given, and mean lifetime reproductive success and "real" lifetime reproductive success calculated.

| | **Ergatoid males** | **Winged males** | **(after leaving the nest)** |
|---|---|---|---|
| **Maximum reproductive success** | n=6 | n=5 | (n=6) |
| inseminated queens (mean ± stdev) | 15.33 ± 8.31 | 13.40 ± 6.66 | (9.33 ± 2.50) |
| min -max | 6 - 30 | 6 – 23 | (5 – 12) |
| lifespan (days) mean ± stdev | 13.83 ± 4.07 | 8.80 ± 2.77 | (7.17 ± 3.97) |
| min- max | 9 - 19 | 6 - 12 | (4 – 15) |
| mean inseminated queens / day | 1.09 ± 0.38 | 1.66 ± 0.88 | (1.48 ± 0.57) |
| **Natural conditions** | | | |
| sperm supply (mean ± stdev) | unlimited | 22 772 ± 5771(n=8) | |
| n transferred sperm / copulation (mean ± stdev) | 1483 ± 972 (n=7) | 953 ± 845 (n=7) | |
| min- max number (in case of sperm transfer) | 165 – 2795 (n=7) | 91 – 2112 (n=7) | |
| lifespan (days) mean ± stdev | 17.43±14.08 (n=51) | 12.25 ± 5.75 (n=12) | |
| min-max | 3 - 60 | 6 - 28 | |
| survival probability S (1-M) | 0.43 ± 0.25 (n=51) | 1 | |
| mean number of males (C) | 1 | 1.45 ± 0.57 (n=53) | |
| **Calculated mean lifetime reproductive success mR** | | | |
| (n inseminated females) | | | |
| mR= mean longevity * insemination frequency | 19.14 ± 5.64 | 20.8 ± 5.13 | |
| **Calculated "real" lifetime reproductive success R** | 8.23 | 14.3 | |
| R = mR *S / C | | | |

## DISCUSSION

We could show that winged and ergatoid males of *C. obscurior* on average have equal maximum lifetime reproductive success under the preconditions that enough females are available for the matings, that males are not killed before they reach reproductive life and that they do not have to compete for females with other males. Considering that winged males are sperm-limited after some days of adulthood in contrast to ergatoid males (Heinze and Hölldobler, 1993; Heinze et al., 1998), and that ergatoid males can grow much older than winged males without rivals, these results are quite striking, but may be explained by a prolonged spermatogenesis due to continuous sexual reproduction in winged males, and, a reduced lifespan in both male morphs, probably due to a trade-off of reproduction and longevity, a phenomenon well known from other species (see e.g. Martin and Hosken, 2004; Partridge and Farquhar, 1981).

However, the factors which were artificially excluded to determine the maximum possible reproductive success in the male morphs, cannot be ignored as they play a major role in *Cardiocondyla.* First, the number of virgin females is usually limited. For example, in 25% of the colonies in which the survival probability of ergatoid males was observed, no single female eclosed during the whole lifetime of the male. Second, the probability for ergatoid males of being killed is extremely high. Both factors only play a subordinate role for winged males, as they can leave the colony to search for females outside of the nest, and as they are not killed by other males due to female mimicry (Cremer et al., 2002). On the other hand, winged males have to compete with other males for virgin queens frequently, because they are peaceful and not able to monopolize the females. But even the effect of competition might be not that drastic, as under laboratory conditions multiple matings can be observed, and it is known from other *Cardiocondyla* species that multiple matings do indeed result in the expression of several patrilines (Schrempf et al., 2005a; J.C. Lenoir, unpubl.; Schrempf and Heinze, unpubl.). Therefore, competing males probably share their reproductive success. Altogether, larvae developing into winged males seem to be in an unexpected better position than larvae developing into ergatoid males.

Even if the probability for winged males to survive and find females outside of the nest is assumed to be very low, they can react flexibly to the conditions in the nest, as they can mate with virgin females within the colony and afterwards leave the nest with a sperm supply still sufficient to inseminate several females in addition. In fact, observations suggest that the emigration time of winged males depends on the availability of virgin queens (Heinze and Hölldobler, 1993; S. Cremer, unpubl.; own observation).

In other species, in which a fighter – flier polymorphism occurs, e.g. in many fig wasps (Cook et al., 1997; Hamilton, 1979; Murray, 1989; Murray, 1990) and bees (Danforth, 1991; Kukuk and Schwarz, 1988), the disperser morph usually only mates outside of the nest. This might be an important reason for the fighter phenotype having higher average fitness than the disperser morph in the bee *Perdita portalis* (Danforth, 1991). Winged males in *Cardiocondyla* take advantage of the fact that they are not attacked by ergatoid males due to female mimicry (Cremer et al., 2002) and thus can maximise their reproductive success – in contrast to the above mentioned species- by mating inside of the nest before leaving it.

In addition to an unlimited sperm supply, another frequently supposed advantage for ergatoid males concerned their ability to grow older in comparison to the winged morph. Indeed, maximum lifespan greatly differs between the morphs. Though, observations revealed that males usually only grow old in those colonies, in which other ergatoid male pupae rarely develop, and these are only very small colonies, in which female sexuals scarcely are produced as well. Thus, the benefit of growing old seems to be not correlated with a higher reproductive success.

When estimating fitness it is of importance to consider that females may have different probabilities to start reproduction and that they may differ in fecundity as well. Moreover, the viability and reproductive success of the offspring in the next generation can differ (indirect fitness; Andersson, 1994; Hamilton and Zuk, 1982, Møller, 1994; Møller and Jennions, 2001). At present, we are unable to integrate these factors into our calculation and assume that females have the same probability to start reproduction and the same reproductive success, independent, with which morph they mate. The variance in the number of sperm transferred to the females during mating was very high and differences between males were not significant. As males of both morphs usually copulate several times in succession with the female, it is likely that all together enough sperm is transferred and that sperm limitation constitutes no problem for *Cardiocondyla* females. Laboratory observations confirm this, as worker or queen pupae can be found in single-queen colonies until the death of the queen, independent whether the queen mated with an ergatoid or with a winged male. Considering in addition that females have a mean lifespan of 26 weeks (Schrempf et al., 2005b) and lay two eggs on average per day, less than 400 sperm cells will be enough to fertilize all eggs.

However, usually no winged males develop in colonies of *C. obscurior* (Heinze and Delabie, in press). This has been discussed as optimal resource allocation of the colonies, because it is probably far less costly to produce the ergatoid male morph, as they are smaller and less heavy than winged males (Cremer and Heinze, 2003). In addition, ergatoid males eclose earlier from the pupal stage and can serve as "unlimited, long-term sperm supply", which is ready to mate whenever females eclose. Thus, if the increase in inclusive fitness for the colony through a winged male, which mates "outside", is less than the costs to produce the winged male, only ergatoid males are expected to occur, what is in fact the case. Moreover, ergatoid males that are killed by rivals during combats are fed to the larvae, so that they are "recycled", what reduces the costs of ergatoid male production in addition. Only if conditions get worse, e.g. due to severe climatic changes, which endanger the survival of the whole colony, disperser males are produced. This has been compared to the life history of some sessile organisms, which switch between vegetative, local growth and dispersal through sexual reproduction (Cremer and Heinze, 2003). Generally, colonies only produce ergatoid males and the young queens, which eclose during this time, most likely remain in the nest to reproduce there as part of the polygynous colony. New colonies are established by budding, which means that a small part of the colony (workers, brood and queens) splits off. Under these conditions, dispersal is limited. In case of winged male production, females may leave the maternal nest more likely to disperse and try to be adopted in another colony or alternatively, even found a new colony on their own.

In social insects, larvae are often forced to develop as workers instead of reproductive queens (Bourke and Ratnieks, 1999; Wenseleers and Ratnieks, 2004; Wenseleers et al., 2003, Wenseleers et al., 2005) and in the determination of the female caste, nutrition frequently plays an important role (Bourke and Franks, 1995; Wheeler, 1986; Wilson, 1971). In *Cardiocondyla*, both, the female caste as well as the male morph seem to be influenced by nutrition. The occurrence of gynandromorphs in *C. emeryi* (male on one side, female on the other; either ergatoid and worker or both winged sexuals; Heinze and Trenkle, 1997) and the effect of a juvenile hormone analogue in *C. obscurior* strongly indicates, that the mechanism for winged morph induction on one hand and for ergatoid and worker development on the other hand, is the same (Schrempf and Heinze, subm). Thus, workers can probably easily prevent larvae from getting winged e.g. by limiting their food intake.

Our study suggests that ergatoid males are of lower “status” than winged males and thus males should prefer to get winged. However, individuals usually cannot change their conditions and have to “do the best” according to their status. Eusociality, on the other hand, may offer polymorphic larvae the unique opportunity to manipulate the conditions they experience by manipulating worker treatment e. g through begging for food (Kaptein et al., 2005). Though, as already mentioned, usually, females in social insects are not able to control their fate, and the same seems to hold for males of *Cardiocondyla*. Thus, the alternative male tactics are a result of a conditional strategy controlled by the colony.

## ACKNOWLEDGEMENTS

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# Chapter 3

# Back to one: consequences of secondary monogyny in an ant with polygynous ancestors*

Alexandra Schrempf and Jürgen Heinze

A long winged and a short winged queen of *C. batesii*

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* Submitted to Evolution

## ABSTRACT

Polygyny, i.e., the presence of multiple fertile queens in an insect society, has long attracted the attention of evolutionary biologists because to some extent parallels the transition from solitary life to sociality. While polygyny has repeatedly evolved from ancestral monogyny (one single queen per colony), little is known about the reversed evolutionary pathway: from polygyny back to monogyny. As queens in polygynous species often lack the morphological adaptations for successful dispersal and independent colony founding and are comparatively short-lived, the transition to derived monogyny is associated with serious difficulties regarding the initiation of new colonies by single queens. According to a phylogenetic analysis, monogyny is derived in the ant species *Cardiocondyla batesii* and *C. nigra.* Here, we show that queens of these species are capable of founding a new colony independently, albeit in a rather unusual way: they mate in the maternal nest, disperse on foot, and forage during the founding phase. Their peculiar wing polymorphism appears to be associated with this reversal to monogyny and independent founding: short-winged queens were significantly more successful in establishing a new colony than long-winged queens, as they invest in fat instead of wing muscles and thus trade off reproduction against dispersal capabilities. Furthermore, queens from monogynous species appear to live longer than queens in related polygynous taxa, suggesting that queen life span is a plastic trait.

*Keywords*: colony founding, queen polymorphism, longevity, trade-off reproduction-dispersal, queen number, *Cardiocondyla*

## INTRODUCTION

Cooperation among biological entities is a powerful force in evolution underlying major transitions, e.g., from prokaryotes to eukaryotes, from single-celled organisms to multicellular organisms, and from solitary animals to animal societies (Maynard Smith and Szathmary, 1997). Reversions of the evolutionary trajectory may occasionally occur (Siddall et al., 1995; Wcislo and Danforth, 1997) but are comparatively rare and their causes and consequences are not well understood.

The evolution of polygyny (presence of multiple reproductives per nest) from monogyny (single reproductive per nest) in social insects shows parallels to the origin of eusociality, in that in both cases individuals give up their reproductive autonomy and are forced to find a compromise with others about the partitioning of reproduction (Keller, 1993; Keller, 1995; Keller and Vargo, 1993). In ants, polygyny has evolved repeatedly from ancestral monogyny wherever ecological conditions disfavoured solitary colony founding (Bourke and Heinze, 1994; Herbers, 1993; Hölldobler and Wilson, 1977; Hölldobler and Wilson, 1990; Ross and Carpenter, 1991).

The switch from one to many queens per nest is typically associated with dramatic changes in dispersal and colony founding strategies (Keller, 1991; Keller, 1993). Whereas young queens of monogynous species disperse and found their own societies solitarily, those of polygynous species stay in their maternal nests or return to it after mating, and new colonies are initiated by "budding" with the help of workers. Non-dispersing, dependently founding queens do not need the large resources required for solitary colony founding and therefore are often much smaller and have less fat reserves than queens of monogynous species (Keller, 1991; Keller and Passera, 1990). Furthermore, because queens from polygynous species produce sexuals faster and probably have a higher extrinsic mortality, they have a considerably shorter life span than queens from monogynous species (Keller and Genoud, 1997; Tsuji and Tsuji, 1996).

All these adaptations impede the reversal from polygyny to monogyny. Among ants, such a reversal occurs in a few slave-making ants, which presumably evolved from polygynous ancestors (Beibl et al., 2005). Young slave-maker queens usurp colonies of other ant species and kill the resident queens (Bourke and Franks, 1995; Buschinger, 1986; Buschinger, 1990; Hölldobler and Wilson, 1990). In this case, the evolution from polygyny to monogyny is easily explained, as the reproductive output of a queen is limited by the number of locally available slave workers (Heinze and Foitzik, in prep.).

A similar reversal from ancestral polygyny to derived monogyny was recently observed in the non-parasitic ant genus *Cardiocondyla.* Monogyny evolved at least once in a clade comprising several species from xeric habitats in Southern Europe and Central Asia (Heinze et al., 2005). Queens of polygynous *Cardiocondyla* are only little larger than the workers and extremely short-lived (Schrempf et al., 2005b). Most matings occur in the nest, and in many species wingless males have completely replaced winged, dispersing males (Heinze and Hölldobler, 1993; Heinze et al., 2005). New colonies are usually founded by budding (Stuart, 1990; Heinze and Delabie, in press). Queen-worker dimorphism is not much more pronounced in the derived monogynous species (Seifert, 2003). The question therefore arises how female sexuals cope with problems arising from the secondary switch to monogyny in particular concerning colony founding. We tested the hypotheses that queens from monogynous *Cardiocondyla* a) maintain the dependent strategy of their polygynous ancestors and found with the help of workers, b) start new colonies jointly with other queens (pleometrosis), and c) found solitarily, but forage during the founding phase (semi-claustral founding). In addition, we examined the role of wing polymorphism, which appears to be common in monogynous *Cardiocondyla* but absent from polygynous taxa (Seifert, 2003).

We show that female sexuals of monogynous *Cardiocondyla nigra* and *C. batesii* found their own nests solitarily after shedding their wings and dispersing on foot and during this period forage for food. Wing reduction appears to be associated with the replacement of wing muscles by fat, i.e., queens trade off dispersal capability vs. increased fecundity and higher survival rate during solitary founding. Finally, we document that the average reproductive life span of queens is considerably longer in monogynous than polygynous *Cardiocondyla* species.

## MATERIALS AND METHODS

### *Study sites and ants*

We collected a total of 988 young queens of *C. batesii* Forel, 1894 from 52 colonies at four different localities near Granada, Spain, in April 2001 (Padul), April 2004, and April 2005. Young queens from Padul, Víznar, Agrón, and from 21 colonies from S$^{ra.}$ Elvira were long-winged ("macropterous", M). In contrast, 25% of young queens from 13 additional colonies from S$^{ra.}$ Elvira were short-winged ("brachypterous", B), and though the remaining queens externally resembled M-queens, a closer inspection showed that they lacked flight muscles (see below; m-queens). All 621 young queens of *C. nigra* Forel, 1905 collected in March 2003 in 27 colonies from Panagia Evangelistria, Kalavasos, and Alassa near Limassol (Southern Cyprus) were short-winged. All 210 long-winged queens of *C. minutior* Forel, 1899 eclosed in laboratory colonies that were reared from several colony fragments collected at CEPLAC, Ilhéus, Brazil, in October 2002. If not mentioned otherwise, experiments were conducted with winged, young queens collected from their nests before dispersal.

### *Founding experiments*

Founding experiments were conducted with single queens (*C. nigra*: 122; *C. batesii*: 110 M from Padul, 44 B, 40 m; *C. minutior*: 110), groups of four queens each (*C. nigra*: 24 groups; *C. batesii*: 24 M, 8 B; *C. minutior*: 12), single queens assisted by three workers each (n = 12 for each species; *C. batesii* only M) and, for *C. minutior,* ten single queens assisted by ten workers each. M-queens from the different populations did not differ in ovarian status and fat content (see below) and the founding ability of M-queens from Padul might be representative also for M-queens from other populations. In an additional experiment two young queens were placed together with approximately 30 workers from their maternal colonies into large plastic boxes, where they had the opportunity to separate into distant nest chambers (n = 3 for each species). Experimental colonies were housed as described previously (Schrempf et al., 2005a). The number of eggs, larvae, pupae, and adults was recorded twice per week. Colony foundation was considered successful once young workers eclosed.

### *Fat content, development of flight muscles, and ovarian status*

Fat content of young queens was determined by gravimetry (Peakin, 1972) immediately after transfer to the laboratory. After drying for 24 hours at 65°C and individual weighing to the nearest 0.1 µg, each female was soaked in diethyl ether for 48 hours, dried again for 24 hours and reweighed to estimate the weight of extracted fat. Queens that disintegrated during this procedure were excluded from the analysis. Fat content could be determined in 10 queens from five *C. nigra* colonies, eight queens from four *C. minutior* colonies, and a total of 59 *C. batesii* queens (8 or 9 queens from different colonies from each of the three collecting sites with only winged queens, and 12 B, 12 m, and 9 M-queens from S$^{ra.}$ Elvira).

Flight muscle development was investigated in semi-thin sections (0.5-1 µm, epoxy resin, stained with 1% Toluidin blue) of the thorax of six young queens of *C. nigra*, five of *C. minutior*, and 15 of *C. batesii* from different populations. Furthermore, we prepared sections of three young *C. batesii* queens from Padul, which had been collected in autumn 2003 before hibernation, and of four freshly eclosed queens from laboratory colonies originating from Padul. Flight muscle and thorax volume were measured in two representative individuals each using Image Analysis 2.1 (Soft Imaging Software GmbH).

Ovary development was investigated by dissecting young queens of *C. batesii* a few hours after collection in the field (10 B-queens, 10 m-queens, and 30 M-queens from different populations). Number and length of ovarioles per ovary, number of developing eggs, and presence or absence of yellow bodies (remnants of previously developed eggs) were recorded. Ovaries were categorized according to the presence of only small egg cells (I), maturing eggs of intermediate size (II), or mature eggs (III). For a comparison between queen morphs, the number of a queen's eggs was multiplied with a size factor (one, two and three according to the ovarian category; in the following referred to as ovarian status).

### *Queen longevity and timing of sexual offspring production*

To determine the longevity of queens, we set up small laboratory colonies with freshly inseminated, young queens. To be able to compare the life span of queens from monogynous species from seasonal habitats with hibernation and polygynous, tropical *Cardiocondyla*, we subtracted the duration of the inactive winter period from the life span of queens from monogynous species. Furthermore, we recorded the time when first sexuals were produced in the experimental colonies.

## Results

### *Founding experiments*

Female sexuals and wingless males of *C. batesii* and *C. nigra* mate inside the nest in autumn. Young queens hibernate in the nest and shed their wings immediately before emigrating in spring to disperse on foot (Heinze et al., 2002). In contrast, female sexuals and both winged and wingless males of *C. minutior* are produced year-round. Solitary queens of *C. batesii* and *C. nigra* were able to found colonies in a semi-claustral way, i.e., they foraged for food until the first workers eclosed. In contrast, queens of *C. minutior* did never succeed in isolation. Significantly more foundresses of *C. nigra* managed to rear their first brood (*C. nigra*: 29 of 122 queens, 23.7%; *C. batesii*: 14 of 194 queens: 7.2%; chi-square-test, $\chi^2 = 12.91$, $p < 0.001$). The difference is caused by the higher success of short-winged queens, which were similarly successful in *C. batesii* and *C. nigra* ($\chi^2 = 1.36$, $p = 0.24$) and more successful than M-queens of *C. batesii* (B vs. M: $\chi^2 = 4.38$, $p = 0.04$; Table 1).

Egg laying rates were significantly higher in B- and m-queens and queens of *C. nigra* than in M-queens and *C. minutior* queens (Mann-Whitney U-test: B vs. m: $U = 153$, $p = 0.14$; B vs. M: $U = 60.5$, $p < 0.001$; m vs. M: $U = 83.0$, $p < 0.001$; *C. nigra* vs. B: $U = 638$, $p = 0.52$; *C.*

*nigra* vs. all other groups: $p < 0.05$; *C. minutior* vs. M: $U = 416$, $p = 0.23$; *C. minutior* vs. all other groups: $p < 0.05$; Table 1, Figure 1). Furthermore, queens of *C. nigra* and B- and m-queens of *C. batesii* started egg-laying earlier than M-queens of *C. batesii* (Table 1), and accordingly, first workers eclosed earlier in colony initiated by queens of *C. nigra* (46 days) and B- and m-queens (54 and 57 days) than in colonies founded by M-queens (72 days). Queens of *C. minutior* started egg-laying already after 3 days, but as they never succeeded in raising brood solitarily no workers eclosed. When accompanied by three workers, the proportion of successful foundresses was much higher in *C. batesii* and *C. nigra*, but *C. minutior* queens succeeded only when assisted by ten workers. Cooperative colony founding failed in both *C. batesii* and *C. nigra*, as queens became aggressive and fed on each others' eggs. Queens even fought when they had the opportunity to separate from each other. Furthermore, workers attacked queens until only one survived. In contrast, four of 12 queen associations of *C. minutior* queens succeeded in raising young workers and queen aggression was never observed.

**Table 1**. Percentage of *Cardiocondyla* queens that were successful in founding a colony either in isolation, with other queens, or assisted by workers, and the start of egg laying in colonies with single queens (mean ± standard deviation).

| | **single queens** | **start of egg laying (days)** | **4 queens** | **1 queen, 3 workers** | **1 queen, 10 workers** |
|---|---|---|---|---|---|
| *C. nigra* | 23.7%<br>(29/122) | 6.9 ± 1.27 | 0%<br>(0/24) | 75%<br>(9/12) | --- |
| *C. batesii* B-queens | 13.6%<br>(6/44) | 6.05 ± 2.29 | 0%<br>(0/8) | --- | --- |
| *C. batesii* m-queens | 10%<br>(1/40) | 6.00 ± 0.81 | --- | --- | --- |
| *C. batesii* M-queens | 3.6%<br>(4/110) | 18.7 ± 7.82 | 0%<br>(0/24) | 41%<br>(5/12) | --- |
| *C. minutior* | 0%<br>(0/110) | 3.04 ± 1.08 | 33%<br>(4/12) | 0%<br>(0/12) | 20%<br>(2/10) |

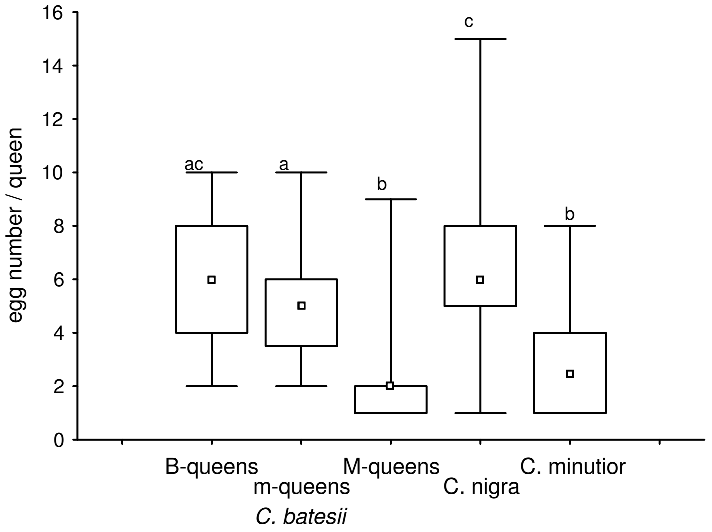

**Figure 1.** Differences in egg laying rates of *Cardiocondyla* queens (median, quartiles, and range). Significant differences are indicated by different letters.

*Fat content, development of flight muscles, and ovarian status*

When correcting for queen weight in an ANCOVA with dry weight as cofactor, B- and m-queens of *C. batesii* had significantly more fat than M-queens ($F = 7.32$, $df = 5$, $p < 0.005$; post-hoc Fisher LSD: B vs. m: $p = 0.39$; B vs. M: $p < 0.005$; m vs. M: $p < 0.005$; comparison between different collecting sites of M-queens: $M_i$ vs. $M_j$: $p > 0.2$ for all comparisons). Queens of *C. nigra* had a similar fat content as B-queens of *C. batesii*, while the fat content of *C. minutior* was even lower than in the least fat M-queens of *C. batesii* (Figure 2).

Flight muscles of young, winged *C. minutior* queens were well developed (18% muscle volume/thorax), but completely replaced by fat in *C. nigra* queens and B-queens of *C. batesii* and undeveloped or strongly reduced in m-queens (3.8%). Flight muscles were well developed in four M-queens (12.8%) and to some extent reduced in five others (9.36%). Flight muscles were already reduced in M-queens collected before hibernation and also in freshly eclosed queens from the laboratory.

All dissected young, winged queens had a filled spermatheca and 2 x 3 ovarioles without visible yellow bodies. Ovariole length did not differ among the different types of *C. batesii* queens (Kruskal-Wallis-ANOVA: $H = 1.35$, $p = 0.51$). M-queens from the different populations did not differ in ovarian status ($H = 6.94$, $p > 0.5$; $H = 4.23$; $p > 0.5$). The ovaries of all B-queens and of 8 of 10 m-queens, but only of 8 of 30 M-queens contained maturing eggs and over all, ovarian development was highest in B-queens, intermediate in m- and lowest in M-queens (B vs. m: $U = 8$, $p = 0.001$; B vs. M: $U = 1.5$, $p < 0.001$; m vs. M: $U = 57$, $p = 0.003$).

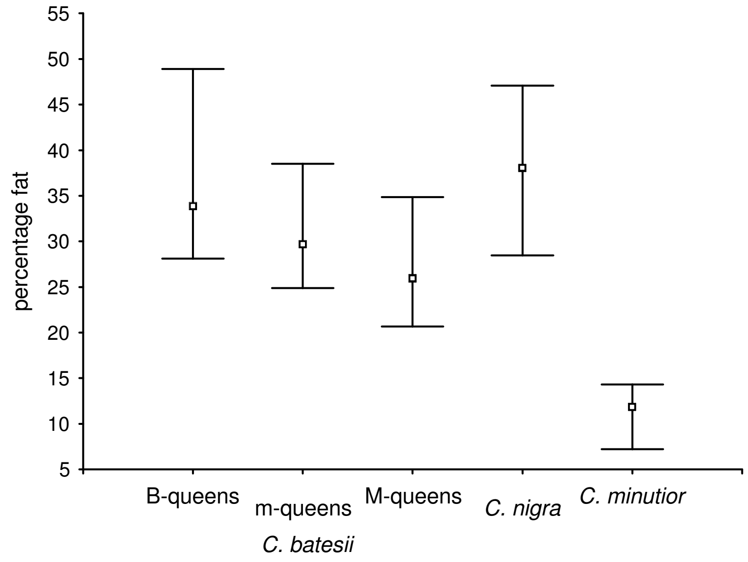

**Figure 2.** Mean, minimum and maximum fat content (% dry weight) of *Cardiocondyla* queens. There is a significant difference in fat content between all morphs and species.

### *Queen longevity and time of sexual offspring production*

It has previously been reported that queens of polygynous *Cardiocondyla*, such as *C. obscurior* (Schrempf et al., 2005b) and *C. emeryi* (J. Heinze, unpubl.), are short-lived compared to other ant queens. *C. obscurior* queens survived for an average of 26 weeks, with a maximum lifespan of 56 weeks. In contrast, queens of *C. batesii* and *C. nigra* survived longer, even when corrected for inactivity during hibernation by subtracting 6 months for field collected queens and additional 4 months for each artificial hibernation in the laboratory. Queens of *C. nigra* survived on average 31.9 weeks ($n = 26$; maximum lifespan 66 weeks), and queens of *C. batesii* on average 56.1 weeks ($n = 23$, maximum 112 weeks; all three species: Kruskal-Wallis-ANOVA: $H= 27.2$, $p < 0.01$; Mann-Whitney U-tests: $p < 0.05$ for all comparisons between *C. obscurior, C. nigra*, and *C. batesii*).

Sexual offspring eclosed in the autumn of the first year in 13 of 26 *C. nigra* colonies and two of 23 *C. batesii* colonies. The other colonies reared sexuals in the autumn of the second year. In *C. obscurior*, first sexuals were reared approximately six weeks after the queens had mated.

## DISCUSSION

With the evolutionary reversion from polygyny to monogyny, queens of the ant genus *Cardiocondyla* successfully "re-invented" a peculiar way of solitary colony founding. Like in polygynous *Cardiocondyla*, queens of monogynous *C. batesii* and *C. nigra* mate with nestmate males and shed their wings in the maternal nest. However, in contrast to polygynous species, young queens are fiercely attacked by workers when they start reproducing in the presence of their fertile mothers. Furthermore, they are mutually intolerant and fight until only one of them survives per nest. Young queens of *C. batesii* and *C. nigra* are therefore forced to emigrate after hibernation and to found their nests independently in spring.

Instead of evolving a larger body size with a more voluminous thorax that would allow rearing their first young completely with histolysed body tissues, queens of *C. batesii* and *C. nigra* remained little larger than the queens of their polygynous relatives (Seifert, 2003) and forage during solitary founding ("semi-claustral founding"; Brown and Bonhoeffer, 2003). In addition, a large percentage of queens of *C. batesii* and *C. nigra* are short-winged and the no longer needed wing muscles are replaced by fat, which increases the amount of nutrients available for brood rearing. Short-winged queens, with a fat content comparable to that of other semi-claustrally founding ants (Keller and Passera, 1989), were best in establishing their own colonies independently, while the long-winged queens of polygynous *C. minutior* had the lowest fat content and did never succeed without worker assistance.

Queen polymorphism is usually interpreted as evidence for alternative dispersal strategies, with solitarily founding, long-winged queens and stationary, dependently founding, flightless queens (Buschinger and Heinze, 1992; Heinze and Tsuji, 1995; Rüppell and Heinze, 1999). In contrast, short-winged queens of *C. nigra* and *C. batesii* appear to be more successful in solitary founding than long-winged queens. This clearly demonstrates the trade-off between the development and maintenance of a flight apparatus on one side and reproduction on the other known from solitary insects. In several grasshoppers, crickets, and aphids, the large-winged morph is significantly less fecund and lays eggs later than the flightless morph (Dixon and Howard, 1986; Ritchie et al., 1987; Roff, 1984). *C. batesii* queens lacked flight muscles already when they eclosed, suggesting that muscle reduction does not result from histolysis but from an inhibition of muscle growth as in other insects (Mole and Zera, 1994; Tanaka, 1993; Zera and Denno, 1997).

Wings are less costly than wing muscles (Roff, 1986), and the fitness differences between winged and wingless insects without wing muscle are therefore negligible (Zera and

Denno, 1997; Zera and Mole, 1994). This might explain why the founding success of long-winged, flightless m-queens of *C. batesii* did not differ from that of short-winged B-queens. At present it is unclear why some long-winged M-queens of *C. batesii* have retained developed flight muscles despite of the considerable fitness costs. Wing dimorphism would be stable if under some conditions long-winged queens dispersed on the wing. Though almost all queens shed their wings before leaving the maternal nest and long-winged queens did not fly even when placed in a wind tunnel (A. Schrempf, unpubl.), winged queens might eventually be drifted farther away by winds than short-winged queens and might thus serve as colonizers of empty habitat patches.

Finally, average queen longevity appears to be longer in monogynous than polygynous *Cardiocondyla* species, though it is still very short compared to queens of monogynous ants with similar colony size, such as *Temnothorax*, which live three to four times longer (Keller and Genoud, 1997). Monogyny and polygyny in Cardiocondyla appear to be associated with different types of habitat and a simple comparison of life spans between seasonally and continuously producing queens is therefore not possible. Our correction for the inactive hibernation period might have underestimated the true difference between the taxa. In any case, queen life span appears to be a plastic trait that evolves with changing life history. A similar point has previously been made concerning the life span of socially parasitic queens that kill the host queen and switch from iteroparity and long life to semelparity and short life (Bekkevold and Boomsma, 2000; Heinze and Tsuji, 1995). The prolongation of queen life span in monogynous *Cardiocondyla* is essential, as queens have to build up large workforce before they can rear sexuals. In contrast, queens of polygynous *Cardiocondyla* species are assisted by workers from the maternal nest *ab ovo* and produce sexual offspring almost immediately after mating.

At present we can only speculate about the causes of the transition from polygyny to monogyny in *Cardiocondyla*. Polygynous species occur predominantly in the tropics and produce sexuals in low numbers year-round. The large number of invasive *Cardiocondyla* species (Heinze et al., in press; Seifert, 2003) suggests that they easily adapt to habitat disturbances, in which polygyny might be favoured because it decreases generation time (Tsuji and Tsuji, 1996). Reverting this argument, deserts, semi-deserts, and steppe habitats might be stable enough for *Cardiocondyla* to build up population densities close to the carrying capacity, which, following Tsuji and Tsuji (1996), would favour monogyny. Habitat stability might also explain the loss of long-range dispersal and wing reduction (Roff, 1990).

## ACKNOWLEDGEMENTS

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# Chapter 4

# Inbreeding and local mate competition in the ant *Cardiocondyla batesii**

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A queen together with an ergatoid male of *C. batesii*

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## ABSTRACT

The ant species *Cardiocondyla batesii* is unique in that, in contrast to all other ant species, both sexes are flightless. Female sexuals and wingless, ergatoid males mate in the nest in autumn and young queens disperse on foot to found their own colonies in spring. The close genetic relatedness between queens and their mates ($r_{qm}$ = 0.76 ± SE 0.12) and the high inbreeding coefficient (F = 0.55; 95%CI 0.45 - 0.65) suggests that 83% of all matings are between brothers and sisters. As expected from local mate competition theory, sex ratios were extremely female-biased, with more than 85% of all sexuals produced being young queens. Despite the common occurrence of inbreeding, we could not detect any adult diploid males. Though the probability of not-detecting multiple mating was relatively high, at least one third of all queens in our sample had mated more than once. Multiple mating to some extent counteracts the effects of inbreeding on worker relatedness ($r_{ww}$ = 0.68 ± SE 0.05) and would also be beneficial through decreasing diploid male load, if sex was determined by a single locus complementary system.

*Keywords:* Formicidae, inbreeding, intranidal mating, multiple mating, local mate competition

## INTRODUCTION

Sex ratios in the Hymenoptera often deviate strongly from 1:1. This has been explained by two different factors: local mate competition (LMC), when related males compete for access to female sexuals (Hamilton, 1967), or, in social species, worker manipulation of sex allocation in response to the asymmetric relatedness to their sexual brothers and sisters (Boomsma, 1989; Bourke and Franks, 1995; Crozier and Pamilo, 1996; Nonacs, 1986; Trivers and Hare, 1976). In many parasitoid wasps and fig wasps, daughters and sons of one mother mate in a single, confined patch, such as the carcass of a host insect or a fig. In such a situation, mothers benefit most from producing just enough males to guarantee the insemination of all their daughters and produce progeny with a highly female-biased sex ratio (Herre et al., 1997; Werren, 1987).

In contrast, LMC appears to be of limited importance in the social Hymenoptera. Though it was soon brought up as an alternative cause of female bias (Alexander and Sherman, 1977), the necessary preconditions of LMC are rarely met in ants, bees, and wasps.

Instead, sib-mating appears to be typically avoided, perhaps because it leads to the production of diploid males owing to single locus complementary sex determination (Bull, 1983; Cook, 1993a). Fertilized eggs, which are heterozygous at this sex determination locus, give rise to diploid females, while unfertilized eggs with a single sex allele and also fertilized eggs with two identical sex alleles develop to haploid and diploid males, respectively (Beye et al., 2003; Bourke and Franks, 1995; Cook, 1993a; Cook and Crozier, 1995; Whiting, 1967). Haploid males are the normal male sexuals in Hymenoptera, but diploid males are often sterile and constitute additional costs in the social Hymenoptera because they do not work (Bull, 1983; Ross and Fletcher, 1985b). In ants with reduced genetic variability, diploid male load is indeed a significant factor for the failure of colony founding (Ross and Fletcher, 1986; but see Pamilo et al., 1994).

Several traits in the morphology and the behavior of sexuals and, on the colony-level, in the pattern of reproduction reduce the probability of mating among close relatives. For example, even in ants, where the workers are always wingless, sexuals are typically winged and disperse from their maternal nests before mating in large mating populations. Male or female wingless sexuals are known from many ant species, but exclusive winglessness of both sexes of a species has never been observed (Heinze and Keller, 2000; Heinze and Tsuji, 1995; Starr, 1984). In some species, mating occurs in the nest (intranidal mating), but here, too, the probability of mating among related individuals is typically reduced through pre-mating dispersal of the winged males, the adoption of alien males and / or the presence of multiple fertile queens (Boomsma et al., 2005; Hölldobler and Bartz, 1985; Hölldobler and Wilson, 1990; Passera and Keller, 1994; Peeters and Crewe, 1986; Starr, 1984). Furthermore, in many species colonies produce exclusively either male or female sexuals (split sex ratios; Boomsma and Grafen, 1990) and the males leave their maternal nests earlier than female sexuals.

Significant deviations from random mating and presumed local mate competition have been described for a couple of species. However, they usually result from limited dispersal, strong population sub-structuring and highly localized nuptial swarms with random mating among sexuals from only a couple of colonies and not from regular sib-mating as in parasitoid wasps or fig wasps (Chapuisat et al., 1997; Cole and Wiernasz, 1997; Foitzik and Heinze, 2001; Hasegawa and Yamaguchi, 1995; Pedersen and Boomsma, 1998; Sundström et al., 2003).

*Cardiocondyla batesii* Forel, 1894 appears to be a very unusual ant in all the above-mentioned respects, because males are always wingless ("ergatoid") and mate with winged female sexuals in their maternal nests in fall. Young, inseminated queens shed their wings after hibernation and disperse on foot to found their own colonies independently, i.e., without the help of workers. Histological analyses of the wing muscles and the regular occurrence of short-winged female sexuals in some populations confirm that female sexuals cannot fly (Heinze et al., 2002; Schrempf and Heinze, unpubl.). Furthermore, all excavated colonies contained only a single fertile queen (monogyny), and workers never tolerated the presence of more than one fertile queen per laboratory nest (Schrempf and Heinze, unpubl.).

Due to this unique combination of life history traits, *C. batesii* provides an exciting system for investigating the effects of possible brother-sister mating and strong local mate competition on the social and genetic structure of its colonies. Here we describe the genetic structure of colonies and populations of *C. batesii* and in particular address the following questions: whether local mate competition is associated with extremely female-biased sex ratios in this ant, whether diploid males are reared, and how strongly inbreeding affects nestmate relatedness.

## MATERIALS AND METHODS

### *Field collection and sampling*

Colonies of *C. batesii* are relatively small (10 to 120 workers) and nest in cavities in the soil down to a depth of more than 1m. In spring, many adults and brood can be found in the uppermost nest chambers, which makes the collection of complete or nearly complete colonies much easier during this season. Colonies were excavated and the positions of their nests were recorded at four different sites in the vicinity of Granada, Spain (Baza 37°31´N, 02°04´E; Padul

37°02´N, 03°40´E; Sierra Elvira 37°15´N, 03°45´E; Guadix 37°23´N, 03°08´E) in April 2001, October 2001, and June/July 2002. Each collecting site measured about 1500m$^2$ and distances between the sites ranged between 30 and 100 km. Colony density was high, with up to one nest per m². Both in spring and fall, winged female sexuals were found in the upper nest chambers of some colonies (mean 15.1 ± SD 15.5; min 2, max 53) together with workers and a single, fertile, dealate queen. In fall, several colonies contained ergatoid males.

Additionally, we collected founding queens that dispersed on foot in spring. Colonies and founding queens were transferred into standard artificial nests in the laboratory (Heinze and Ortius, 1991) and reared under temperature conditions simulating the natural seasons (winter: 12 hrs 8°C / 12 hrs 12°C; summer: 12 hrs 24°C / 12 hrs 27°C; 6 to 13 hrs light). For genetic analyses, ten to 20 workers each (total: 379 workers) from 34 summer colonies (8, 14, 5, and 7 colonies from the four populations) and 12 males collected in fall were frozen at -20°C shortly after collection. After polyandry had been detected (see below), an additional ten young workers each and a total of 24 males from nine laboratory-reared single-queen colonies were genotyped. Young workers in these colonies were definitely offspring of a single mother.

### *Microsatellite and allozyme analyses*

For allozyme electrophoresis, the gasters of all frozen individuals were homogenized in 20 µl buffer (0.1 M TRIS, 0.002 M EDTA, 0.05 mM NADP; pH 7.0). We applied 12 µl of the homogenate to 7.5% vertical polyacrylamide gels (gel buffer: 0.5 M TRIS/HCl; pH 8.0; running buffer: 0.2 M glycine, 0.025 M TRIS; pH 8.3). Proteins were separated by electrophoresis at 10 V/cm for 120 min, and enzymes were stained following standard methods (Harris and Hopkinson, 1978). Glucose phosphate isomerase (GPI) was slightly variable (three alleles), but other enzymes (malate dehydrogenase, esterase) showed no consistent variability in 40 individuals from different colonies. Following a method based on selective hybridization (Gautschi et al., 2000; Tenzer et al., 1999), eight microsatellite loci were isolated from ants from summer colonies. Two loci showed some variability with four (Card 8) and 11 (Card 21) alleles, respectively. The other loci did not reveal any variation in our sample (Table 1).

DNA for microsatellite analysis was isolated from heads and thoraces of all 469 frozen workers, 36 males, and 14 queens using a Puregene® DNA Isolation Kit (Gentra Systems, Minneapolis, MN), as described by Foitzik and Herbers (2001). Standard PCR reactions were carried out in 20 µl reaction volumes. The amplified PCR-Products (primers labeled with Tet and Fam dyes) were visualized on an ABI Prism 310 Genetic Analyzer. For 438 workers, the genotypes of both microsatellite loci were available. For 406 workers, all queens and all males, the genotypes at all three loci (microsatellites and GPI) were available. In many summer colonies queens could not be collected because of the depth of their nests, and in others they were kept alive for further behavioral investigations. In these cases, the genotypes of queens and their mates were reconstructed from the genotypes of their offspring. Subsequent genotyping of 14 queens confirmed that we had correctly reconstructed their genotypes from those of their workers.

**Table 1.** Primer sequences, number of alleles ($N_A$), size range, repeat motif, and primer pair-specific annealing temperature ($T_A$) of eight microsatellite loci developed for the ant *Cardiocondyla batesii.* EMBL accession numbers were obtained only for the first two microsatellite loci, which showed considerable variability

| Locus name | Primer sequences (5´to 3´) | $N_A$ | Size range | Repeat type | $T_A$ (°C) | Accession no. (EMBL) |
|---|---|---|---|---|---|---|
| **Card 8** | F 5´- TCG CCG TCT ATT CTG TCG TTA –3´<br>R 5´- CTA TTA TCG GCA ATG TGC –3´ | 4 | 126 - 134 | $(AC)_{13}$ AT $(AC)_3$ | 54 | AJ 568023 |
| **Card 21** | F 5´- GAA TCG TGA CGA AGC ATA C –3´<br>R 5´-GTA ATG GCC AAC GCC TCG C –3´ | 11 | 154 - 174 | $(AG)_{18}$ | 58 | AJ 568022 |
| **Card D1** | F 5´- AGT CGT GTT ACC GTT CCG AAC-3´<br>R 5´-CTC GGA GAC GTC GAA CTA AGT T-3´ | 1 | 212 | $(AC)_5GC(AC)_6GCACGC$<br>$(N)_{21}$ $(AC)_{10}$ | 55 | |
| **Card 19** | F 5´-GTG CAG GGC GTT ATA GGG CGA-3´<br>R 5´-ACA GCT GTC ATT TCG CGA TCG-3´ | 1 | 114 | $(GA)_4$ $(N)_3$ $(GA)_{14}$ | 56 | |
| **Card C8** | F 5´-GTG TAT ATA CAT ACA TAC ATA-3´<br>R 5´-TAG TGA CGT AAA GTC GCT C-3´ | 1 | 276 | $(AC)_{66}TC(AC)_{31}$ | 45 | |
| **Card 2.2** | F 5´-TCT GGA CTC AGT GCC AAT TAC-3´<br>R 5´-CTA GTC TGT AAT AGA AAC GC-3´ | 1 | 112 | $(GGC)_6$ GGT $(GGC)_2$ | 45 | |
| **Card B7** | F 5´-CTG AAT GGC GAA TGG ACG C-3´<br>R 5´-GGT CGA GGT GCC GTA AAG C-3´ | 1 | 208 | $CT_{30}$ | 59 | |
| **Card B11** | F 5´-AGG CTA CTA ACA TGC CGT GT-3´<br>R 5´-AAT GGA TTC ACG AAT AGA GC-3´ | 1 | 160 | $(CT)_{24}$ | 50 | |

### *Population genetic analysis*

Regression relatedness (Queller and Goodnight, 1989) among workers and between queens and their presumed mates was calculated using the program RELATEDNESS 4.2 (Goodnight and Queller, 1994). Groups were weighted equally and standard errors were estimated by jackknifing over colonies. Coefficients of population subdivision and inbreeding were estimated from worker genotypes in a three-level analysis with the program Genetic Data Analysis 1.1 (Lewis and Zaykin, 2001), based on the algorithms by Weir and Cockerham (1984). In this analysis, the lowest level represents individuals within colonies (however, as workers in colonies are related, they do not represent independent samples), the next level colonies within subpopulations, and the highest level subpopulations within populations.The values obtained by GDA are Weir's f (correlation between pairs of genes within individuals, compared to random genes within the colony), Weir's F (correlation between pairs of genes within the individual, compared to random genes within the subpopulation), $\theta_S$ (correlation between pairs of genes within colonies, compared to random genes within the subpopulation) and $\theta_P$ (correlation between pairs of genes within subpopulations, compared to random genes within the population). For population analysis, F gives information about the amount of inbreeding due to non-random mating within subpopulations, $\theta_P$ about allele frequency differences between subpopulations, taking variable population sizes into account (see also (Sundström et al., 2003). Confidence intervals were obtained by bootstrapping 5000 times over loci. The frequency of sib-mating α was estimated from $F = \alpha/(4-3\alpha)$ (Pamilo, 1985; Suzuki and Iwasa, 1980).

Mating frequencies of queens were inferred by directly comparing queen and worker genotypes. In eight cases, in which it was not clear from the worker genotypes whether a heterozygous queen had mated once or a homozygous queen had mated twice, we assumed the former scenario. The mating frequency calculated by hand may therefore be underestimated.

In general, the effective mating frequency can be given by the harmonic mean of the number of mates per queen over all queens in the sample, assuming equal paternity among males (e.g., (Pamilo, 1993; Pedersen and Boomsma, 1999; Ross, 1993). A more accurate estimate of the pedigree-effective mate number is $m_{e,p} = 1/\sum p_i^2$, where $p_i$ is the proportional paternity contribution of the i<sup>th</sup> male (Boomsma and Ratnieks, 1996; Starr, 1984). The unbiased estimation of $\sum p_i^2$ corrected for sampling error after Pamilo (1993) with the observed male contributions $y_i$ is given by $\sum p_i^2 = (N\sum y_i^2-1)/(N-1)$ ($y_i$ = number of detected fathers, N = number of analyzed offspring). The probability of missing a double-mated queen because two unrelated males have an identical multilocus genotype is $d = \prod_{j=1}^{k}\left(\sum_{i=1}^{aj} p_{ij}^2\right)$, where $p_{ij}$ denotes the frequencies of $a_j$ alleles at each of k loci (Boomsma and Ratnieks, 1996). With inbreeding, the non-detection error because of identical male genotypes at all loci can be calculated by $m_{e,p} = \left(\bar{\pi}\left(1-\prod_{k=1}^{l}\left(F+(1-F)(1-H_{\exp,k})\right)\right)\right)^{-1}$, where $\bar{\pi}$ is the sum of squared paternity contributions corrected for non-sampling error, $F$ is the inbreeding coefficient, and $H_{\exp,k}$ is the expected heterozygosity at the $k^{th}$ locus in the absence of inbreeding (JS Pedersen, personal communication).

In addition, we estimated the mating frequency of queens from their female offspring with the program MateSoft 1.0 (Moilanen et al., 2004), using the broad deduction option for maternal genotypes, as multiple mating appeared to be common. The estimated pedigree effective mate number ($m_{e,p}$) was subsequently corrected for non-detection error from identical male genotypes taking inbreeding into account (see equation above).

As null alleles may also cause heterozygote deficiency, we estimated the maximum frequency of potential null alleles as $r_0 = D / (2-D)$, where $D = (H_{exp}-H_{obs}) / H_{exp}$ (Brookfield, 1996; Chakraborty et al., 1992). The number of individuals, which are expected to be homozygote for the null allele and therefore do not yield amplification products in PCR, is $r_0^2$.

## RESULTS

### *Population structure and heterozygote deficiency*

The value of $\theta_P$ (0.12; 95% confidence interval 0.01 - 0.23) suggested a moderately high genetic differentiation among the different populations. The inbreeding coefficients F, both averaged over all populations (0.55; 95% CI 0.45 - 0.65) and estimated separately for each population (Table 2), revealed a considerable heterozygote deficiency. They did not differ significantly among the four populations (ANOVA; F = 0.99, df = 3, p = 0.45). Exclusion of GPI genotypes, which did not vary in populations 1 and 3, gave a similar result (F = 1.17, p = 0.42). The high inbreeding coefficient corresponds to 83.0 % brother-sister matings (CI 76.6% - 88.1%).

Heterozygote deficiency may result from non-amplifying null alleles. However, explaining the high inbreeding coefficients in *C. batesii* from the occurrence of null alleles alone would require null allele frequencies of 38% and 36% at the two microsatellite loci Card 8 and Card 21, respectively. Null alleles have normally much lower frequencies of below 15% (Jarne and Lagoda, 1996). Furthermore, with such high null allele frequencies, at least 13 of the genotyped 36 males and 61 of the 469 genotyped workers are expected to give no PCR product. In contrast, microsatellite DNA could be amplified in all males, and Card 8 could not be amplified only in three workers. Card 21, which is very sensitive to low DNA quality, gave no amplification product (or a product, which could not be evaluated) in 28 workers. Assuming that all these workers were homozygous for null alleles gives null-allele frequencies of 0.08 (Card 8) and 0.24 (Card 21), respectively. Correcting the expected heterozygosity by including hypothetical null allele homozygotes as an additional class of genotypes still gives positive inbreeding coefficients (F = 0.48 and 0.23, corresponding to 78.7 and 54.4% sib-mating). However, as all loci are similarly affected by inbreeding and the F-values differ more strongly after correction for hypothetical null-alleles, it appears that null alleles do not contribute considerably to the heterozygote deficiency.

**Table 2.** Inbreeding coefficients in the ant *Cardiocondyla batesii,* estimated by microsatellite analysis and enzyme electrophoresis on workers. The notation follows that given by the program GDA, based on Weir and Cockerham (1984). F describes the amount of inbreeding due to non-random mating in the parental generation, $\theta_P$ shows differences between subpopulations. Values for f (individual in the colony) are expected to be zero or negative, those for $\theta_S$ (colony within subpopulation) are positive, when individuals are related.

| **Locus** | **F** | **F** | $\theta_S$ | $\theta_P$ | **Relatedness R ± SE** |
|---|---|---|---|---|---|
| GPI | 0.18 | 0.31 | 0.15 | 0.03 | 0.16±0.11 |
| Card 8 | 0.05 | 0.56 | 0.54 | 0.01 | 0.68±0.09 |
| Card 21 | -0.02 | 0.56 | 0.57 | 0.17 | 0.72±0.05 |
| **All three loci** | **0.01** | **0.55** | **0.54** | **0.12** | **0.68±0.05** |
| upper 95% CI | 0.11 | 0.65 | 0.70 | 0.23 | 0.78 |
| lower 95% CI | -0.08 | 0.45 | 0.38 | 0.01 | 0.58 |
| **Population** | | | | | |
| Sierra Elvira | 0.03 | 0.45 | 0.43 | | 0.60±0.09 |
| Padul | 0.00 | 0.39 | 0.39 | | 0.63±0.07 |
| Guadix | 0.18 | 0.55 | 0.45 | | 0.64±0.11 |
| Baza | -0.1 | 0.67 | 0.70 | | 0.84±0.10 |

### *Queen mating frequency*

The worker genotypes matched the assumptions of monogyny and monandry in 19 of 34 colonies (55.9%; excluding two presumably "alien" workers from one colony, see below), monogyny and double paternity in 12 (35.3%; excluding three "alien" workers from one colony), and monogyny and triple paternity in three colonies (8.8%). Double mating was also found in three of nine single-queen laboratory colonies (33.3%), supporting the view that genetic heterogeneity in field colonies is usually not due to serial or simultaneous reproduction by multiple queens. Combined for all populations, the mean number of fathers detected per colony was 1.49 ± SD 0.63 (uncorrected pedigree-effective mate number, $m_{e,p} = 1.35$; corrected for sampling error 1.48). The likelihood of missing a double-mated queen because of the probability that two unrelated males share the same multilocus genotype was $d = 0.12$ when estimated from the overall allele frequencies at all three loci and 0.03 when calculating for each queen separately. However, this non-detection probability is considerably increased through regular inbreeding, resulting in 43 % of the males being identical at all loci. Therefore, the corrected mating frequency (based on the mean number of fathers) was $m_{e,p} = 2.63$.

The average estimated and corrected mating frequency over all groups obtained from MateSoft was $m_{e,p} = 3.42$ (average $p_i$ over all groups = 0.52). The average number of matings detected based on the frequency distribution of the number of patrilines per group was $k = 1.83$ (corrected for inbreeding = 3.23) and only slightly higher than the minimum average number of matings estimated from the smallest number of matings found per group ($k_{min} = 1.80$; corrected for inbreeding = 3.18). The power to correctly deduce the queen genotypes was > 0.95 for all offspring groups except for one group, which was therefore left out from the analysis. As more than one queen genotype may occasionally be compatible with offspring data, MateSoft calculates the probabilities of the alternative genotypes from allele frequencies and / or the allele segregation among the offspring. In case any of the queen alternatives implies a segregation of the alleles among the offspring with very low probability (less than 0.2), we deleted these genotypes from the dataset. Furthermore, we excluded queen alternatives of 14 queens, for which the actual genotypes were known. The mating frequency calculated by hand is less than that calculated by MateSoft, because in the first case queens were always assumed to be heterozygous and single-mated rather than homozygous and double-mated.

The effective mating frequencies as estimated by MateSoft did not differ among populations (ANOVA: $F = 1.51$, $df = 39$, $p > 0.23$). Nevertheless, the four populations appeared to differ significantly in the frequency of multiply mated queens. Queens from Sierra Elvira were more often multiply mated (87.5%: seven of eight colonies) than queens from Padul (35.7%: five of 14 colonies, Fisher's exact test: $p=0.03$; including the laboratory colonies: 34.8%: eight of fifteen colonies, $p=0.01$) and Baza (16.7%: one of six colonies, $p=0.01$). Two of five queens (40.0%) from Guadix were also multiply mated (Figure1). Overall populations, colonies with singly-, doubly- and triply-mated queens did not differ in worker number (ANOVA: $F = 0.87$, $df = 2$, $p > 0.43$; Scheffé test: $p > 0.44$, 0.80, and 0.99; Figure 2).

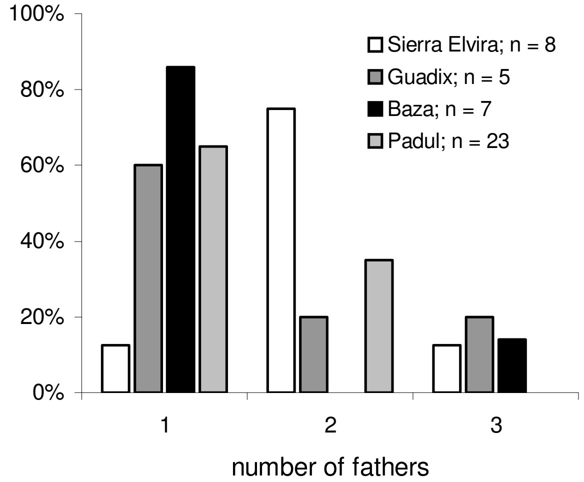

**Figure 1.** Frequency of multiple paternity in four different populations of the ant *Cardiocondyla batesii* (including laboratory colonies).

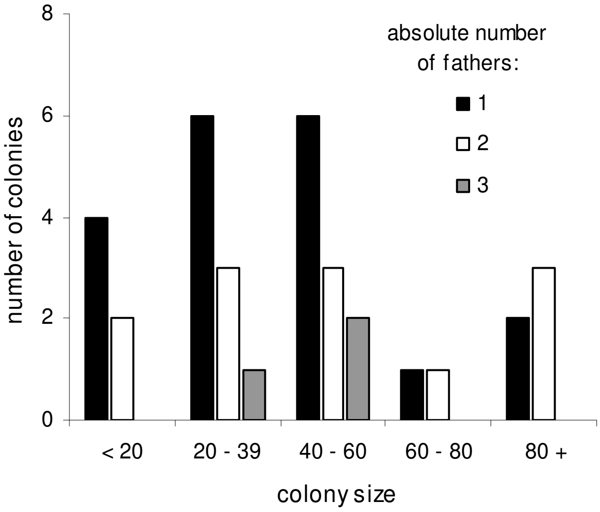

**Figure 2.** Frequency of multiple paternity in different size classes of *C. batesii* summer colonies (34 colonies).

### *Colony structure and relatedness*

Worker genotypes were consistent with monogyny in 32 of 34 colonies (94.1%). In two colonies, several workers (two out of ten and three out of twelve; 22.5%) did not share a single allele with the queen but obviously belonged to a different matriline. As our laboratory observations indicate that it is unlikely that several fertile queens can coexist within a single nest (polygyny) (Schrempf and Heinze, unpubl. results), the occurrence of multiple matrilines might be explained by queen replacement or the accidental adoption of stray foragers from neighboring colonies. However, the latter is not likely, since all foreign workers in each colony belonged to a single matriline. Furthermore, the genotypes of the alien workers did not fit to genotypes of neighboring colonies. In contrast, laboratory experiments suggested that founding queens may occasionally take over alien colonies (Schrempf and Heinze, unpubl. results).

The overall relatedness among nestmate workers was $b_{ww} = 0.66 \pm$ SE 0.07 (excluding the "alien workers" from two colonies 0.68 ± 0.05). Relatedness was not significantly different between the four populations (ANOVA: $F = 1.07$, $df = 3$, $p = 0.38$; Table 2). The regression relatedness of the genotyped queens ($n = 14$) to their presumed mates was $r_{qm} = 0.70 \pm 0.27$, and that of the presumed mates to the queens was $r_{mq}= 0.29 \pm 0.17$. Including reconstructed queen genotypes in the analysis and thereby increasing the sample size ($n = 43$) leads to a $r_{qm}= 0.76 \pm 0.12$ and $r_{mq}= 0.26 \pm 0.09$. These values are significantly different from zero (t-test, $t = 6.33$, $p < 0.001$ and $t = 2.89$, $p < 0.001$). The estimated $r_{qm}$ is slightly higher than the regression relatedness of sisters to brothers (0.5; $t = 2.17$, $p < 0.05$), while $r_{mq}$ is not significantly different from that of brothers to sisters (0.25; $t = 0.11$, $p > 0.5$). In nine of 43 cases (20.9%), the alleles of one mate differed from those of the queen (two single, four double, three triple matings), suggesting that the mating partners occasionally came from different colonies.

### *Sex ratios*

Numerical sex ratios (relative proportion of female sexuals among all reproductives) were always highly female-biased (field-colonies in fall: 0.94 ± 0.04; laboratory reared colonies: 0.87 ± 0.09, Table 3). For field colonies, the coefficients of variability V (s/mean) were 43% for males and 65% for female sexuals. Laboratory colonies give similar values, when one colony with an extraordinarily large number of males is excluded (males: $V = 56\%$, female sexuals: $V = 79\%$).

In all laboratory colonies, one or two males emerged a few days before first female pupae eclosed. In some colonies, one or a few additional males emerged some days later together with the first female sexuals (the total number of males is given in Table 3). The rarity of adult males is in accordance with local mate competition theory and also suggests that inbreeding does not lead to a substantial production of large numbers of adult diploid males. Furthermore, genotyping did not reveal any adult diploid males. Based on the frequency of heterozygosity among nestmate workers, 23 of 36 investigated males would have been heterozygous at one of the three loci had they all been diploid.

**Table 3.** Sexuals present in field and laboratory colonies of the ant *Cardiocondyla batesii* and numerical sex ratio (relative proportion of female sexuals among all reproductive) in fall. Mating is presumed to occur in fall and female sexuals disperse after hibernation in spring.

| | **Young queens** | **Ergatoid males** | **Numerical sex ratio** |
|---|---|---|---|
| **field (fall)** | 7 | 1 | 0.875 |
| | 28 | 1 | 0.965 |
| | 12 | 1 | 0.923 |
| | 5 | 0 | 1 |
| | 10 | 1 | 0.909 |
| | 10 | 1 | 0.909 |
| | 7 | 1 | 0.875 |
| | 38 | 2 | 0.950 |
| | 32 | 2 | 0.941 |
| | 13 | 1 | 0.929 |
| | 8 | 1 | 0.889 |
| | 17 | 1 | 0.944 |
| | 39 | 1 | 0.975 |
| | 32 | 1 | 0.970 |
| | 29 | 1 | 0.967 |
| **mean** | **19.13** | **1.07** | **0.935** |
| **SD** | **12.35** | **0.46** | **0.038** |
| **laboratory (fall)** | 5 | 1 | 0.833 |
| | 35 | 4 | 0.897 |
| | 10 | 1 | 0.909 |
| | 6 | 2 | 0.750 |
| | 4 | 3 | 0.667 |
| | 30 | 2 | 0.938 |
| | 28 | 3 | 0.903 |
| | 20 | 1 | 0.952 |
| | 58 | 10 | 0.853 |
| | 18 | 1 | 0.947 |
| **mean** | **21.40** | **2.80** | **0.865** |
| **SD** | **16.98** | **2.74** | **0.092** |

## DISCUSSION

Our data show that a considerable percentage of female sexuals of the ant *Cardiocondyla batesii* mate multiply and that more than 80% of all matings are between brothers and sisters. The inbreeding coefficient is the highest as yet reported from social Hymenoptera. Though DNA could be amplified in all haploid males, we cannot rule out that the inbreeding coefficient is inflated to some extent by null alleles. Nevertheless, the F-values remained significantly positive even after a correction for null alleles. As expected from local mate competition theory (Hamilton, 1967), sex ratios were highly female-biased. All three phenomena - local mate competition, inbreeding, and multiple mating - are remarkable in themselves, but their combination within a single ant species makes *C. batesii* truly exceptional.

Inbreeding with local mate competition and strongly female-biased sex ratios are common in solitary haplodiploid animals. Female ambrosia beetles, parasitoid wasps, and fig wasps adaptively adjust the sex ratio in their progeny by rearing more sons in the presence of reproducing conspecific females than when alone (e.g. Herre, 1985; King, 1986; Peer and Taborsky, 2004). In ants, evidence for local mate competition affecting sex ratios is rare, owing to the prevalence of pre-mating dispersal (Boomsma et al., 2005; Hölldobler and Bartz, 1985).

Local mate competition in small, localized mating swarms may promote female-biased sex allocation in *Messor aciculatus* (Hasegawa and Yamaguchi, 1995). Sex ratios are also more female-biased than predicted from relatedness asymmetries alone in *Technomyrmex albipes*, an ant with locally mating, wingless replacement reproductives (Yamauchi et al., 1991), and several socially parasitic ants with intranidal mating, such as *Myrmoxenus kraussei* (Bourke, 1989; Winter and Buschinger, 1983). Of particular interest is the situation in *Cardiocondyla obscurior*, a facultatively polygynous relative of our study species. *C. obscurior* queens produce only very few ergatoid males, as long as they are the only reproductives in a colony, but strongly increase the production of such fighter males when other fertile queens are present in their nests (Cremer and Heinze, 2002; deMenten et al., 2005).

Ergatoid males of *C. obscurior* always engage in deadly fighting, even in monogynous societies. In contrast, about one third of the sampled *C. batesii* colonies contained several mutually tolerant males, and male fighting appeared to be very rare (unpubl. results). It appears that queens produce just enough males to ensure the insemination of all their daughters. Ergatoid *Cardiocondyla* males differ from other Hymenopteran males in that their spermatogenesis continues throughout their lives (Heinze et al., 1998; Heinze et al., 1993). Very few males are therefore sufficient to inseminate all female sexuals produced in a colony. The number of males varied considerably less than the number of female sexuals, which is in accordance with the assumption that colonies invest first in a few males and, depending on resource availability, in a varying number of female offspring (constant male hypothesis, (Frank, 1987). Though queen mating frequency varied considerably between colonies, it is quite unlikely that this variation alone explains our sex ratio data. According to split sex ratio theory (Boomsma and Grafen, 1990), workers from monandrous colonies produce a highly female-biased sex ratio, whereas workers from polyandrous colonies invest mostly in males, because the relatedness asymmetry of workers to female and male sexuals decreases. This results in bimodally distributed colony-level sex ratios, but sex ratios in *C. batesii* were always highly female-biased. The number of colonies for which colony size, mating frequency and sex ratio are exactly known is too small, however, to investigate whether sex ratios meet predictions about the variation of sex ratios under inbreeding with varying mating frequency.

The genetic data match conclusions based on the morphology and behavior of sexuals. Female sexuals apparently mate in their maternal nests before dispersing on foot, and all young queens that were collected outside their nests in spring were inseminated (Heinze et al., 2002). It is, therefore, quite surprising that approximately 1/5 of all matings involve unrelated partners. Though the wingless, small-eyed ergatoid males are obviously not well adapted for dispersal, they (and / or the winged female sexuals) might easily cross the short distance between neighboring nests to outbreed. Such behavior has as yet not been observed in the field, though Marikovsky and Yakushkin (1974) incorrectly cite Morley (1954) for ergatoid male dispersal in *Cardiocondyla*. However, Morley (1954) mentions neither ergatoid males nor *Cardiocondyla*. LMC should nevertheless be strong, as males apparently first mate inside the nest before dispersal.

In spite of the high inbreeding coefficient we did not detect any diploid males. Even if our result is based on only a small number of analyzed males, the female-biased sex ratio supports the assumption that adult diploid males are absent or rare. Diploid males might either be detected and eliminated at a very early stage of development, as in honeybees (Woyke, 1963), or are not produced at all. In a system with regular inbreeding, however, it seems more likely that a sex determination mechanism has evolved, which is not susceptible to inbreeding. Such mechanisms, e.g. genomic imprinting, have previously been suggested for parasitoid wasps and socially parasitic ants with regular sib-mating (Beukeboom et al., 2000; Buschinger, 1989; Dobson and Tanouye, 1998; but see Stahlhut and Cowan, 2004).

Even with the rather low resolution of our genetic markers, we were able to detect multiple mating in about 1/3 of all queens from both natural and definitively monogynous laboratory colonies. The correspondence between field and laboratory data indicates that queen replacement, though it might occasionally occur in the field, does not strongly affect the genetic colony structure. The effective frequency of multiple mating, both calculated by hand and with the program MateSoft, indicates that queens mate on average with more than two males. Laboratory observations of males indicate that all males copulate with all female sexuals, since they almost continuously show sexual behavior. Field colonies contained mostly single males, but the high frequency of multiply mated queens and our laboratory results suggest that not all males are produced at the same time. In laboratory colonies, one or two males were produced before the young queens eclosed, and several males eclosed later. Some of them might leave the colony after mating with their sisters and enter alien colonies to mate with unrelated queens, as well.

Although frequent multiple mating has been reported in several species of ants with large colony sizes (e.g., *Formica*, Chapuisat, 1998; Pamilo, 1993; Sundström, 1993; *Acromyrmex,* Bekkevold et al., 1999; Boomsma et al., 1999; *Pogonomyrmex*, Cole and Wiernasz, 1999; Gadau et al., 2003), female sexuals of related genera of *Cardiocondyla* with small colonies typically mate only once (e.g. *Harpagoxenus,* Bourke et al., 1988; *Temnothorax*, Foitzik et al., 1997; *Leptothorax*, Hammond et al., 2001; *Protomognathus*, Foitzik and Heinze, 2001). Multiple mating in *C. batesii* lowers nestmate relatedness to 0.68 and thus counteracts the effects of inbreeding on relatedness. The frequency of multiple mating appeared to differ slightly between populations, but more data are needed to corroborate this result and investigate the consequences of varying mating frequency.

Hypotheses that explain polyandry through benefits from higher genetic variability are not easily applicable in *C. batesii*, because they have only a small colony size, simple division of labor, and live in a uniform habitat. *Cardiocondyla* workers are sterile (Heinze et al., 1993) and due to inbreeding and local mate competition, their interests converge with those of the queen towards a highly female-biased sex ratio. The sperm-limitation hypothesis (Cole, 1983) is also not supported because of the small colony size of *C. batesii*. Furthermore, our results show that single-mated queens produce similar worker numbers as multiply-mated queens. Polyandry may be a strategy of reducing diploid male load in species with complementary sex determination (Page and Metcalf, 1982; Pamilo et al., 1994), but as suggested above, *C. batesii* might have evolved a sex determination mechanism, which is not sensitive to inbreeding.

Finally, multiple mating is typically considered to be selected against because it is costly to queens due to increased energy expenditure, time loss, and higher risks of predation and parasite transmission (Boomsma and van der Have, 1998; Bourke and Franks, 1995; Keller and Reeve, 1994; Sherman et al., 1988). In *C. batesii*, these risks are probably negligible, because female sexuals mate in the safety of the nest. Because there are nearly no costs of multiple mating, it might simply not be selected against even if it were not associated with any particular fitness benefits.

## ACKNOWLEDGEMENTS

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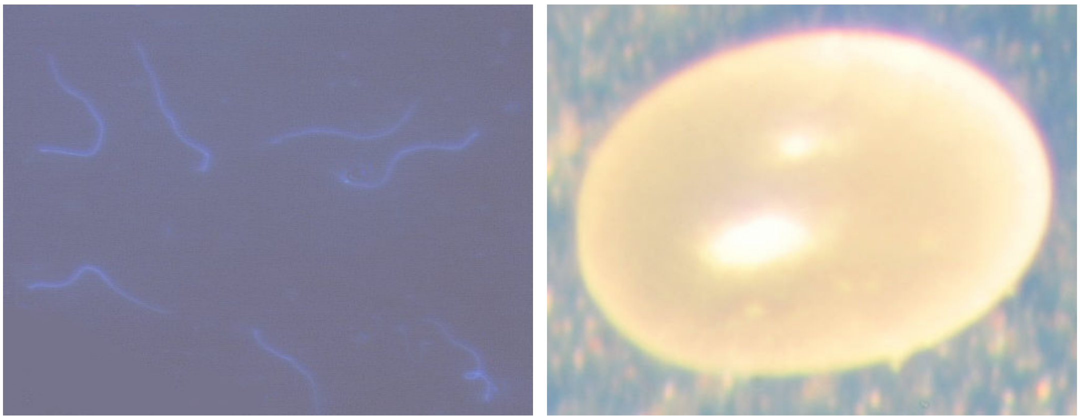
# Chapter 5

# Exclusion of complementary sex determination, inbreeding depression and sex ratio adjustment in the ant _Cardiocondyla obscurior_*

Alexandra Schrempf, Serge Aron and Jürgen Heinze



Spermatozoa of an ergatoid male (stained with DAPI) and an egg of a queen of *C. obscurior*

* Manuscript to be submitted to Heredity

## ABSTRACT

In many haplodiploid Hymenoptera, sex is determined at a single polymorphic locus (single locus complementary sex determination, sl-CSD). Individuals which are heterozygote at this locus develop as females, whereas hemizygote and homozygote individuals develop as haploid and diploid males, respectively. Diploid males are typically sterile or unviable. As inbreeding is expected to result in an increase in homozygosity and thus in diploid male production, other mechanisms than sl-CSD have been proposed to act in regularly inbreeding species. Several authors suggested complementary sex determination with multiple loci (ml-CSD) or genomic imprinting as the most likely alternatives. In this study, we examine the sex determination mechanism of the inbreeding ant species *Cardiocondyla obscurior.* After ten generations of inbreeding we can exclude sl-CSD or ml–CSD with a few loci as mechanism of sex determination. Nevertheless, inbreeding depression occurred and lead to a shift in sex ratio towards a male bias and high brood mortality. Both seem to be attributable mainly to male sterility, although haploid males in Hymenoptera cannot experience inbreeding depression directly, but only indirectly via maternal effects. Finally, we could show that females adjust the sex ratio according to inbreeding conditions as expected due to relatedness asymmetry.

*Keywords*: sex determination system, inbreeding depression, complementary sex determination, sex ratio, haplo-diploidy, Hymenoptera

## INTRODUCTION

In Hymenoptera, the common mode of sex determination is parthenogenetic arrhenotoky, where males develop from unfertilised eggs and are haploid, while females develop from fertilised eggs and are diploid. Different models have been proposed for the mechanism of sex determination, but only two of them have been confirmed by studies so far: single locus complementary sex determination (Whiting, 1939; Whiting, 1943) and genomic imprinting (Beukeboom, 1995). Whereas the latter was shown for *Nasonia vitripennis* (Dobson and Tanouye, 1998), single locus complementary sex determination (sl-CSD) seems to be widespread in haplodiploids. Individuals which are heterozygous at the sex locus develop into females, whereas hemizygous individuals develop into males. Homozygosity at the sex locus usually results in the production of inviable or sterile diploid males (Cook, 1993a; Cook, 1993b; Stouthamer et al., 1992; Whiting, 1943). Although some diploid males have been shown to produce viable sperm, sperm cells are usually diploid and result in sterile triploid progeny (Duchateau and Marien, 1995; Krieger et al., 1999; but see Cowan and Stahlhut, 2004, for fertile diploid males).

Diploid males have been detected in 34 species of Hymenoptera (Stouthamer et al., 1992), and underlying sl-CSD has been demonstrated e.g. in the parasitic wasp *Bracon herbetor* (Whiting, 1943), *Diadromus pulchellus* (Périquet et al., 1993) and the bumble bee *Bombus terrestris* (Duchateau et al., 1994). The probability that a mating is “matched”, so that both partners share the same allele at the sex locus, is dependent on the amount of alleles, and estimated ranges typically are from ten to 20 alleles (Cook and Crozier, 1995). However, in species that prevalently mate with their siblings as e.g. many parasitoids, sl-CSD would result in a large number of diploid males due to a fifty percent probability of a matched mating. Thus, sl-CSD is unlikely to operate in these. Indeed, prolonged inbreeding experiments do not lead to high levels of diploid male production in some species, and Crozier (1971) suggested that in these sex may depend on multiple loci instead of one single locus (multiple locus complementary sex determination, ml-CSD). He argued that as long as occasional outcrosses occur, diploid male production would remain low even under inbreeding. A number of generations of inbreeding is necessary to detect ml-CSD, because diploid males are produced more slowly and production is dependent on the number of sex loci. Only individuals which are homozygous at all loci will develop as diploid males. Up to now, ml-CSD has only been explicitly tested in two studies, and in these, CSD could be rejected (*Goniozus nephantidis*, Cook, 1993b; *Nasonia vitripennis*, Skinner and Werren, 1980). Later on, genomic imprinting was shown to act as mechanism in *Nasonia*, and this may be an alternative for all other inbreeding species, as well. Here, one or more loci are differentially imprinted in paternal versus maternal development (Beukeboom, 1995; Poiré et al., 1993), and the few diploid males, which can be found, apparently arise by mutation.

In social insects (ants, bees, wasps), sl-CSD has been shown in the honey bee *Apis mellifera* (Beye et al., 2003; Mackensen, 1951; Woyke, 1965) and the bumble bee *Bombus terrestris* (Duchateau et al., 1994). Diploid male production has been shown to increase colony mortality (Ross and Fletcher, 1986) and to decrease colony growth rates as diploid males are produced at the expense of workers (Plowright and Pallett, 1979; Ross and Fletcher, 1986).

In this study, we investigate the sex determination system of the ant species *C. obscurior*. Generally, the mechanism of sex determination in the Hymenopteran suborder *Formicidae* has not been examined up to now, although diploid males are known from 19 species and sl-CSD was suggested for the fire ant *Solenopsis invicta* due to a high amount of diploid males (Hung et al., 1972; Hung and Vinson, 1976; Hung et al., 1974; Ross and Fletcher, 1985a; Ross and Fletcher, 1986). However, detailed studies have not been performed, and the number of diploid males would be consistent with both, two-loci-CSD with 14 alleles or sl-CSD with 86 alleles (Cook and Crozier, 1995).

The ant genus *Cardiocondyla* is known to inbreed consistently, as it is characterized by mating inside of the nest between related individuals. For example, in *C. batesii*, genetic analysis revealed that more than 80% of the matings are among siblings (Schrempf et al., 2005a), and high levels of inbreeding were also shown to occur in *C. nigra* (Schrempf and Heinze, unpubl.). Although the degree of inbreeding may vary among the different species, e.g. dependent on the number of reproducing queens in a colony, sl-CSD as sex determination mechanism seems unlikely for all of them. So far, no exceptional (e.g. bigger) males have been found, and the few genetic data collected revealed no diploid males, as well.

We performed inbreeding experiments for ten generations and investigated sex ratio, mortality of brood and ploidy level of males in *C. obscurior*. Genetic analysis of field colonies with newly established microsatellite markers confirm that the species is highly inbred, despite of being facultative polygynous (unpubl. result).

## MATERIALS AND METHODS

### *The study species Cardiocondyla obscurior*

Colonies of *Cardiocondyla obscurior* were collected in Bahia, Brazil in September 2000 and reared in the laboratory till the start of the experiment (August 2002) at 30°C-25°C temperature and day-night (12h/12h) cycle. In this species, several queens can reproduce together in one colony. Young queens and males, both offspring of the colony, mate inside the nest with each other. Males can be both ergatoid wingless or winged and the latter leave the colony after mating inside the nest. When we started the experiments, colonies could be more or less inbred depending on the relatedness and number of queens in the colonies at the time of collection. Under "normal" conditions, colonies produce only ergatoid males and sex ratio is adapted to local mate competition and thus female biased (Cremer and Heinze, 2002). Under stressful conditions, however, colonies produce winged males as well, and sex ratio tends towards 0.5. In the following experiments, all colonies produced winged and ergatoid males due to the detailed investigation of the colonies and the removal of pupae twice per week, which acts as a stress factor.

### *Detection of sl-CSD and two-loci ml-CSD (first generation of inbreeding)*

Twelve single queen colonies were established in October 2002 by mating newly emerged virgin females with males from alien colonies. Matings were performed in colonies with 20 workers. Some days later, queens started egg-laying, and, after approximately four weeks, first young workers eclosed in this set–ups. Later on, sexuals were produced as well. These single queen colonies were used to create inbred (brother-sister pairs) and control set-ups by transferring a queen pupae and a male pupae (brother or unrelated) to a colony of 20 orphaned, unrelated workers, where the sexual pupae eclosed and mated with each other. Two of the single queen colonies produced only a few female sexuals, and in one, male and female pupae developed at a different time, so that an inbred line was not possible to create.

Twelve control (queen and alien male) and 16 inbred colonies were established (inbred: from nine different single-queen colonies; control: from ten different single-queen colonies). Number of worker pupae and eggs was recorded twice a week, and worker number was kept constant by removing all additional workers (beyond 20) to standardize the set-ups. Furthermore, all sexual pupae were removed and sex ratio was recorded. Workers in ant colonies are females and are important to be considered in the sex ratio as well, but as it would be detrimental for the colonies to remove all worker pupae, we compared the mean number of pupae counted twice per week of the different set-ups. In case of diploid male production (viable or non-viable), the number of worker pupae should decrease (as 50% of workers are expected to become diploid males). Additionally, a shift of the sex ratio of reproductives is expected towards a male bias (because 50% of queens will be male). Set-ups were controlled till the death of the queen (mean: 31 weeks) and, if possible, a new generation was established afterwards. As sex ratio is expected to be different under winged and ergatoid male production, allocation of winged and ergatoid males was compared between set-ups.

### *Detection of multiple locus CSD (generations 2-10)*

Sexual pupae of the first generation were inbred for nine more generations over a period of three years (n= 238; 10 to 37 set-ups per generation). Additionally, control set-ups were created as well (n= 118; 6 to 18 set-ups per generation). The last generation (ten) was only controlled for a shift in sex ratio (not till the death of the queen). In all colonies, growth was not limited any more, and number of worker pupae and eggs was recorded only irregularly in colonies growing well. All sexual pupae were removed and recorded twice per week. In case a colony was exceptional, e.g. did produce a lot of male pupae and / or number of worker pupae was low, it got quickly obvious during this observations. Then, additionally egg number and worker pupae number was recorded.

### *Diploid male production*

In some colonies, sex ratio got male biased, and in most of them, the proportion of males increased in further generations. From colonies with extreme bias, male pupae were collected and flow cytometry was conducted to detect the ploidy level of the males. Furthermore, we performed flow cytometry with eggs of those colonies, to compare the secondary sex ratio with the primary sex ratio. For the method, see Roisin and Aron (2003).

### *Fertility of inbred males and females*

Males and virgin queens of the above mentioned “exceptional“ colonies were allowed to mate with unrelated sexuals to detect whether inbred individuals are sterile, and whether an “outbreeding“ generation cancels out the effect of inbreeding on sex ratio and brood mortality. In addition, two winged and four ergatoid males of the exceptional colonies were dissected to discover “abnormalities” and to control whether they contain a visible amount of sperm.

### *Lifespan of queens*

To compare the queens´ age of inbred and outbred set-ups, only those were considered which were found dead in the colonies in order to avoid distortion of data by taking erroneously colonies into account in which queens were lost. Furthermore, we considered only queens which survived the critical founding stage with a minimum lifespan of five weeks.

### *Data analysis*

Set-ups with less than five sexual pupae were discarded from sex ratio analysis, as the number did not differ between inbred and control set-ups (16 of 118 controls, 33 of 238 inbred set-ups; $\chi^2$: 0.01; p= 0.94). One worker pupae eclosed in all colonies at least, confirming that all queens were mated. Before data were pooled, heterogeneity tests (Brown-Forsythe–test of homogeneity of variance) were performed to determine whether pooling is statistically valid. As the number of worker pupae was not different between inbred and control set-ups (see below), expected sex ratios (of sexuals) for the inbred set-ups under sl-CSD and two-loci ml-CSD can be calculated from the observed sex ratio in the control crosses, both for the assumption of viable and unviable diploid males (for calculation see Beukeboom et al., 2000). Deviations from the expected sex ratio were tested using a chi-squared test with Yates correction. Sex ratios are given as female proportion.

In general, under sl-CSD, 50% of all matings are expected to be matched, and in these, 50% of the fertilized eggs will develop as diploid males, thus, the probability that a diploid offspring will be male is 25%. Under the assumption of ml-CSD with two loci, the diploid must be homozygous at both separate loci to develop as male. In this case, only one–fourth of matings will be matched, and in these, one-fourth of diploid offspring will be homozygous at both loci, thus, 6.25 % of fertilized eggs will be diploid males. In case diploid males are viable, the number of only male brood producing colonies should increase due to sterility of diploid males.

Multiple locus CSD with more than two loci should lead to an increase in sex ratio towards male bias and a decrease of worker number, but more slowly than under sl-CSD and two-loci ml-CSD. For example, in case of ten loci, each with two alleles, only one single diploid of 1000 is expected to be male in the first generation ($1/2^{k}$; k = number of sex loci). In case of a matched mating, the number of diploid males cannot exceed 50% of the diploid eggs (equal to sl-CSD), so that in any case female offspring is expected to occur.

## RESULTS

### *Detection of sl-CSD or tow-loci ml-CSD (first generation of inbreeding)*

Inbred (I, n = 15) and control groups (C, n = 9) did neither differ in the number of sexuals, workers and eggs produced in the colonies nor in the longevity of the queens (Kolmogorov-Smirnov n.s. for all data.; $n_C$= 9, $n_I$ = 15; mean number of sexuals C: 23.0, I: 26.3; t = -0.56, p =

0.58; mean number of worker pupae: C: 7.14, I: 7.17, t = -0.07, p = 0.93; mean egg number: c: 8.86, I: 8.61, t = -0.46, p = 0.64; mean queen age $n_C = 8$, $n_I = 13$: C: 30.8 weeks, I: 31.5 weeks; t = -0.12, p = 0.9). The number of sexuals produced in the colonies correlated significantly with the longevity of the queens (Spearman rank correlation: n=21, R = 0.56, p = 0.008), but brood sex ratio did not correlate with the queens´ age or number of sexuals produced (Spearman rank correlation: n=21; R = 0.02, p = 0.92 and R = 0.17, p = 0.45). All colonies produced winged and ergatoid males. Allocation of winged males and ergatoid males did not differ between inbred and control set-ups (ratio winged males / total; Kolmogorov-Smirnov: n.s.; $n_C = 9$, $n_I = 15$, t-test: t = 1.67, p = 0.11).

Heterogeneity tests revealed that sex ratio data were sufficiently homogeneous to be pooled for each group for subsequent analysis (control group: F = 1.29, p = 0.25; inbred group: F = 1.66, p = 0.1). In case of sl-CSD or two-loci ml-CSD, at least seven or three of the matings should be matched and result in diploid male production, respectively. However, all inbred colonies performed well, and in all, sex ratio was female biased. The lack of diploid male production is further supported by the comparison of sex ratio data: expected values for sex ratio under sl-CSD and two-loci ml-CSD are given in Table 1, based on data of the control group, and under the assumption of 25% (sl-CSD) and 6.25% (two-loci ml-CSD) diploid male production.

**Table 1.** Expected number of males and females under sl-CSD and two-loci ml-CSD based on data of the control group. The observed sex ratio values after one inbreeding generation were neither consistent with sl-CSD nor with two-loci ml-CSD, but differed also significantly from the control group.

| | **Number of males and females** | **Sex ratio (female proportion)** | **Chi-squared-test with Yates correction** |
|---|---|---|---|
| Control crosses | 116 / 87 | 0.43 (n=9 crosses, 203 sexuals) | <0.0001 |
| Observed results in inbred set-ups | 148 / 246 | 0.62 (n= 15 crosses, 394 sexuals) | |
| Expectations based on viable diploid males under sl-CSD | 266 / 127 | 0.32 | < 0.0001 |
| Expectations based on unviable diploid males under sl-CSD | 252 / 142 | 0.36 | < 0.0001 |
| Expectations based on viable diploid males under two-loci ml-CSD | 236 / 158 | 0.40 | < 0.0001 |
| Expectations based on unviable diploid males under two-loci ml-CSD | 232 / 162 | 0.41 | < 0.0001 |

Data were neither consistent with sl-CSD nor with two-loci ml-CSD. In inbred set-ups, sex ratio (females / total) is significantly more female biased than in control groups (Figure 1). Whereas in six of nine control colonies sex ratio is slightly male biased, this is the case in none of the inbred set-ups (chi-squared-test with Yates-correction: $\chi^2 = 5.94$, $p = 0.014$). This is contrary to expectations and reveals sex ratio adaptation to inbreeding.

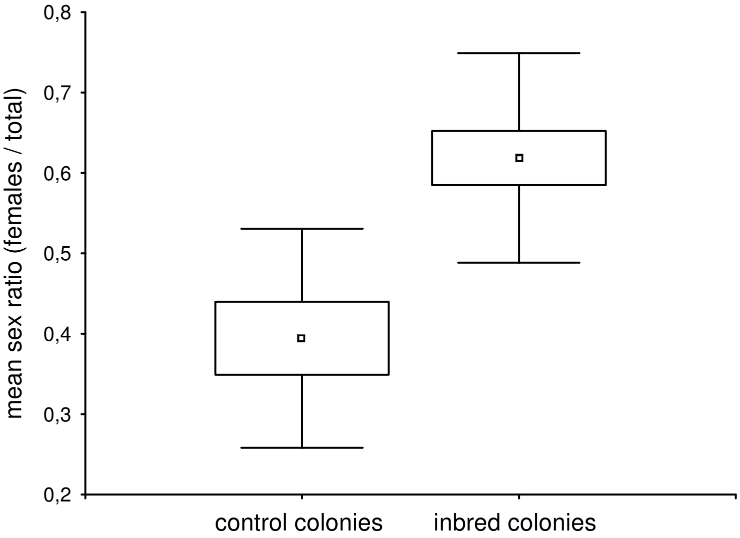


**Figure 1.** Mean (± sdev) sex ratio (female proportion) of inbred and control set-ups of the first generation; sex ratio of inbred set-ups is significantly more female biased than sex ratio of control set-ups (Kolmogorv-smirnov: n.s.; mean C: 0.40; I: 0.62; t-test: $t=-4.02$, $p<0.01$, $n_C=9$, $n_I=15$).

### *Detection of ml CSD (generations 2-10)*

There are no differences in the sex ratio of the control set-ups over all nine generations (ANOVA, n=99, F = 1.24, p = 0.28; mean: 0.637 ± 0.12). However, sex ratio is more female biased compared to the first generation, as colonies were no more restricted to 20 individuals, thus producing more often ergatoid males than winged males. The same trend towards an even more female biased sex ratio is visible in inbred set-ups, as well. Overall, there are no differences in sex ratio of different generations of the inbred set-ups (ANOVA: n=190, F = 1.73, p=0.09, mean: 0.64 ± 0.23). However, there are some exceptional colonies (total: 32 of 205; 15.6%) which produce a male biased sex ratio (Figure 2). In two of this 32 colonies, sex ratio was only slightly male biased and in the following generations again female biased, thus, those colonies are not considered to be exceptional in analysis. Without the remaining 30 colonies, overall sex ratio got more female biased (mean 0.72 ± 0.13).

In comparison with the control group, the sex ratio of the inbred group is not different over all nine generations (t-test: $n_C=99$, $n_I=190$: t=-1.57, p=0.88), but significantly more female biased when taking the outstanding colonies not into account (t-test: $n_C=99$, $n_I=160$: t=-5.14, p<0.001).

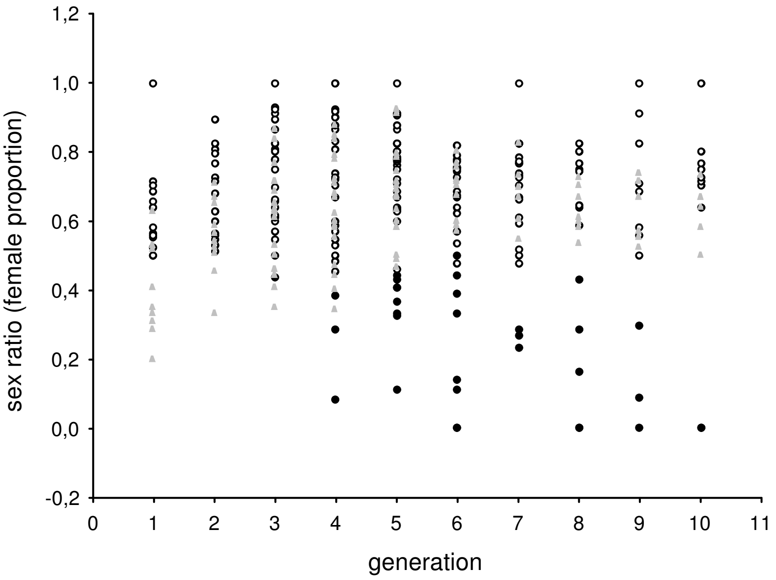


**Figure 2.** Sex ratio of inbred and control colonies of all ten generations. Grey triangles show sex ratio of control colonies, white circles inbred colonies (without problems) and black circles inbred colonies with male biased sex ratios and problems in raising brood, workers and/or female sexuals.

The exceptional colonies belong to three of the nine lineages (=single queen source colonies 2, 7 and 10). The first of these colonies occurred in generation three; however, the colony performed well, although sex ratio was slightly male biased (0.438). Only in the next generation it got obvious that the colony had a problem since the number of worker pupae declined drastically as did the number of female sexuals (sex ratio 0.25 and 0.083).

The same trend was visible in nearly all (except one) other colonies with male biased sex ratio: sex ratio remained male biased or even got more male biased, as long as no further generation was possible to breed (most often due to the lack of female sexuals). Only in one set-up the sex ratio got female biased again, whereas in three other set-ups with sexuals of the same “source colony“, sex ratio got even more male biased (Figure 3). Despite the extreme shift in sex ratio, some of the colonies produced a high number of sexuals (up to 285!). In all those colonies, temporarily, egg mortality was high (up to 87%) and larvae and pupae number low. Moreover, sex ratio got more and more male biased over time (Figure 4).

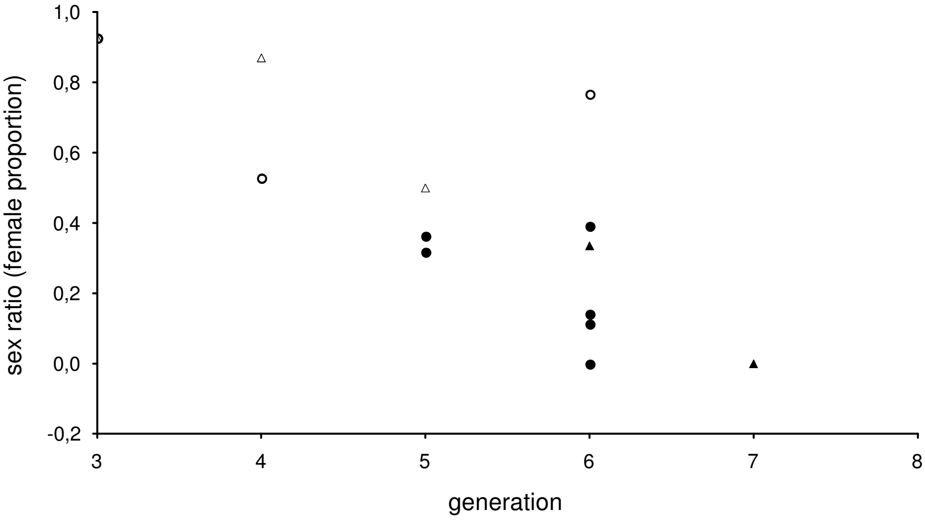


**Figure 3.** Sex ratio course of exceptional colonies of two different lines (circles and triangles, each). Black colonies have limited worker number and high brood mortality. In the sixth generation, one of four colonies of the "circle line" got female biased again, although the "source" colony of the fifth generation had obviously problems.

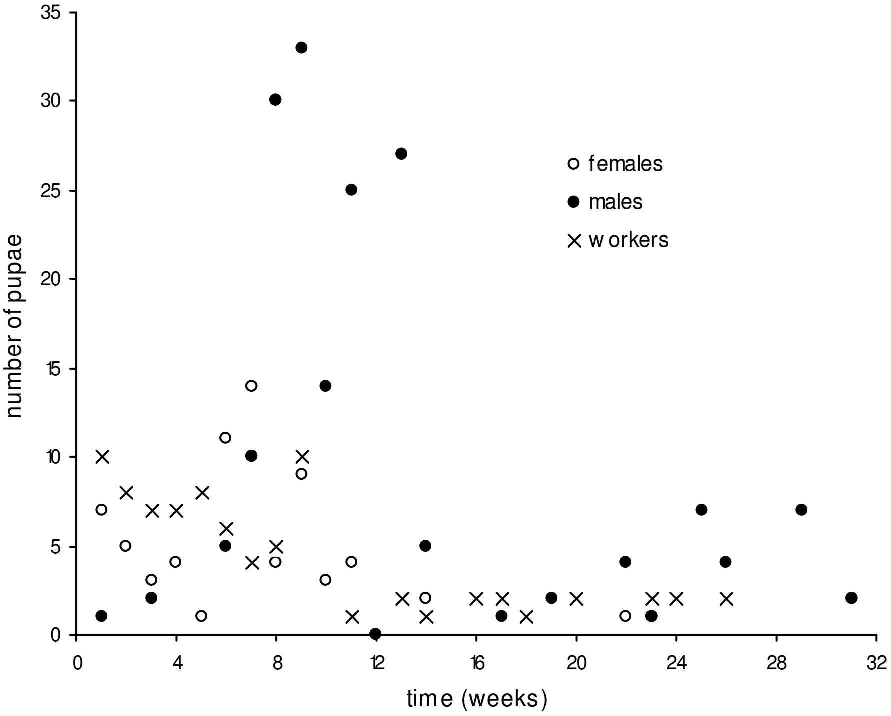


**Figure 4.** Production of males, females and workers of an "exceptional" colony which produced a high number of sexuals over time (starting point: six weeks after establishment). After seven weeks, mainly males are produced, and worker production decreases, as well.

Other colonies had problems to raise larvae at all, and only a low number of workers and male pupae eclosed. In this context it is important to notice, that in all colonies at least a very few worker pupae occur, thus, queens are mated for sure, and diploid male sterility (what would result in all male broods) can be excluded.

Assuming that those exceptional colonies are due to diploid male production under ml-CSD, and supposing that all loci got homozygous and that 50% of fertilized eggs are male, sex ratio should be shifted from an average of 0.7 to 0.54 (unviable diploid males). However, sex ratio of those colonies is on average 0.24 (n = 30).

### *Diploid male production*

Flow cytometry revealed that only one of 67 male pupae of colonies with extremely male bias was diploid (winged male; seventh generation). All other males were haploid. This might suggest that diploid males in *Cardiocondyla* have a very low viability. If so, primary sex ratio should reveal much more diploid eggs than visible in the secondary sex ratio. Out of 77 eggs from five colonies, 40 could not be classified because they had too few cells or were too young. However, sex ratio of the remaining 37 eggs reveals that 26 eggs are haploid and only eleven eggs are diploid. This corresponds to a sex ratio of 0.3 and is in accordance with the mean secondary sexual sex ratio of 0.19 of the five colonies, if one considers that in all colonies workers are produced as well. Thus, unviable diploid males are unlikely, and the single diploid male pupae seems to be the exception rather than the rule.

### *Fertility of males and females*

One single of nine inbred males which were allowed to mate with an alien control female seemed to be infertile, as the female behaved as a virgin queen (e.g. started egg laying late, only a few eggs, only some male pupae, no worker pupae). Alternatively, it could be that partners did not mate with each other. All other females produced worker pupae, thus they mated with the inbred male. In two cases, the colony performed well, and sex ratio was slightly female biased (both 0.56). In five set-ups, sex ratio was strongly male biased (on average 0.16), and in the other case, only a few worker pupae developed and no sexuals were produced at all. Two queens of inbred set-ups which were allowed to mate with alien control males had normal fertility and produced female biased sex ratios (0.57 and 0.7). Testes and seminal vesicles of four dissected males were filled with sperm, and no abnormalities could be detected. In one ergatoid male, sperm storage organs seemed to be not that stuffed than usually, and in another one, sperm storage organs seemed to be completely empty.

### *Lifespan of queens*

Control queens live on average 28.2 weeks, and this is similar to queens of inbred colonies during the first four generations. From the fifth generation on, mean lifespan of inbred queens is significantly reduced (Figure 5). In two set-ups in which control females were mated with inbred males, queens already died after eight weeks.

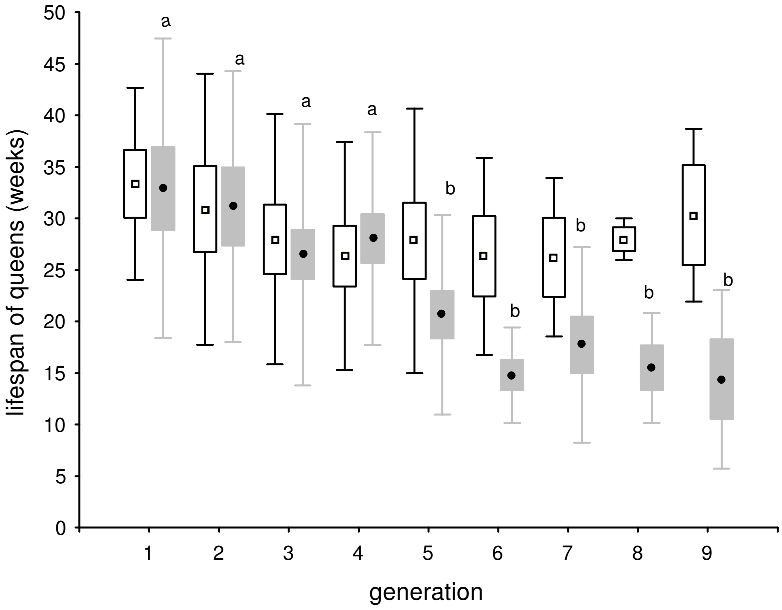


**Figure 5.** Lifespan of queens in control groups and inbred set-ups. In control groups (white bars), queens live on average 28.2 weeks. Lifespan is independent of the generation (n=74; Kolomogorov-smirnov: n.s., ANOVA: F=0.37, p=0.93). Lifespan of queens of inbred set-ups (grey bars) is significantly dependent on the generation of inbreeding (n=124; Kolomogorv-Smirnov: $p<0.05$; Kruskal-Wallis-ANOVA: H = 23.47, $p<0.01$; Mann-Whitney-U: generations 1-4, all combinations: n.s., generations 1-4 versus 5-9: all combinations: significant, generation 5-9 all combinations n.s.). Age of queens is reduced for more than three month in highly inbred set-ups (mean of 29 weeks in generations 1-4; mean of 16.6 weeks in generation 5-9). Significant differences within the inbred group are indicated by different letters.

## DISCUSSION

The results strongly support the hypothesis that in *Cardiocondyla obscurior* a sex determination mechanism which is not based on heterozygosity evolved. Moreover, the study suggest the ability of kin discrimination and a rare example for the separate effect of inbreeding and local mate competition on sex ratio.

### *Sex determination and inbreeding depression*

The absence of CSD was suggested for other inbreeding - mainly parasitic - species earlier, as e.g. in the genus *Epimyrma* (Buschinger, 1989), but it has never been tested explicitly up to now. After one generation of inbreeding in *C. obscurior,* females did not suffer decreased colony founding ability, fecundity or longevity, and sex ratio data were not in agreement with expectations under sl-CSD and two-loci ml-CSD. Thus, both can be ruled out as sex determination mechanism.

In case sex is determined by a multi-locus system with a high number of loci, many generations of inbreeding are necessary to detect diploid males. However, stated by Crozier and Bull (Bull, 1981; Crozier, 1971; Crozier, 1977), selection would be too weak to maintain heterozygosity at many loci, unless they had pleiotropic effects. According to Cook (1993a), ten generations of inbreeding should be more than adequate to detect at least ml-CSD with 15 loci, provided that one mother-son mating goes ahead. This was not possible in our set-ups, as females that already laid eggs are unattractive for males. Nevertheless, the ten generations of inbreeding we conducted should be sufficient to proof a system with ten loci at least. Although a shift in sex ratio towards a strong male bias actually occurred in some colonies, only one single male was diploid, and even primary sex ratio was strongly male biased. Furthermore, in rare cases, the effect of male bias disappeared in the next generation of inbreeding, what contradicts expectations of ml-CSD and diploid male production. Thus, reasons for the male bias seem to be others. It could be argued that these colonies produced a male biased sex ratio, e.g. due to split sex ratios. But as brood mortality was very high in those colonies (and only in those), this possibility is very unlikely.

Sex ratio distorters were shown to be another reason for changes in sex ratio. For example, in an autoparasitoid wasp, increased male offspring occurs due to paternal genome loss (Hunter et al., 1993). In eggs of this species, one set of chromosomes is lost following fertilization and resulting in primary males. A similar effect was shown by a supernumerary chromosome, the paternal sex ratio chromosome PSR, in *Nasonia* (Beukeboom and Werren, 2000). Although sex ratio distorters are expected to occur independently of inbreeding and should be visible in control groups as well, the transmission is heritable (even if heritability is low and variability high) and inbreeding could lead to selection for them, what would explain the progressive increase in male offspring. Nevertheless, as above mentioned, sex ratio distorters cannot explain the high number of eggs failing to hatch.

Results rather indicate that some sib mated females suffer inbreeding depression after four or five generations of inbreeding, and that this in turn leads to sperm sterility or limited sperm production of their sons, as haploid males can only be affected indirectly by inbreeding, e.g. through maternal effects. In colonies with problems in raising brood at all, a high ratio of the male sperm cells were probably unviable or, alternatively, sperm supply in females was very low because the males did not produce and / or transfer enough sperm to the female. In colonies, in which the worker number decreased and sex ratio got male biased over time, a proportion of viable, motile sperm was available to fertilize the eggs at the very beginning, however, the number of viable sperm or sperm supply in general decreased and got rare later on.

Sperm sterility or low sperm supply correlated to the fact that most control queens which mated with inbred males produced male biased sex ratios as well. Contrary, inbred females which mated with control males had normal fertility and sex ratio was female biased. The high egg mortality might be better explained by the fact that eggs fertilized with sterile sperm are not able to emerge, and it contradicts the explanation that males are not able to produce or transfer enough sperm, as unfertilized, haploid eggs should hatch normally. Anyway, a combination of both is likely (and suggested by the dissections), as inbreeding probably affects both, sperm number and sterility.

Finally, high female egg mortality due to inbreeding might be another explanation for the observed results, but in haplo-diploid species the expression of lethal genes is expected to be visible in haploid males first and hidden in heterozygous females, thus, higher egg mortality for haploid males should be observed (Antonlin, 1999).

Taking all results together, sperm sterility is the most plausible explanation. A study of bulb mites revealed that sterility and preadult mortality is increased due to inbreeding (Radwan, 2003), and Konior and colleagues (2004) could in addition show that males produced by sib mating had a significantly lower sperm competition success than outbred males. However, as mentioned above, haploid males cannot suffer inbreeding depression directly as they develop from unfertilized eggs, and thus, the effect of inbreeding can be only indirectly transferred via the mother.

Moritz (1986), who observed that inbreeding in *Apis* lead – among other things – to the production of less sperm, suggested that this may be due to non-heritable, maternal effects on the egg or due to cellular components which are transferred via the cytoplasma to the egg, and we believe that this could be an explanation for the observed results in *Cardiocondyla* as well.

Wilkes (1947) reported on a similar effect of inbreeding on male fertility in the wasp *Microplectron fuscipennis* (Wilkes, 1947), but according to him, the change in male fertility is not due to inbreeding *per se* but heritable and responds to selection (similar to sex ratio distorters mentioned above, Henter 2003). Evidence for this or other reasons for male bias in *M. fuscipennis* is still unknown (Henter, 2003).

Salin and colleagues (2004) found a significantly male – biased sex ratio in the parasitoid *Aphidius rhopalosiphi* after inbreeding, which was consistent with expectations under sl-CSD and viable diploid males. However, they did not find diploid males and sex ratio was not consistent with expectations of unviable males. Unfortunately, this study as well as the study by Wilkes gave no data concerning brood mortality.

In *Cardiocondyla*, lifespan of females is significantly reduced in queens which were inbred for at least five generations. This could be a direct effect of inbreeding depression on females (see e.g. Henter, 2003; Radwan, 2003) or a side-effect of mating with inbred males (or a combination of both), as control queens mated with this males suffer increased mortality as well. Inbreeding depression may not only influence sperm viability, but also seminal fluids. Recent studies strongly suggest an influence of the latter on longevity of queens (Schrempf et al, 2005b).

At the moment we cannot conclude whether genomic imprinting or something different is the mechanism of sex determination, but sl-CSD and ml-CSD with only a few loci can be rejected for sure. Inbreeding depression occurs after four or five generations of sib-mating nevertheless. Studies in the related species *C. batesii* revealed that about 80% of matings are among sibs, but that the remaining 20% (that is each fifth mating) are among unrelated individuals (Schrempf et al, 2005a). This could be similar in *C. obscurior*, and obviously, selection therefore would not be strong to prevent inbreeding depression after four or five generations of inbreeding. Diploid males, on the other hand, would occur already after one generation of inbreeding in case of sl-CSD or ml-CSD with only a few loci. Thus, selection to another mechanism of sex determination has obviously been strong.

### *Sex ratio adaptation*

In the remaining, “normal“ inbred colonies, sex ratio is significantly more female biased than in the control groups, and in principle, there are two possible explanations for this: sex ratio adjustment to inbreeding, or, alternatively, outbreeding enhancement.

Crossing between too distantly related individuals (with increased differences in genetic variation) may disrupt well adapted alleles within the genome and thus result in outbreeding depression (Lynch, 1991; Peer and Taborsky, 2005) – with similar effects as inbreeding depression (and thus a bias in sex ratio towards male, see above). Such phenomenon was shown for example in regularly inbreeding beetles, in which outbred offspring suffered reduced hatching rates (Peer and Taborsky, 2005). In our study, however, the only differences which could be detected between inbred and outbred colonies concerned the proportion of sexuals produced. Egg number as well as worker number did not differ between the set-ups. Under the assumption, that e.g. sperm cells and eggs are not that compatible between outbred pairs, not only a male bias, but also a decline in worker number should be observed. Moreover, the fact that colonies were reared in the laboratory for several years and that they are from only two different populations makes outbreeding depression unlikely.

Instead, the higher female bias in inbred colonies suggests firstly, that– as already suggested previously– queens and not workers control the sex ratio in *Cardiocondyla* (Cremer and Heinze, 2002; deMenten et al., 2005), secondly, that there is a separate effect of inbreeding beside local mate competition (LMC) on sex ratio, and finally, that queens are able to recognise kin.

Inbreeding in Hymenoptera leads to an increase of relatedness between mothers and daughters, but not between mothers and sons (due to haplodiploidy). Therefore, sex ratio is expected to be shifted towards females, in case queens are able to adjust the sex ratio after sib mating (Herre, 1985). Under worker control, sex ratio would be expected to strive for even investment in males and females contrary (0.5), as relatedness between brothers and sisters increases under inbreeding conditions (see e.g. Chapman et al., 2000; Crozier and Pamilo, 1996; Tsuji and Yamauchi, 1994) and workers do not benefit from biased sex ratios as in outbred populations. Our study clearly shows that sex ratio in inbred set-ups is more female biased than in control set-ups, thus suggesting that sex ratio is adjusted by the queen. For that, individuals have to distinguish kin from non-kin. Odour is known to play a major role in the ability to distinguish relatives from non-relatives in social insects, and both, environmental factors and genetic determinants can influence odour (Obin, 1986; Stuart, 1987; Waldman et al., 1988). Most often, discrimination happens among nestmates and non-nestmates by the use of environmental factors. In our experimental design, sexual pupae eclosed together in all set-ups. Thus, colony odour is unlikely to play a role, and in turn, common growth in the colonies cannot be used by sexuals as a sign to distinguish related from unrelated individuals. Most likely, genetic determinants allow queens to distinguish between kin and non kin in *Cardiocondyla*, and further studies may give more information.

Theory predicts that local mate competition (LMC) select for female biased sex ratio in organisms, in which mating takes place between the offspring of one or a few mothers in isolated broods (Hamilton, 1967). In *Cardiocondyla*, LMC is expected to be high in case only ergatoid males are produced, as they do not disperse, and recent studies confirmed this (Cremer and Heinze, 2002; de Menten et al., 2005). Contrary, under winged male production, LMC is relaxed, and in fact, it got obvious that sex ratio tends towards an even ratio. However, as the investment into the different male morphs was the same in inbred and control colonies, the intensity of LMC can be assumed having been the same as well. Under the given level of LMC, sex ratio got even more female biased under inbreeding conditions. In previous studies, effects of inbreeding and LMC have often been equated or could not be disentangled, respectively, as both lead to a female biased sex ratio. Herre (1985), however, could show that sex ratio in fig wasps is adjusted to both the intensity of inbreeding and LMC (on population level). We could proof the same for a social insect species on colony level: queens of *C. obscurior* are able to precisely adjust sex ratio to both, the level of LMC (dependent on the male morph) and inbreeding.

## ACKNOWLEDGMENTS

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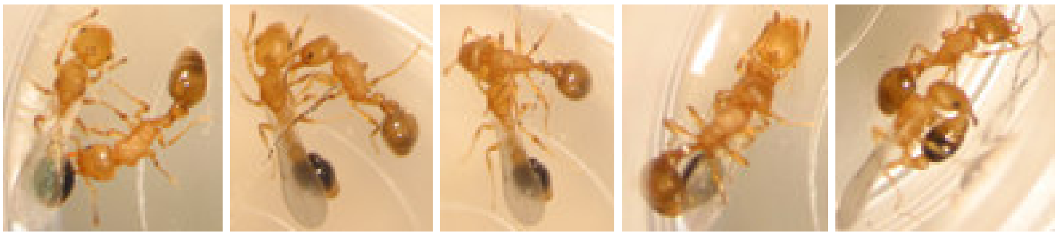
# Chapter 6

# Sexual cooperation: mating increases longevity in ant queens*

Alexandra Schrempf, Jürgen Heinze and Sylvia Cremer



Mating of an ergatoid male with a queen of *C. obscurior.* After antennal contact, the male mounts the female and copulates. Afterwards, the female is cleaning herself.

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## ABSTRACT

Divergent reproductive interests of males and females often cause sexual conflict (Chapman et al., 2003; Parker, 1979). Males of many species manipulate females by transferring seminal fluids that boost female short-term fecundity while decreasing their life expectancy and future reproductivity (Thornhill and Alcock, 1983; Wolfner, 1997). The life history of ants, however, is expected to reduce sexual conflict: whereas most insect females show repeated phases of mating and reproduction, ant queens mate only during a short period early in life and undergo a lifelong commitment to their mates by storing sperm (Hölldobler and Wilson, 1990). Furthermore, sexual offspring can only be reared after a sterile worker force has been built up (Hölldobler and Wilson, 1990). Therefore, the males should also profit from a long female lifespan. In the ant *Cardiocondyla obscurior,* mating indeed has a positive effect on the lifetime reproductive success of queens. Queens that mated to either one fertile or one sterilized male lived considerably longer and started laying eggs earlier than virgin queens. Only queens that received viable sperm from fertile males showed increased fecundity. The lack of a trade-off between fecundity and longevity is unexpected, given evolutionary theories of aging (Kirkwood and Rose, 1991). Our data instead reveal the existence of sexual cooperation in ants.

*Keywords*: sexual cooperation, longevity, trade-off reproduction-longevity, evolutionary theories of aging, social insects

## INTRODUCTION

Sexual conflict arises because the reproductive interests of males and females in many species differ greatly; females benefit most from a high lifetime reproductive success, while males attempt to gain maximum fitness from each single mating event. During copulation, males can enhance their own short-term benefits by transferring substances that increase female egg-laying rates (Herndon and Wolfner, 1995; Manning, 1962), but have a negative effect on their re-mating probability, lifespan and lifetime reproductive output (Chapman et al., 1995; Chapman et al., 1998; Chen et al., 1988; Fowler and Partridge, 1989; Price et al., 1999; Prout and Bundgaard, 1977; Thornhill and Alcock, 1983). Conflict between the sexes is high in promiscuous mating systems (Johnstone and Keller, 2000; Rice, 2000; Snook, 2001) and decreases when, as in monogamous animals, the same partners are likely to re-mate later in life and thus have converging interests. Experimentally enforced monogamy in the normally promiscuous fruit flies, in fact, led to the evolution of less harmful males (Holland and Rice, 1999).

Lifelong commitment between two partners is rare in nature (Arnqvist and Nilsson, 2000), but is typical for social insects. In termites, the queen and the king live together for their entire lifespan inside a well-protected "royal cell" in the heart of the nest (Boomsma et al., 2005). In ants, males and virgin queens mate during a short period early in life, after which the males die. The queens store the sperm of their mates in a spermatheca and use it to fertilize eggs throughout their entire lives, which in some species can last decades (Hölldobler and Wilson, 1990). During the first months or even years after mating, the sperm is exclusively used to produce large numbers of workers, which form the "soma" of the colony. Like somatic cells of a multicellular organism, workers are typically sterile and do not directly contribute to future generations but, instead, rear the queen's sexual offspring - the equivalent of the germ line in multicellular organisms. Sexuals are produced only after this first period of colony growth, and the sexual output of mature colonies depends on the size of the worker force that has been built up (Bourke and Franks, 1995). Both ant queens and males therefore benefit from mating only after the time lag of a long ergonomic phase; males would not profit from increasing the short-term fecundity of the queens at the cost of their longevity. Hence, the two specific life-history parameters of ants – a lifetime pair bond and late reproduction – are expected to reduce sexual conflict and, in its place, to promote cooperation between males and queens (Boomsma et al., 2005; Bourke and Franks, 1995). In the stable associations of social insects, both sexes should benefit from a long female lifespan, which allows a lengthy period of sexual reproduction once the colony has reached sexual maturity.

## MATERIALS AND METHODS

We tested the effect of mating on lifespan and reproductive success of queens of the ant *Cardiocondyla obscurior*. We compared three types of queens: virgin queens (VQ n = 34), queens that mated with a single fertile male (of the wingless male morph; Hölldobler and Wilson, 1990; MQ n = 47), and queens that mated with a single male (of the wingless male morph; Hölldobler and Wilson, 1990) that was previously sterilized by X-ray irradiation (minimum 60 Gy; 2.95 ± standard deviation 0.12 Gy/min; SQ, n = 18). These latter queens had experienced copulation and stored sperm in their spermathecae. This sperm, however, was not viable (i.e. motionless and in clumps, as revealed by dissections of the spermathecae of five queens) and did not fertilize eggs. Eggs laid by such queens, and also by virgin queens, were therefore expected to develop into males as a result of haplodiploid sex determination. Each queen was housed in an individual nest box with 20 workers, which corresponds to the average natural colony size. The number of workers was kept constant over the course of the experiment, during which colonies were kept on a high nutrition diet (i.e., fed three times a week *ad libitum* with honey and pieces of dead cockroaches). The number of eggs and the survival of the queen were checked twice per week. At the same time, short observation scans were performed, revealing that all three groups of queens stayed permanently in the central nest area containing the brood. Furthermore, no other behavioural differences between queens (e.g., in general activity or brood care), or between workers in the handling of these queens, could be detected in the different colonies.

## RESULTS AND DISCUSSION

As predicted, colonies of queens that had mated with a fertile male produced both female and male offspring, whereas both virgin queens and queens that had mated with a sterilized male produced exclusively male offspring. Egg-laying rates were three times higher in the queens mated with fertile males compared to both groups of male-producing queens (Figure 1). Independent of the large differences in egg-laying rates, queens that had mated with either a fertile or sterilized male lived approximately 50% longer than virgin queens (26 *versus* 18 weeks, Figure 2). In addition, they started laying eggs earlier than virgin queens (Figure 3). Therefore, mated queens experienced a twofold increase of their lifetime reproductive period: first, through an earlier onset of reproduction (by about 2 weeks); and second, through a longer lifespan (by approximately 8 weeks, Figure 3). Even though fecundity was equally low in virgins and queens that had mated with a sterilized male, the longer egg-laying period of the latter resulted in a 1.5 fold higher reproductive output (estimated total egg number laid during the lifetime: VQ: 93; SQ: 146). Because that queens that mated with fertile males also showed increased fecundity, their lifetime reproductive success (estimated total egg number: 499) was much higher than that of the two groups of male producing queens.

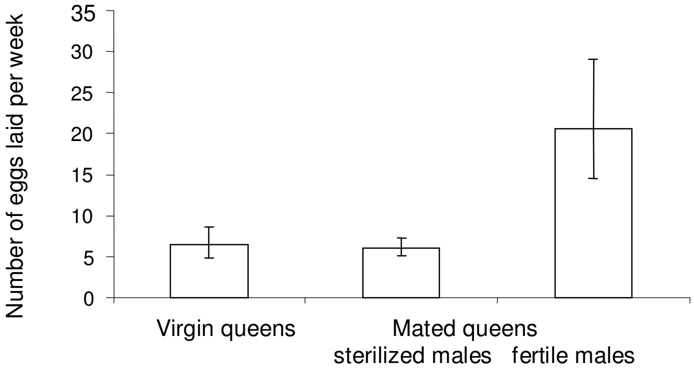

**Figure 1.** Fecundity of *Cardiocondyla* queens: the egg-laying rate of queens receiving viable sperm during copulation was higher than that of virgin queens and queens that mated with a previously sterilized male transferring non-viable sperm (ANOVA on ln transformed number of eggs laid per week: F=112.1, p<0.001; Post-Hoc Bonferroni t-Test all pairwise: VQ-SQ: p=n.s., VQ-MQ and SQ-MQ: p<0.001; mean eggs/week and sample size: VQ: 6.4 (*n*=13), SQ: 6.1 (*n*=18), MQ: 20.6 (*n*=21)). Graphical presentation of the back transformed means ± standard deviations.

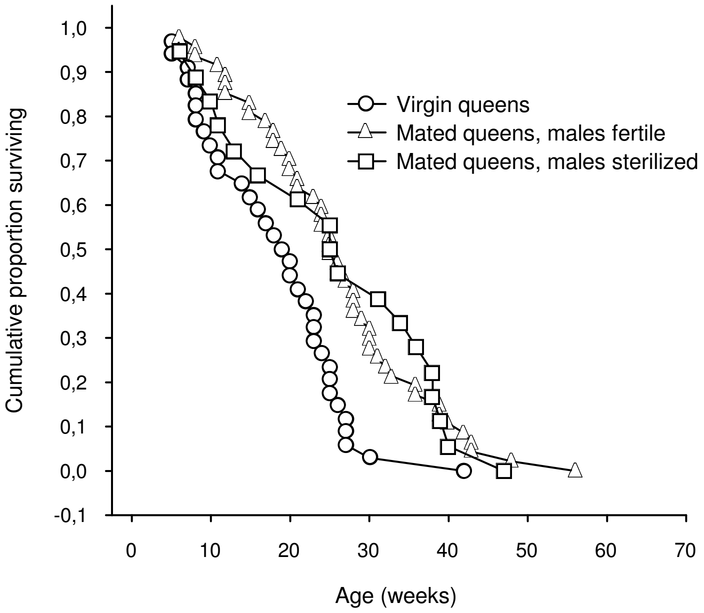

**Figure 2.** Lifespan of *Cardiocondyla* queens: mated queens lived longer than virgin queens, irrespective of whether their mate transferred viable or non-viable sperm (survival analysis for multiple samples: $\chi^2$=11.0997, p<0.004; Cox's F-Test: VQ-SQ: p=0.013, VQ-MQ: p<0.001, SQ-MQ: p=n.s; mean queen life span in weeks and sample size: VQ: 18.2 (*n*=34), SQ: 25.8 (*n*=18), MQ: 26.0 (*n*=47)). The graph shows the proportion of surviving queens (Kaplan-Meier).

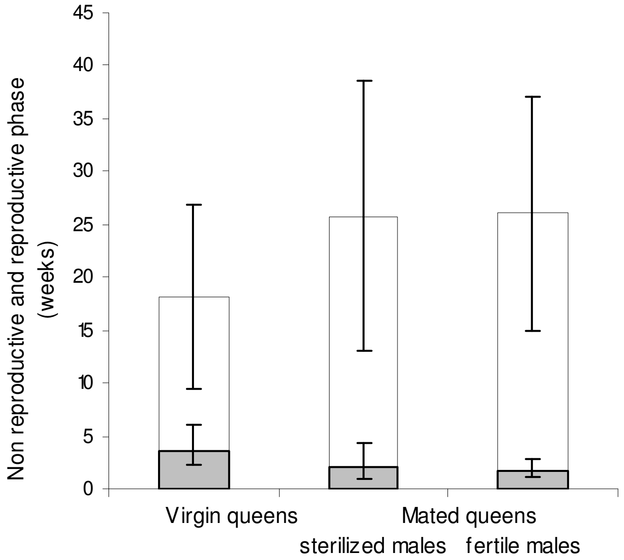

**Figure 3.** Duration of the reproductive phase in *Cardiocondyla* queens: the onset of reproduction was more delayed in virgin queens compared to queens that mated with either one fertile or one sterilized male (ANOVA on ln transformed queen age at first egg-laying: F=6.2, p<0.01; Post-Hoc Bonferroni t-Test all pairwise: VQ-SQ: p=0.01, VQ-MQ: p<0.001, SQ-MQ: p=n.s; mean day of first egg-laying and sample size: VQ: 25.7 (*n*=15), SQ: 13.9 (*n*=18), MQ: 12.5 (*n*=14)). Because egg-laying continued until queen death, queen lifespan is equivalent to the end of the reproductive phase (for statistics, mean values and sample size see Fig.2). The graph shows the means ± standard deviations of the start (back transformed data) and the end of egg-laying in the queens' lifespan (given in weeks).Gray: nonreproductive phase. White: reproductive phase.

The low egg-laying rate of virgin queens matches previous observations in other ants (Vargo and Porter, 1993). Similarly, virgin females of many nonsocial insects are characterized by a lower fecundity and delayed oviposition compared to mated females (Eberhard, 1996). Virgin females of solitary insects as well as self-fertilizing nematodes, typically live longer than mated individuals (Chapman et al., 1998; Dean, 1981; Gems and Riddle, 1996; Partridge, 1986; Quiring and McNeil, 1984; Tatar et al., 1993). In contrast to this widespread negative effect of mating on longevity, virgin *Cardiocondyla* queens lived shorter than mated queens, even if males were infertile as a result of irradiation. The egg-laying rate of these latter queens was as low as that of virgins, which suggests that fertilization of eggs by viable sperm is necessary for a *Cardiocondyla* queen to reach maximum fecundity.

The long lifespan of mated queens cannot result from potential preferential care of workers directed toward highly fecund queens (laying fertilized eggs) because queens mated with sterilized males lived as long as the queens mated with fertile males but produced the same low number of (unfertilized) eggs as the short-lived virgins.

The difference between the egg-laying rates of both types of mated queens of *C. obscurior,* and the similarity of their lifespan, further indicate that no trade-off exists between investment in reproduction and longevity. *Cardiocondyla* queens pay no apparent cost of high fecundity in terms of longevity – an observation not only indicated by comparisons between groups of queens but also further supported by the absence of a significant correlation between individual egg-laying rates and lifespan within any of the groups (Spearman Rank correlation VQ: $r_s = 0.44$, $p > 0.12$; SQ: $r_s = -0.19$, $p > 0.45$; MQ: $r_s = -0.11$, $p > 0.78$). Moreover, both groups of mated queens started laying eggs earlier than virgin queens and also lived considerably longer.

Our data on longevity and reproduction in *C. obscurior* conflict with predictions from evolutionary theories of aging (Kirkwood, 1977; Kirkwood and Rose, 1991; Williams, 1957) and, more generally, of life history evolution (Roff, 1992; Stearns, 1992) given that a trade-off between these two life-history parameters is consistently found in many organisms (Bennett and Harvey, 1988; Partridge, 1986; Westendorp and Kirkwood, 1998). For example, fruit flies selected for a long lifespan had reduced early fecundity (Rose and Charlesworth, 1980), whereas flies with experimentally lowered egg-laying rates lived longer than control females (Partridge et al., 1987). It has previously been shown that queens of social insects typically live much longer than sterile workers (Hölldobler and Wilson, 1990; Keller, 1998). However, this alone does not allow us to conclude that reproduction causes increased longevity because reproducing individuals differ from non-reproductives in numerous life-history aspects (e.g. morphology, physiology and behaviour), including their sheltered way of life in the centre of the nest (Hartmann and Heinze, 2003; Keller and Genoud, 1997). A low extrinsic mortality rate generally promotes internal longevity (Stearns, 1992) in queens (Keller and Genoud, 1997) and even in non-reproducing indoor workers (Chapuisat and Keller, 2002). Previous research on aging in social insects did not distinguish between the effects of reproduction itself and those of the low extrinsic mortality rate on the longevity of reproducing individuals. In contrast, our study compares the lifespan of two groups of reproducing individuals that differ in fecundity but otherwise share the same life history (e.g., their reproductive and risk avoidance behaviour). The study therefore gives the first convincing evidence that the longer lifespan of reproducing individuals among social insects is independent of the reproductive investment, but can instead be associated with the mating act or substances received through mating itself.

Whereas our study describes the positive effect of single mating *versus* non-mating on both female lifespan and lifetime fecundity, previous work has shown that, compared to female crickets mating with unattractive males, females mating with preferred males had either increased reproductive success or increased longevity (while the onset of oviposition was delayed; Wagner and Harper, 2003). In crickets and several other species, a protein-rich spermatophore is transferred as a nuptial gift to the females during copulation. Multiple mating (as opposed to single mating) either increased or did not affect female longevity in such species (Arnqvist and Nilsson, 2000; Wiklund et al., 1993). These results did even held true when spermatophores were experimentally removed (Wagner and Harper, 2003; Wagner et al., 2001). In species without nuptial feeding, in contrast, lifespan generally decreases with increased mating rate (Arnqvist and Nilsson, 2000; Chapman et al., 1995). Ant males do not transfer nuptial gifts to the females, and multiple mating is rare (Hölldobler and Wilson, 1990). Nevertheless, we predict a similar positive effect of multiple mating on lifespan, as observed in our study with single mating, because both the lifetime pair bond (now with several partners) and the late onset of reproduction after colony maturation remain important unchanged life history parameters promoting sexual cooperation. We further suggest that sexual cooperation might not be restricted to social insects but could be generally found throughout the animal kingdom wherever partners show a lifetime commitment to each other.

At present, it is unknown whether *Cardiocondyla* males transmit substances that lengthen female lifespan, directly or indirectly, for example by activating the production of antioxidants in the female body. Recent work on honeybees revealed higher expression levels of such compounds in the environment of the spermatheca in mated than in non-mated females (Collins et al., 2004). These compounds are assumed to ensure the survival of the stored sperm throughout the long reproductive phase of the queens, and it remains to be tested whether they might similarly be involved in prolonging the life of the queens.

## ACKNOWLEDGEMENTS

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# GENERAL DISCUSSION

The two male morphs in the ant species *Cardiocondyla obscurior* are the result of a conditional strategy under the control of the colony. The workers react – independent of the presence of a queen - to environmental conditions, and, by treating larvae differently, determine the morph of the male (Chapter 1).

In general, polyphenisms in insects are modulated by the environmental conditions, the larvae experience during a sensitive period of their development (Nijhout, 2003b). As larvae often cannot move among patches, parents (usually the mother) are able to influence the phenotype of the offspring in a non-genetic, indirect way, for example by laying their eggs at different places and thus altering the environment they provide. An example for the influence of males can be found in the dung beetle *Ontophagus taurus*, where paternal provisioning co-vary with the morph the larva will express as an adult (Hunt and Simmons, 1998).

Another, direct way of offspring manipulation can evolve through “maternal effects”, when females alter the nature of the egg, or program a developmental switch in their offspring in response to predictive environmental cues (“trans-generational phenotypic plasticity”; Fox, 2000; Fox et al., 1999). By doing so, populations can successfully adapt to variable environmental conditions. For example, seed beetle females that oviposit on low quality substrate produce larger eggs than on high quality substrate (Fox et al., 1997). In a recent study, Pienaar and Greeff (2003) suggested that a similar effect may be responsible for a male dimorphism in *Otitesella* fig wasps.

However, after deposition of the eggs, parents generally have no further influence on the larvae. Conversely, in social insect colonies, the environment of the larvae can be changed continually, and larvae are extremely vulnerable to control during the entire developmental time. In *Cardiocondyla*, the morph is determined at the end of the second larval stage (of three stages). It is well known that early differentiation allows more profound differences between the castes or morphs (Michener, 1974; Wheeler, 1986). On the other hand, a more flexible reaction is possible when the sensitive period is late in larval life. The intermediate stage of determination in *Cardiocondyla* allows a quick reaction to changes in environment, but still enough time to express the different phenotypes.

Caste in social insect colonies is frequently controlled and inhibited by the queen, e.g. directly through different composition of eggs, or through control of worker behaviour and pheromones, respectively (Wheeler, 1986).

In polygynous tramp species such as *C. obscurior*, inhibition is unlikely to occur as several queens reproduce together in a single colony and young queens are produced year-round, probably as a result of receiving more nutrition from the workers. Our study has shown that male morph determination (and caste determination) is not under the control of a queen, because under certain conditions winged males are produced independently of the presence / absence of a queen. Instead, as already discussed, workers control the morph of their brothers by treating larvae differently, thus, representing a “sisterly effect”.

Interestingly, the colonies differ significantly in their sensitivity to changes (e.g. temperature), in that some colonies react more quickly and strongly to changes than others (Y. Du, unpubl.). The reasons for this remain to be investigated. In the field, no winged males have been found so far (see e.g. Heinze and Delabie, in press), and it is unclear under which circumstances they are produced. In the laboratory, winged male production can be easily induced through temperature decreases of only 3°C (Y. Du, unpubl.). However, temperatures in the field are not constant as in the laboratory, and a reaction of field colonies to similar temperature changes is highly unlikely. The most important factor for winged male production in nature is probably density. Where habitats are saturated and competition between colonies is high - following local growth through colony founding by budding – dispersing propagules (winged males and dispersing queens) are expected to be produced to reach new habitats and create new colonies. This was obviously not the case at our study sites, where colonies were collected regularly and, consequently, density was diminished.

Despite the lack of winged males under natural conditions, the comparison of the reproductive potential of the different individuals suggests that males should preferably be winged (Chapter 2). This result is surprising, as generally winged males are considered to be disadvantaged in comparison to the fighter males (Anderson et al., 2002). However, female mimicri by males (Cremer et al., 2002) together with the fact that they mate inside the nest before they disperse makes them an at least equal opponent to the ergatoid males, as the latter are not able to recognise them as rivals. Even if the survival probability of the males and the number of competing males are not considered, the maximum and mean fitness of winged males and ergatoid males is equally high. Again, this is contrary to expectations, as ergatoid males theoretically can produce an infinite number of sperm and can grow older than winged males. However, for the colony it is cheaper to produce ergatoid males, as they are not as heavy as winged males and consequently consume less energy resources. Moreover, as they do not disperse, they remain in the colony available to mate with newly developed virgin queens.

Ergatoid males are produced continuously in all colonies, independent of the occurrence of another adult male. Thus, males have to fight frequently and the possibility of being killed is high. As dead males are fed to the larvae, the costs of continuous male production for the colony can probably be neglected. Moreover, it may act as a guarantee to prevent old and / or weak males inseminating the females in the colony which could result in diminished offspring quality and, in turn, reduced colony efficiency.

Despite of being eusocial, conflict in social insect colonies can and does occur, for example, conflict over male production (where workers posses ovaries; Bourke, 1988; Trivers and Hare, 1976) sex allocation (Bourke and Franks, 1995; Crozier and Pamilo, 1996), and also over caste fate of developing individuals (Bourke and Ratnieks, 1999; Wenseleers and Ratnieks, 2004; Wenseleers et al., 2003; Wenseleers et al., 2005). However, where caste (or morph) is determined by factors such as the amount of food, the passive larvae usually have no ability to control their own fate and conflict is suppressed by social control. Although some studies have shown that larvae may try to manipulate the workers to influence their fate (Creemers et al., 2003; Kaptein et al., 2005), in the male sex of *Cardiocondyla*, individuals obviously cannot influence their morph.

The polymorphism found in females of some monogynous *Cardiocondyla* species is probably also environmentally determined, because daughters of a short winged queen can be long winged (A. Schrempf, unpubl.) and different queen morphs can be found in one single colony. The different queen morphs appear to represent a typical dispersal and founding dimorphism of short winged, depending founding queens and long winged, dispersing queens in ant females (see General Introduction). However, all queens behave in the same way, and morphs do not represent different alternative tactics as known from other queens. Instead, the occurrences of the different morphs appear to be linked directly to the reversion from polygynous ancestors to monogyny and independent colony founding (Chapter 3). Queens that were still morphologically adapted to dependent founding (like their ancestors) establish new colonies without the help of workers and forage during the founding phase, independent of their morph. Short winged queens invest into fat instead of flight muscle mass in addition and are much more successful in founding new colonies than long winged queens. Thus, queens trade off between dispersal and fecundity, a phenomenon well known from many non-social insect species (Dixon and Howard, 1986; Ritchie et al., 1987; Roff, 1984). However, long winged queens were never observed to use their wings, and it is unclear whether the polymorphism is indeed a stable phenomenon or represents only a transitory stage to brachyptery in all queens.

In general, the evolution of the loss of flight capability in insects has been correlated with the stability of patches (Darlington, 1943; Roff, 1986; Roff, 1994; Southwood, 1977). In *Cardiocondyla*, the stability of patches may have lead to the transition from short-lived, polygynous colonies to long-lived, monogynous colonies. Then, the renunciation of wing muscles may have been the easiest and less costly method to exploit new limited energy resources in a habitat, in which wings are not important anyway.

In these monogynous species, many sexuals are produced seasonally once per year in contrast to the continuous reproduction of sexuals in the tramp-species. Thus, one ergatoid male would probably be unable to inseminate all females, and several ergatoid males (brothers) can occur simultaneously in one colony, without fighting against each other (Heinze et al., 2005). Genetic analysis suggests that young queens mate with all possible males within a colony, as many queens use the sperm of different males to fertilize their eggs. Despite the high levels of inbreeding (as expected from intranidal mating in monogynous colonies), some of the matings were recognized to be among unrelated individuals (Chapter 4). This is surprising, as queens leave their colonies only after being inseminated and after hibernation in the nest, and ergatoid males are not adapted to dispersal. However, some small (probably young) colonies produce only a few ergatoid males and no winged queens. Such ergatoid males may leave the nest and enter alien neighbouring colonies. In the laboratory, ergatoid males can be transferred easily from one colony to another, and in *C. elegans* and *C. emeryi,* ergatoid males were observed to run around outside of the nest (Bolton, 1982; J.C. Lenoir, pers. comm., J.L. Mercier, pers. comm.).

Sex ratios of colonies that produced both sexes were female biased in concordance with expectations of local mate competition (LMC), which has been shown formerly in *C. obscurior* (Cremer and Heinze, 2002). However, in case of intranidal mating, inbreeding can occur simultaneously with local competition for mates, and both factors can contribute independently to female biased sex ratios (Frank, 2004; Herre, 1985; Reece et al., 2004). So far, the only evidence for the separate effect of the two factors came from a work by Herre (1985) by a comparison of different fig wasp species. In Chapter 5, we were able to show similar effects at colony level in *Cardiocondyla*. The sex ratio of inbred and control colonies, which experienced the same amount of local mate competition, were compared, and – assuming that no other factors as e.g. outbreeding depression were responsible for the differences in sex ratio (see discussion Chapter 5) - it became clear, that sex ratio was adjusted to inbreeding in addition to LMC, which lead to an even higher female proportion. In the case of winged male production, the effect of LMC is reduced and sex ratio tends towards an equal fraction of males and females, while the effect of inbreeding remains constant. In fig wasps, sex ratio adjustment can be explained either by adjustment to an average level of inbreeding in the population or as a direct response of the females to sib-mating (Greeff, 1996; Reece et al., 2004). Reece and colleagues (2004) could show that the females were unable to discriminate between kin, and thus, females may adjust their sex ratios in response to the average level of inbreeding.

However, while Herre (1985) analysed sex ratio adjustment for a given level of LMC between different inbred species, this study compared sex ratio adjustment of inbred and control colonies within one species. Thus, sex ratio adaptation to inbreeding in *Cardiocondyla* requires that queens – which control sex ratio (Cremer and Heinze, 2002; deMenten et al., 2005) - recognise that they mate with a related male. So far, it is not clear how they distinguish kin from non-kin. Kin discrimination can occur via genetic cues, or indirect via environmental cues (Mintzer and Vinson, 1985; Obin, 1986). In *Cardiocondyla*, a genetic component is most probably involved. Environmental cues (e.g. colony odour), which play a major role in nestmate recognition, appear unlikely to serve as a sign of relatedness, as all mating partners eclosed together within the same colony.

Several authors have suggested that inbreeding in haplo-diploid systems is less detrimental than in diploid species, because selection against haploid males that express deleterious or lethal mutations should lower genetic loads (Antonlin, 1999; Crozier, 1985). However, inbreeding depression in female limited traits is still possible.

We were not able to detect any negative effects of inbreeding in natural colonies of the genus *Cardiocondyla* despite their high inbreeding coefficients (Chapter 4; *C. nigra, C. obscurior,* Schrempf and Heinze, unpubl.). Moreover, a mechanism of sex determination different from sl-CSD, which is considered to be ancestral (Bull 1983; Crozier, 1971), has evolved, as otherwise diploid male production should be high in inbred colonies. We were able to exclude single locus complementary sex determination (sl-CSD) and multiple-locus complementary sex determination (with few loci) as mechanisms of sex determination in *C. obscurior*, and it is likely that all other species of this genus also lack CSD. Firstly, because in no single species have exceptional males been found so far, secondly, because sex ratio was female biased even in monogynous species, where inbreeding is higher than in polygynous species and finally, because phylogeny suggests that *C. obscurior* is one of the ancestral species within the genus *Cardiocondyla* (Heinze et al., 2005). Further investigations are necessary to detect the underlying mechanism of sex determination. The most likely mechanism may be genomic imprinting, as it is so far the only mechanism shown to exist as an alternative to sl-CSD (Dobson and Tanouye, 1998).

After five generations of inbreeding in the laboratory, inbreeding depression occurs, causing high brood mortality, male sterility and reduced female longevity. Such effects have never been observed in natural colonies. Genetic analysis of *C. batesii* revealed that about 20% of all matings in natural colonies were among non-related individuals (and similar results were obtained for *C. nigra* and *C. obscurior*, Schrempf and Heinze, unpubl.), and this may be sufficient to prevent inbreeding depression. As individuals are able to recognise kin (see above), they may outbred whenever it is possible.

However, inbreeding depression in haplo-diploid systems can affect males only indirectly via the mothers, as they eclose from unfertilized eggs. Male sterility thus has to be transferred from the mother to their sons, and this could happen for example through maternal effects.

In the female sex, inbreeding leads to reduced longevity. This can be either a direct effect of inbreeding (Herndon and Wolfner, 1995; van Oosterhout et al., 2000; Vermeulen and Bijlsma, 2004) or, alternatively, an effect of mating with sterile males (see below). As inbreeding does only affect female longevity, but not other traits as e.g. female egg laying rate, an indirect effect on lifespan through males is conceivable, as we were able to show that mating has important influences on the lifespan of queens.

According to the prediction that males in social insects can only gain high reproductive success in cases females live long and have a high reproductive output, mating has a positive effect on female longevity, and this effect is independent of female fecundity (Chapter 6).

The longer life span may either result from physiological or endocrinological changes in the queen triggered by the mating act itself, or alternatively through the transfer of specific male substances such as antioxidants in the seminal fluid. Seminal fluids can contain toxic substances in species with sexual conflict (Chapman, 2001; Chapman et al., 1995; Kalb et al., 1993; Snook, 2001; Wolfner, 1997; Wolfner, 2002), and consequently the opposite may have evolved in the case of sexual cooperation.

Coming back to inbreeding, the probability that not only the sperm viability of males, but also the production of seminal fluids is affected, is high. Then, the reduced lifespan of queens could easily be explained by changes in the composition or amount of seminal fluids transferred to females by the males. Conversely, in case that lifespan in *Cardiocondyla* queens is prolonged due to physiological changes of mating, the reduced lifespan in inbred females has to be due to direct negative effects of inbreeding on females – independent of male sterility.

Despite the lack of inter-sexual conflict, intra-sexual conflict can lead to competition between males, where a female mates with more than one male. To gain maximal reproductive success, males e.g. can prevent females from remating by making them unattractive for other males or by the use of mating plugs (Mikheyev, 2002; Robertson, 1995; Robertson and Villet, 1989; Tram and Wolfner, 1998), they can try to destroy and remove sperm from competitors (Harshman and Prout, 1994; Price et al., 1999; Singh and Singh, 2001) and finally, promote success of their sperm in direct competition. In *Cardiocndyla*, females seem to be attractive to males independently whether they mated or not- as long as they did not shed their wings (after several days), and mating plugs also could not be detected. Though, nothing is known concerning sperm displacement or success of sperm in direct competition. In the future, it will be of interest to investigate competition between the sperm of the two male morphs, as this may also have an effect on their fitness.

## Conclusions

In *Cardiocondyla*, it appears that the switch to intranidal mating lead to the evolution of alternative reproductive tactics due to locally competing males. Accompanied with this, sex ratio got female biased, and, in addition, mating between relatives lead to inbreeding. Inbreeding in turn contributed additionally to female biased sex ratios and lead to the evolution of a sex determination mechanism which is not based on heterozygosity. All of this probably evolved in concert, as it has been suggested for mating system, sex ratio and inbreeding depression in parasitoids (Antolin, 1999). *Cardiocondyla* is an exceptional ant genus, which shares local mate competition, inbreeding and alternative reproductive tactics in males with many non-social insects, but still shows all features of eusociality.

# SUMMARY

The ant genus *Cardiocondyla* is characterised by the occurrence of “workerlike”, ergatoid males that mate inside the nest. In many species of this genus, “normal”, winged males occur in addition, and a dimorphism of wingless and winged males is exhibited. The two male morphs represent alternative reproductive tactics with aggressive territorial males and peaceful disperser males. The latter mate inside the colony and then leave after several days to establish further matings with alien queens. The male morph is determined environmentally and thus the result of a conditional strategy.

Generally, in the male dimorphic species *C. obscurior* only ergatoid males are produced. The bigger and more “expensive” winged male develops only in case where environmental conditions become worse. Investigations have shown that the larvae themselves are insensitive to changes in environmental conditions, but that workers determine the winged male morph by treating the larvae differently. The colony is able to react to environmental changes quickly, as the sensitive phase of morph determination is late (at the end of the second larval instar). After approximately two and a half weeks, adult winged males can already eclose and disperse to reach new habitats. At an individual level, males would appear to be better winged, as they are not recognized by ergatoid males as competitors (due to female mimicry) and thus avoid being killed, and still have the opportunity to mate inside the nest before dispersing. However, larvae appear to have no possibility to influence their fate as it is usual in an eusocial community.

Local mating within the colony is accompanied with high levels of inbreeding. Inbreeding in haplo-diploids is less detrimental than in diploid organisms, as deleterious and lethal alleles are purged in haploid males. Exceptions are Hymenoptera with a complementary sex determination system, because in these, inbreeding leads to diploid male production. The study demonstrates that the inbreeding level in *Cardiocondyla* is very high. Inbreeding in addition to local mate competition, leads to even more female-biased sex ratios. As expected, another sex determination system than single-locus complementary sex determination or multi-locus complementary sex determination (with few loci), based probably not on heterozygosity, evolved in *Cardiocondyla.*

Nevertheless, inbreeding depression can be seen after several generations of sib mating in the laboratory. Genetic data suggest that events of outbreeding alternate with inbreeding in the colonies, and this may prevent inbreeding depression in nature. As queens appear to be able to recognize kin from non-kin, they may prefer to mate with unrelated males when a selection is available. Furthermore, multiple mating may promote outbreeding, and the costs of intranidal mating in terms of risk of pathogens and predators can be neglected. Moreover, mating has no

negative effect, as it can be seen in many species with sexual conflict, but instead prolongs the lifespan of the queens.

A dimorphism in the female sex of long winged and short winged queens is not representative of the typical alternative reproductive tactics of dispersing and territorial queens, but appears to correlate with the switch from polygynous, dependent founding ancestors to monogyny. All queens found their colonies independently after shedding their wings, but short winged queens invest into fat instead of flight muscle mass and consequently are more successful in establishing new colonies.

# ZUSAMMENFASSUNG

Die Ameisengattung *Cardiocondyla* zeichnet sich durch arbeiterinnenähnliche, ergatoide Männchen aus, die sich im Nest verpaaren. In vielen Arten der Gattung treten neben diesen ergatoiden Männchen auch noch die „normalen“, geflügelten Ameisenmännchen auf, so dass ein Männchendimorphismus zu beobachten ist. Die beiden Morphen verfolgen unterschiedliche alternative Reproduktionstaktiken: ergatoide Männchen sind aggressiv und territorial, während geflügelte Männchen friedfertig sind und die Kolonie verlassen. Bevor sie das tun, paaren auch sie im Nest. Nach einigen Tagen fliegen sie aus, um sich außerhalb des Nestes mit Weibchen aus fremden Kolonien zu verpaaren. Die Männchenmorphe ist nicht genetisch festgelegt, sondern wird durch Umwelteinflüsse gesteuert; es handelt sich also um eine konditionale Strategie.

In der Art *C. obscurior*, in der es beide Männchentypen gibt, werden normalerweise nur ergatoide Männchen produziert. Die geflügelten Männchen sind größer und „teurer“ und entwickeln sich nur, wenn die Bedingungen in der Kolonie schlecht sind. Untersuchungen haben gezeigt, dass die Larven nicht direkt auf die Veränderungen reagieren, sondern dass die Arbeiterinnen in der Kolonie die Larven auf eine andere Weise behandeln. Die Kolonie kann sehr schnell auf etwaige Veränderungen reagieren, weil die sensible Phase, in der bestimmt wird, in welche Morphe sich die Larve entwickelt, erst am Ende des zweiten (von insgesamt drei) Larvenstadiums liegt. Insgesamt dauert es etwa nur 2.5 Wochen, bis die geflügelten Männchen schlüpfen und sie die Kolonie verlassen können.

Für die Männchen selbst wäre es besser, die geflügelte Morphe auszubilden, weil diese von den ergatoiden Männchen nicht als Konkurrent erkannt (aufgrund von Weibchenmimikry) und somit nicht von ihnen getötet wird, und dennoch die Möglichkeit hat, sich im Nest mit Jungköniginnen zu paaren. Die Männchen haben jedoch, wie es in eusozialen Sozietäten üblich ist, keinen Einfluss auf ihre eigene Entwicklung.

Durch die Paarung im Nest tritt Inzucht in unterschiedlich starkem Grad auf. In Arten mit haplo-diploider Geschlechtsbestimmung ist das weniger schwerwiegend als in diploiden Arten, weil alle schädlichen Allele durch die haploiden Männchen eliminiert werden können. Eine Ausnahme stellen diejenigen Hymenopteren dar, in denen die Art der Geschlechtsbestimmung auf einem komplementären Mechanismus beruht, weil Inzucht dann zur Produktion von diploiden Männchen führt. Die Studie zeigt, dass der Grad der Inzucht in *Cardiocondyla* sehr hoch ist. Das führt dazu, dass der Anteil an Weibchen im

Geschlechterverhältnis noch höher wird als er bereits aufgrund von lokaler Paarungskonkurrenz ist. Wie erwartet, kann ein komplementärer Mechanismus der Geschlechtsbestimmung mit nur einem einzigen Locus oder mehreren wenigen Loci ausgeschlossen werden, und es hat sich wahrscheinlich ein anderer Mechanismus entwickelt, der nicht auf Heterozygosität beruht.

Trotzdem tritt im Labor nach einigen Generationen von Inzucht eine Inzuchtdepression auf. Die genetischen Daten zeigen jedoch, dass auch immer wieder Paarung zwischen nicht verwandten Individuen stattfindet, und es ist wahrscheinlich, dass dadurch in natürlichen Kolonien eine Inzuchtdepression verhindert wird. Außerdem scheinen Königinnen verwandte von nicht-verwandten Individuen unterscheiden zu können, so dass sie im Falle einer Wahl bevorzugt mit unverwandten Männchen paaren können. Ebenfalls zur Auszucht beitragen kann die Tatsache, dass sich Weibchen oft mit mehreren Männchen paaren. Die Paarung im Nest birgt keine Kosten und Risiken für die Weibchen, was Räuber oder Krankheitserreger betrifft, und sie verlängert das Leben der Weibchen. Dies ist ein großer Unterschied zu anderen Arten, in denen ein sexueller Konflikt zwischen den Geschlechtern auftritt und sich die Paarung negativ auf die Weibchen auswirkt.

Bei zwei verschiedenen Typen Weibchen, Königinnen mit langen Flügeln und Königinnen mit kurzen Flügeln, handelt es sich nicht um alternative Reproduktionstaktiken mit einer territorialen und einer Ausbreitungsmorphe. Vielmehr scheint dies mit der Entwicklung von polygynen Vorfahren hin zu monogynen Kolonien zusammenzuhängen. Alle Königinnen gründen ihre Kolonien ohne der Hilfe von Arbeiterinnen, nachdem sie ihre Flügel im Nest abgeworfen haben. Königinnen mit kurzen Flügeln investieren in Fett anstelle von Flugmuskel, so dass sie erfolgreicher neue Kolonien gründen können.

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