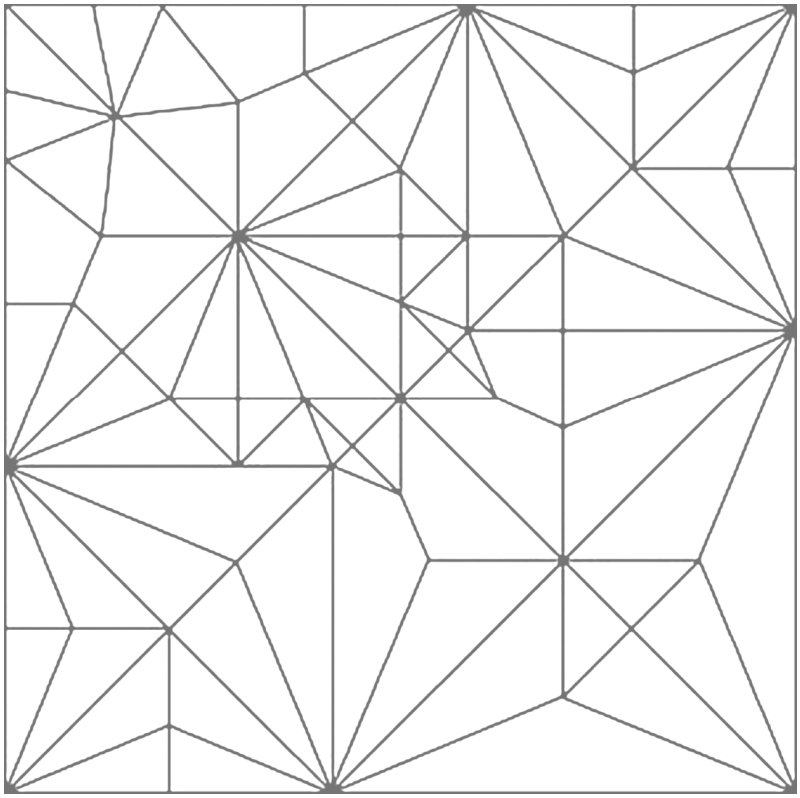
Natural History, Plastic Traits and Reproduction in Ants



Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.) der Fakultät III der Universität Regensburg

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Can love of insects make a difference?
I am not sure. But I would like to believe that it does.

Thomas Eisner

Acknowledgments

Ant science rocks! I am very grateful to Bob Johnson for showing me the road some ten years ago and to Jürgen Heinze for guiding me along these past three years. Because of my limited abilities this would not have been possible without Chris R. Smith (I want that child to be a long haired child), Thomas Schmitt, Mischa Dijkstra and John Wang.

Various help came from different sources, namely Helmut Durchschlag, Alex Schrempf, Yannick Wurm, Laurent Keller, Alex Wild, Adam Kay, Birgit Fischer, Barry Bolton, Corrie S. Moreau, Andi Trindl and Gudrun Herzner. Many thanks to Kathrin Stangel for much of the microsatellite lab work. Cheers to my almost-Doktorvater Jürgen Gadau. And of course Masaki: どうもありがとfor helping out whenever I needed it.

Many more people to thank: All my labmates, especially Bartosz for the discussions and Katrin for the fun times on the island. Maria and Tina for all the help with everything. The staff at the Southwestern Research Station. Special thanks to Robert the cook: A friend of the devil is a friend of mine.

Because nature is what keeps me happy: I am deeply obliged to the one great consciousness of which we are all part, whatever that might be, and its little critters. Thanks for all the stories. And sorry for the killing.

I also have to acknowledge my little hill for the socialization and whatnot: My (full) sister, the queen, and her mate.

Vera, I will always be your friend.

Table of Contents

Publications

This thesis is based on the following manuscripts:

Oettler J, Schmitt T, Herzner G, Heinze J (2008). Chemical profiles of mated and virgin queens, egg-laying intermorphs and workers of the ant *Crematogaster smithi*. Journal of Insect Physiology 54: 672-679

Oettler J, Dijkstra MD, Heinze J (to be submitted). One ant can make a difference: the adaptiveness of queen-worker intermorphs in *Crematogaster smithi*.

Oettler J, Heinze J (to be submitted). Polyphenisms of female reproductives in the tramp ant *Technomyrmex vitiensis*.

Smith CR, Oettler J, Kay A, Deans C (2007). First recorded mating flight of the hypogeic ant, *Acropyga epedana*, with its obligate mutualist mealybug, *Rhizoecus colombiensis*. J. Insect Science 7:11

Oettler J, Heinze J (manuscript). Phylogeny of the ant genus Cardiocondyla: Evolution of male morphology and life history strategies.

Oettler J, Wang J (report). Sexual cooperation: Genomic response to sex in *Cardiocondyla obscurior* ant queens.

Part I: General introduction

Chapter 1

Background and main findings of this thesis

Evolution of castes

Eusociality will challenge science forever. It is defined as the division of the reproductive labor, i.e. the partition into reproducing and non-reproducing units cooperating in the same colony at the same time. Moreover, extant species may contain up to a million non-reproducing individuals that are subdivided into task cohorts based on an inert specialization to perform particular tasks. This specialization can be either expressed as subtle behavioral plasticity (e.g. Oettler & Johnson in review) or is associated with morphological adaptations (Hölldobler & Wilson 1990). Ants in particular have realized division of labor to such an extreme that it enables them to inhabit almost every conceivable ecological niche.

Convergent evolution of eusociality - numerous times in the insects and at least nine times in the hymenoptera - suggests plastic genomic pathways that may show universal similarities. The ultimate selective forces that have lead to and maintained eusocial structures (in the Formicidae for the last 115-135 million years, Brady et al 2006) have been thoroughly addressed since 1964 (cf. Gardner & Foster, 2008) and are at times still subject to an active debate (Wilson & Hölldobler 2005, Foster et al 2006). While this has been subject to numerous studies, fundamental questions remain. How did flexibility evolve, i.e. what are the mechanisms that can potentially evolve by few mutational changes and that may lead to developmental plasticity? Most parsimonious, we can assume that independent lineages rely on the same (or similar) conserved genomic pathways responsible for expressed plasticity. However, only the termites and ants are strictly eusocial, while some species within the corbiculate bees reverted to solitary free-living strategies. In addition most species within the Sphecidae and Vespidae are solitary. Thus we have to assume a *potential* plastic genome as an ancestral trait which has been subject to selection under specific environmental conditions and which led to differential expression of plasticity of closely related lineages.

Since no exceptions occur within the termites and ants we also have to assume genomic or ecological constraints in these lineages that prevent reversal from the eusocial road associated with the degree to which plasticity is expressed (“point of no return” Wilson 1971) once eusociality has evolved. The genomic plasticity is a priori present (see below) and selection simply takes advantage of this flexibility. I want to emphasize the separation of selection on eusociality and plasticity in contrast to the model by Wilson and Hölldobler (2005) which assumes alleles “that induce cooperation and possess phenotypic plasticity which includes a non-genetic worker caste”.

Polyphenism and polymorphism in ants

One important recent finding is that not all plasticity found in ants (and the honeybee) is true polyphenism in the sense that different phenotypes have the same genetic background. There is evidence in species with natural, and experimentally created, diversity showing that different patrilines or matrilines are associated with behavioral (*Apis mellifera*, Frumhoff & Baker 1988, Robinson & Page 1988; *Solenopsis invicta*, Krieger & Ross 2002; *Eciton burcellii*, Jaffe et al 2007; *Acromyrmex versicolor*, Julian & Fewell 2004) and morphological specialization (*Pogonomyrmex badius*, Rheindt *et al.* 2005; *Vollenhovia emeryi*, Ohkawara *et al.* 2006) and caste determination (*Pogonomyrmex* inter-lineages, Julian *et al.* 2002, *Cataglyphis cursor*, Pearcy *et al.* 2004; *Wasmannia auropunctata*, Fournier *et al.* 2005). It is important to highlight that the likelihood to express one phenotype is associated with but not per se determined by the genetic background and to my knowledge exceptions occur in all cited cases (no exceptions are reported for *Wasmannia*

auropunctata). Future work will require the combination of insights in caste development and task partitioning of mono- and polyandric and mono- and polygyne species.

Phenotypic plasticity and division of the reproductive labor

The outcome of plasticity is fascinating. Reproductive division of labor results in different phenotypes, each of them exhibiting distinguishable behavior. Queens may experience an entirely different world than workers. This is exemplified by the life cycle of *Acropyga epedana*, an ant living in obligate mutualism with mealybugs (*Rhizoecus colombiensis*) which provide honeydew, i.e. sugar-rich fecal excretes, and which are in return cared for by the colony (Chapter seven). A virgin queen leaves the nest carrying a fertilized female mealybug between the mandibles, mates with a male at a mating aggregation, and walks or flies off after mating to find a suitable nest site. This being accomplished she starts to dig into the soil to escape the threat of the environment, relying on a thick cuticle to minimize the effects of abrasion (cf. Johnson 2000). In those rare instances where the queen and the mealybug survive this initial phase, she has to dig to the roots of grass to deposit the bug which begins to feed on the root sap and produces offspring itself. The queen will then begin to lay eggs and to raise a first generation of workers. Eventually, the workers take over the entire non-reproductive labor and continue to guard and nurse the mealybugs to benefit from their fecal droplets. In addition, workers start to tend and feed the queen-produced eggs, larvae, pupae and eventually also the mother queen. This division is accompanied with a variety of morphological and physiological adaptations. None of these workers will ever experience the epigaic ‘outer’ world and they only have weak pigmentation and are photophobic. Workers probably also lack cuticular hydrocarbons to protect the body from water loss as we found them to die soon after excavation when exposed to light and air. Much like the metamorphosis of holometabolous insects, the dichotomy of queens and workers enables them to occupy different environments.

Physiological constraints of plasticity are also demonstrated by the differential expression of cuticular hydrocarbons (CHC) in the three female castes of *Crematogaster smithi* (Chapter four). The hydrocarbon signature in ants - and social insects in general - has been shown to function as a key recognition cue for nestmate and kin recognition (cf. Howard & Blomquist 2005) and also as a reliable and ‘honest’ signal to display the fitness of the reproductive. *C. smithi* colonies contain a single inseminated queen, a few hundred workers and 0-16 intermorphs (Chapter five). Intermorphs are highly fertile but not capable of producing diploid offspring as they are unable to mate. We analyzed the CHCs of workers, intermorphs, mated and virgin queens using various common statistical approaches and argue that the observed differences are due to a combination of morphology, physiological activity and age. One interesting result of this study is that we did not find a signature that discriminated fully functional queens from the egg-laying intermorphs (but see Chapter three / Analysis of cuticular compounds). This indicates that in *C. smithi* it may be rather quantitative than qualitative aspects that underlie fertility recognition and that small quantitative changes of single substances escape the sensitivity of our statistical methods.

Phenotypic plasticity and evolution of queen and male traits

To make things even more complex, the common view of the dichotomy of morphological queens and workers is vanishing (Heinze 2008). While we can still differentiate between functional reproductives and non-reproductives this idea is impracticable when it comes down to the morphology. Queens may attain a worker-like morphology and vice versa and may have more than one discrete reproductive caste. Chapter 6 describes the two reproductive queen castes of *Technomyrmex vitiensis* whose occurrence is associated with different reproductive functions and stages in the life cycle of the colony. This species contains smaller intermorphic wingless queens that occur in established colonies in great numbers allowing for rapid spread of the colony by budding. Intermorphic queens are slightly larger than workers and have a more advanced anatomy in that they have a spermatheca for the storage of sperm. In addition *T. vitiensis* produces a

'regular' winged queen morph that might function as a dispersal morph, a trait described in detail for the close related *T. brunneus* (Tsuji *et al.* 1991). Several species of the *T. albipes* group feature this dualism of queens and intermorphic queens, indicating that plastic traits are evolutionary stable and persist over time after the reproductive split of a population leading to two new species.

Analogically, some species of the new world myrmicine genus *Pogonomyrmex* (and its sister group *Ephebomyrmex*, Heinze *et al.* 1992, Johnson *et al.* 2007) contain intermorphs (RA Johnson pers com), hence plasticity - again - is an ancestral trait, exploited when advantageous. A mapping of queen polyphenism on the phylogeny of *Pogonomyrmex* (Strehl 2005 PhD Thesis) is recommended, as *Pogonomyrmex* is one of the best studied ant genera to date.

Compared to queen plasticity (reviewed in Rüppel 2000) plasticity in males is a rare trait in the Formicidae. Exceptions occur and the most prominent male dimorphism to date, found in the genus *Cardiocondyla*, is described in Chapter eight within a phylogenetic context. Wingless males in this genus are common to all species for which males are known, although winged males may co-occur in a number of species. A previous phylogeny based on mtDNA failed to generate a comprehensive picture, thus I enlarged the dataset in terms of taxa and nDNA markers. The presented topology, again, has its weakness and some basal nodes remain unresolved resulting in ambiguous polytomy. Nevertheless, this phylogeny sufficiently supports our main predictions: The co-occurrence of winged and wingless males is the ancestral condition and the loss of wings in males is an adaptation associated with intranidal mating of (mostly) close related virgin queens and males. Some species within the genus have completely lost the winged male caste while others have regained winged males secondary, indicating costs of simultaneous reproductive strategies.

Phenotypic plasticity and conflict in ants

As predicted, conflict over reproduction among individuals of a reproductive unit is common in monomorph genera such as *Polistes* (Tibbetts & Reeve 2000) and polyphenic species (Hammond & Keller 2004). Conflict among individuals of a social unit and conflict resolution has been highlighted since Trivers and Hares seminal work (1976) that synthesized Hamilton's kin selection theory (1964) and Fisher's sex ratio theory (1930) and led to clear cut predictions about conflict. The focus has been brought to the selfishness of the individual. We tend to view individual workers as being independent entities. In ants, bees and wasps workers theoretically have the potential to enhance their own inclusive fitness. This is realized directly by fertile workers that produce haploid males, and indirectly by workers which selectively adjust the sex ratio towards females or males, increasing their own inclusive fitness that way.

Conflict between cooperating individuals is theoretically a very unstable condition in evolution that could lead to a waste-of-energy arms race between members of a group. On a species level conflicts are restricted by interspecific fitness as the unit still is the one entity that is subject to selection by the means of intra and interspecific competition. Thus there is only a narrow "space for potential conflict" (Fig 1) where workers and queens can compete with each other at low efficiency costs without reducing their chances of survival beyond a given threshold at which the species' fitness would suffer.

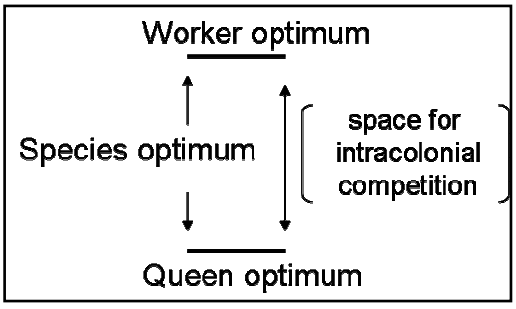

Figure 1. Limitations to intracolonial conflict.

Chapter 5 of this thesis addresses this conflict over reproduction among individuals of the monogyne-monoandric *Crematogaster smithi*. As mentioned above, this species contains intermorphs that are specialized in producing large numbers of haploid eggs (Heinze *et al.*1995, 1998, 2000). Our genetic analysis showed that these eggs do only rarely, if at all, develop into males. Instead the presence of intermorphs is associated with a more female-biased sex ratio. Hence, we suggest that the surplus of available eggs produced by intermorphs is reinvested in the more costly sex (females), although direct evidence is still missing.

The genomics of plasticity

In general, polyphenism, i.e. discrete phenotypes within a species sharing a single genotype, is a common trait in the animal kingdom. Phenotypic diversification by external cues can result in sex determination (Crocodilia, temperature-dependent sex determination, Janzen & Paukstis 1991; blue headed wrasse fish, gender-dependent sex determination, Warner 1984), predator-induced dimorphism (*Daphnia*, Agrawal *et al.*1999), habitat camouflage dimorphism (Lepidoptera, Wiklund & Tullberg 2004, Brakefield & Reitsma 1991), feeding preference dimorphism (larval tiger salamanders Hoffmann & Pfenning 1999, spade-foot toad tadpoles Pfennig 1992) and season-related sexual strategies (Aphidae, Brisson & Stern 2006), just to name a few prominent examples.

All these phenotypes result from external conditions that alter the differential expression of genes. A very appealing example is found in the ants: the pathways leading to either queens or workers (and the pathway leading to male dimorphism in *Cardiocondyla*, Schrempf & Heinze 2006) are determined by cues such as nutrition and temperature (Hölldobler & Wilson 1990, Wheeler 1991). While only queens have the power over primary sex determination, both queens and workers can determine secondary sex ratio and morph development by influencing egg deposition and larval rearing conditions under varying spatio-temporal regimes.

Social insect polyphenisms and the involved underlying hormones have been studied since Carlisle and Butler (1956) have described a primer pheromone produced by honeybee queens. This 'queen substance' inhibited ovary development in honeybee workers but also affected *Formica fusca* ant worker development. Carlisle and Butler already recognized the commonality of development and compared the physiological reaction to the 'queen substance' to a similar mechanism in decapode crustaceans. This substance was then isolated and described a few years later (Butler *et al.* 1959) and the discovery triggered physiology-based research on caste development.

But with the advent of genomic tools the proximate pathways have just recently been addressed. A broad taxonomic approach is now feasible with the completion of the genome projects on *Drosophila* (Drosophila 12 genomes consortium 2007), *Tribolium* (Tribolium genome sequencing consortium 2008), honeybee (Apis genome consortium 2006) and many more to come in the near future. Studies on the differential expression of candidate genes have targeted various insects which in the near future might or might not reveal similarities of evo-devo patters. These organisms with distinct polyphenism are butterflys (*Bicyclus anyana*, cf. Beldade *et al.* 2005), pea aphids (*Acyrthosiphon pisum*, cf. Brisson *et al.* 2007), termites (*Cryptotermes secundus*, Weil *et al.* 2007), locusts (*Schistocerca gregaria*, cf. Kang *et al.* l 2004), honeybees (*Apis mellifera*, cf. Whitfield *et al.* 2002) and ants (Abouheif & Wray 2002, Goodisman *et al.* 2005, Wang *et al.* 2007, Ingram *et al.* 2005). A first comparative study by Abouheif and Wray (2002) demonstrated that the patterns underlying the development of wings is conserved in *Drosophila* and ants but that the expression patterns of these differ across closely related species.

We are crossing the threshold to discover the proximate subjects to selection and evolution of caste on a fine scale. The honeybee genome and the fire ant EST library are first promising step stones. Nevertheless, regulatory mechanisms of caste development appear to be very complex and so far only few studies on ants have been conducted. Based on the striking differences in RNA expression obtained from comparing larval and adult workers (Goodisman *et al.* 2005) and adult queens and workers (Gräff *et al.* 2007) we can expect to find extreme complexity in caste development. How to make sense of such a vast amount of information about a multitude of genes

and regulatory pieces that are connected in pleiotropic networks and that cascade each other? How are we expected to understand the genomics of caste development? Thus far not a single study has been conducted on a whole genome-based EST description of genomic expression at different developmental stages across castes (but the scientific community will have this available very soon). To target genes and regulatory genomic interactions involved in caste development it will be necessary to combine expertise. We will need to conduct more studies on development and behavior to dissect the roles of candidate genes using a broad taxonomic framework assuming that conservative mechanisms account for much of the observed polyphenisms in social insects.

Chapter nine of this thesis addresses the genome-wide response to sex in *Cardiocondyla obscurior* queens as a first step in applying EST-based technology to ant behavior. Our results show that thousands of genes immediately change their expression in response to sex, indicating unsurprisingly that sex has dramatic effects.

Chapter 2

Evolution of this thesis

This thesis, being quite very heterogeneous in terms of species, topics and methodology, has evolved over the past few years. Hence, a brief history of how I came to do these different things is just appropriate.

When I started my PhD in 2005 the idea was to focus on the intermorphs of *Crematogaster smithi*. Back in the early 1990's Stefan Cover (MCZ, Harvard) and Jürgen Heinze discovered that the intermorphs do not occur in every nest that they excavated. This probably was the point when they realized that these intermorphs are not just some arbitrary freak caste but that they might have some adaptive value instead. Jürgen kept working on this species (Heinze *et al.* 1995, 1998, 2000) but the collection is very time consuming and success limited to those few rainy weeks in summer. In the beginning I did neither know the species nor anything about intermorphs. But from past experience I was familiar with the area where *C. smithi* occurs. In summer 2005 I went to collect colonies in the Chiricahua Mountains, one of my all time favorite places. Collecting essentially is the never-ending process of spending all morning flipping small rocks in hope to find the occasional worker which would lead me to a colony. Naturally, this has a severe impact on the environment. Located at ca. 1,800m elevation, the meta-population is protected to some extend from the severe heat of the lowlands, but the Chiricahua Mts are what they are: A desert habitat with very low precipitation. Moisture is trapped underneath rocks and pebbles and many animals rely on these microclimate conditions. Even when one tries to carefully relocate the rocks, this whole collecting process has a negative impact on the environment, something an ecologist should not ignore. After the typical initial failure I brought back some colonies and started the analysis of the cuticular profiles.

In summer 2006 I returned but collected only half of what I was aiming for, likely due to two factors. First, the Chiricahuas are – like everything else – subject to climate change, i.e. the summer monsoon rains are less predictable and winter rains also occur less frequently. Winter 2005/2006 was very dry, the oak trees did not produce a full set of leaves the following spring and as a result of this the ground was less shaded. On average, the colonies that I collected in 2006 were smaller, contained fewer sexuals and were also less active than in 2005. Second, in 2005 I already might have collected too many colonies from the known locations where they occur in greater numbers.

The Chiricahuas are one of the biodiversity hot spots of the planet. Formed by volcanic activity some 25-30mill years ago, species from two desert habitats (the Sonaran and Chihuahuan desert) and two mountain ranges (the Rocky Mountains and the western Sierra Madre) meet here, finding refuge from the desert plains. These "sky islands" are characterized in general by high species diversity but low abundance; deserts are a highly competitive place. *C. smithi* has found its niche but occurs only in few places. Even the 30 highly motivated rock-flipping students and 15 instructors of the infamous "ant course" held every other year at the Southwestern Research Station rarely find a nest. Using black-light attraction of male dispersers I attempted to locate additional sites across the Chiricahuas. But apart from some few males that I collected every now and then at various locations, the more dense population seems to be restricted to a small valley at mid-elevation. My collections of mated queens at the night of the main female mating flight (for which I used a mercury vapour light that could be seen for miles) indicated that the yearly number of new colony foundations is very low. In sum it was crucial to give the species a break. I believe that my impact was not justified any more and that the habitat and the species need to recover. Many questions remain and I would love to return and continue these studies.

Thus I needed a new topic/species to work on and Jürgen was fast suggesting *Cardiocondyla*. As I was used to working with 'real sized' ants, such as *Pogonomyrmex* harvester ants or even *C. smithi*, I was not too enthusiastic in the beginning. Not necessarily something I consider a manly genus. Very, very tiny. They do not sting. I had never collected a single sample before, although I have probably seen them in the field somewhere, ignoring them, thinking I would not want to identify the species anyway. Read Seifert's (2003) revision; it becomes very clear what I mean. But soon I found out that *Cardiocondyla* is perfect, despite their small size or even because of it. They clearly have the potential to become the 'Drosophila-like' model organism of ant science. We already know so much about the biology and evolution of the genus, they are easy to keep in the lab, have a short generation time and produce sexuals voluntarily all year round. These ants can be used as a very powerful tool to understand genomics, just because they are a true laboratory species. In this respect they by far out-compete the fire ant *Solenopsis invicta* or the harvester ant genus *Pogonomyrmex*. Eventually we will have a sequenced genome and will be able to address manifold questions concerning the evolution of male and female phenotypic plasticity, processes involved in the hybridization of species, sex and colony founding strategies and of course behavior.

Since I changed my mind late (or more precisely: got shut down by the limits in studying *C. smithi*) my two *Cardiocondyla* projects are not finished and included here as project descriptions. In the very near future we will add new species to the phylogeny of *Cardiocondyla*. Furthermore, we are currently sequencing additional nDNA fragments to get a more robust topology. And, second, the EST (expressed sequence tags) study on queens of *C. obscurior* just started to reveal its full potential.

Although I have a crush for the elegant myrmicine ants (the postpetiolus! Great thing!), along the road of my thesis I also stumbled about two other ants. The dolichoderine *Technomyrmex vitiensis* and the formicine *Acropyga epedana*. Forget everything I just said about *Cardiocondyla*: Each and every species of ants is cool and has its own crazy story to tell. Considering that there are approximately 15,000 species out there, the book of ants will not be written all too soon.

Chapter 3

Methods

Anting and Digging

Ant science still relies on finding the things actively, which can be pure pleasure. You need tools such as a shovel, an axe, a knife or even a spoon. You need to lure, dig hard, poke in every hole, break open everything you can get your hands on and you will find many things you did not expect. Not only ants but also all the other things (with and without extremities) that hide in cavities. Digging can be a very contemplative experience, such as the two hour plus excavations of *Crematogaster smithi* colonies using a teaspoon. Or very exciting, like the tree whacking actions to get a dozen of *Platythyrea* workers.

Analysis of cuticular compounds

When we enter the world of chemical communication we are unable to sense anything. Like a blind person lost in a discotheque. Our primary senses are sight, hearing and touch, accompanied by the weaker senses of taste and smell. Our stronger senses do not play a big role in the sensual array of ants. Instead, they communicate with the world by means of taste and smell and sense their environment with volatile chemicals. Ants may have good vision and they may communicate with tactile signals, but like most insects they mainly rely on the use of chemical signals. These are used as recognition cues and activity mediators for labor and defense, a very early finding. The history of chemical ecology in ants ranges back to the 19th century (cf. Jackson & Ratnieks 2006). Since then substances in the Formicidae have been identified that are correlated with particular biological situations like alarm and trail pheromones, primer pheromones or caste specific recognition cues (D'Ettorre *et al.* 2004). Using gas chromatography coupled with mass spectrometry or neurophysiologic tools we have the opportunity to discriminate signal from noise and describe single substances that play a role in communication - albeit we are still limited in deciphering the code. Obvious questions concern the quality and quantity of the signal and how ants actually perceive them. Citing Meinwald (2003): "If one were to ask whether chemistry right now is anywhere near reaching its ultimate goal with respect to providing a full molecular understanding of chemical communication, the answer must be a resounding NO!"

In *Crematogaster smithi* I sought to detect whether morphological phenotype (queen, worker, intermorph) or physiological phenotype (high versus low fecundity) is associated with a particular chemical phenotype.

Phylogenetic analysis

The understanding of the phylogenetic relationships of taxa is fundamental to understand life. Since Darwin's voyage on board of the 'Beagle' eventually led to a universal concept of speciation, nothing in biology can be viewed without the premise of comprehending the evolution. The field of phylogeny is the most traditional discipline in biology and we are close to comprehend the eukaryotic tree of life (Sanderson 2008). With the advent of DNA sequencing tools it appears that the reconstruction of systematic relationships does not require special training in taxonomy any more. Many morphology-based sister-group relationships within the Formicidae have been questioned by results from DNA-based topologies (Brady *et al.* 2006). Sequencing techniques are advancing (whole genomes, ESTs and barcoding) but most DNA-based studies are still based on few gene fragments. These fragments may be subject to differential speed of evolution and likelihood of base substitutions and bear the risk of suggesting a valid truth. Without a comprehensive understanding of morphology and using only limited DNA sequence datasets one

will risk drawing wrong conclusions about relationships. But in concordance with knowledge of morphology and behavior we can confidentially assess past divergence of species. And the value of morphology-based alpha taxonomy is out of question.

The genus *Cardiocondyla* is such an example where we are able to combine morphology, biology and DNA information to generate a comprehensive phylogeny. I used DNA sequences as an unbiased estimate of a potential topology and discussed the results in respect to morphology and behavior. To assess sequence divergence I used the computational approaches of MrBayes and GARLI. Both programs are still under development and their output remains subject to an active debate within the scientific community. Clearly, sequencing technology is faster than the development of universal analyzing tools and morphology-based alpha taxonomy (unfortunately we cannot clone Seifert).

Kin structure analysis

The evolutionary synthesis combined the mathematical approach of population genetics and Darwin's seminal finding of natural selection (cf. Mayr & Provine 1998). During the past three decades population genetics has become a very powerful tool in deciphering ant biology. It is indispensable in studying kin selection and kin conflict testing the predictions of Trivers and Hare (1976). Due to the hymenopteran haplo-diploid sex determination it is particularly easy to assess the kin structure of a colony. Microsatellites as quasi-neutral markers are especially useful in this context. I used microsatellite analysis to detect worker-worker relationship, paternal contributions to worker genotypes and worker reproduction in *Crematogaster smithi*.

Gen expression data

Ant science develops, slowly but steadily. Still in the early stages, the ant science community started to focus on the role that genes play in ants. Although clearly not a model organism yet (such as *Mus musculus, Caenorhabditis elegans, Drosophila melanogaster* or even *Apis mellifera*) we have started to address pathways underlying developmental patterns. Before long, we will have the first sequenced ant genomes. A first step towards functional biology is the development of the EST-library for the fire ant *Solenopsis invicta* (Wang *et al.* 2007). By using ESTs you may screen many genes simultaneously to obtain information on complex regulations. I used this library for an experiment on sex effects in *Cardiocondyla obscurior* showing a high sequence overlap of these two close related species and the applicability of this technique for the small sized *Cardiocondyla*.

Analysis tools

I used the following programs and applications

Statistica 6	(StatSoft)
SPSS 13.0	(SPSS Inc.)
SAS 9.1.3.	(SAS Institute)
PAST 1.75b	(http://folk.uio.no/ohammer/past/)
Agilent Chemstation	(Agilent Technologies)
Chromas	(Technelysium Pty ltd)
BioEdit	(Tom Hall, Ibis Biosciences)
MrModeltest	(http://www.abc.se/~nylander/)
MrBayes 3.1	(http://mrbayes.csit.fsu.edu/)
Paup 4.0	(Sinauer Ass, Inc. Publishers)
GARLI 0.951	**(**www.bio.utexas.edu/faculty/antisense/garli/Garli.html)
FigTree v1.1.1.	(http://tree.bio.ed.ac.uk/software/figtree/)
Relatedness 4.2	(http://www.gsoftnet.us/GSoft.html)
Matesoft Silver version 1.0	(http://www.bi.ku.dk/staff/jspedersen/matesoft/)
GenePix 6.0	(MDS Inc)
MeV	(http://www.tm4.org/mev.html)
BAGEL	(http://web.uconn.edu/townsend/software.html)
R 2.7.1.	(www.r-project.org, www.bioconductor.org /
bioconductor / limma	www.bioconductor.org/ packages/release/bioc/html/limma.html)

Part II: Phenotypic plasticity: Case studies

Chapter 4

Chemical profiles of mated and virgin queens, egg-laying intermorphs and workers of the ant *Crematogaster smithi*.

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Queen, intermorph and workers of *C. smithi*. The intermorphs are morphologically intermediate (top center). Photograph © Alex Wild 2005 (used with permission)

Abstract

Many colonies of the North American ant *Crematogaster smithi* contain a “third female caste” in addition to queens and workers. These “intermorphs” are morphological intermediate of queens and workers and have well-developed ovaries but lack a spermatheca for the storage of sperm. They are specialized for laying large numbers of unfertilized, viable eggs, most of which serve as food for larvae and adults, though a few may eventually develop into males. Based on the assumption that cuticular hydrocarbons (CHCs) in social insects honestly signal the reproductive status of an individual we investigated the CHC of mated mature queens, virgin queens, intermorphs and workers. We expected intermorphs to show chemical profiles intermediate between those of mated queens and non-reproductive workers. A discriminant analysis of the chemical profiles reliably separated queens, virgin queens, and workers, but failed to distinguish between queens and intermorphs even though workers were apparently capable of doing so.

Introduction

The elaborate division of reproductive labour characterizing insect societies (Wilson 1971, Hölldobler & Wilson 1990) is maintained or at least stabilized by complex communication, mainly via tactile or chemical signals. In ants, reproductive status is commonly reflected in an individual's blend of cuticular hydrocarbons (CHCs). The chemical profiles exhibited on the cuticle of fertile queens and, in species that have lost the queens caste, gamergates (mated workers, Peeters 1991) typically differ from those of non-reproductives in the absolute quantities and relative proportions of particular compounds (Cuvillier-Hot *et al.* 2004, Denis *et al.* 2006, D'Ettorre *et al.* 2004, Dietemann *et al.* 2003, Endler *et al.* 2006, Hannonen *et al.* 2002, Hartmann *et al.* 2005, Heinze *et al.* 2002, Liebig *et al.* 2000). For example, in *Gnamptogenys striatula*, the quantities of 32 substances varied with different degrees of fertility, and two CHCs each occurred either only in reproductives or non-reproductives (Lommelen *et al.* 2006).

Substances characteristic of reproductives have previously been interpreted as manipulative "queen pheromones", which suppress worker reproduction (e.g., Dejean & Passera 1974). In contrast, it is generally agreed today that such substances serve as honest fecundity signals, to which non-reproductives react in their own interest by "voluntarily" refraining from egg laying (Keller & Nonacs, 1993). Most research on the association between variation in cuticular hydrocarbons and fecundity is as yet correlative. Direct evidence comes from two studies showing that the antenna of *Pachycondyla inver*sa workers responds to a specific alkene characteristic of reproductives (D'Ettorre *et al.* 2004) and that workers of *Myrmecia gulosa* can discriminate between cuticular extracts of fertile queens and infertile workers (Dietemann *et al.* 2003).

Here we report on variation in CHCs among female castes of the myrmicine ant *Crematogaster smithi* (Creighton 1950). This species exhibits an unusual caste system that differs from the typical queen-worker dichotomy of other social insects in the occurrence of a peculiar third female caste (Heinze *et al.* 1995, 1999, 2000). These "intermorphs" of *C. smithi* (in previous papers referred to as "large workers"; we here use the morphology-based terminology of Buschinger & Winter 1976) are intermediate between regular workers and queens in morphology and behaviour. Almost half of all colonies collected in the Chiricahua Mountains, Arizona contained between one and ten intermorphs (median = 2). Their ovaries were usually well-developed with numerous maturing oocytes but without a spermatheca for the storage of sperm (Heinze *et al.* 1995, 1998). Egg-laying rates of queens, intermorphs, and workers differed significantly (Queens: 2.1 eggs / day / mg fresh weight; Intermorphs: 0.97; workers: 0.05; Heinze *et al.* 1995). It appears that most of these unfertilized, intermorph-laid eggs, though viable, are normally eaten by the queen and larvae and can therefore be considered as a storable food source. However, genetic data suggest that a minor proportion of unfertilized eggs laid by intermorphs and / or workers may develop into male offspring (Heinze *et al.* 2000). Thus, it appears that workers can discriminate queen-laid eggs from those produced by workers and intermorphs based on chemical cues as has been shown before in other ants (D'Ettorre *et al.* 2004, Endler *et al.* 2006).

The complex reproductive system of C. smithi, with highly fertile queens, which lay both diploid and haploid eggs, virgin queens, which when prevented from mating and kept in isolation lay unfertilized eggs, intermorphs, which at a similar rate produce unfertilized eggs, and workers of very low fecundity, provides a unique system for investigating the relationships among fertility, mating status, and chemical signalling. We therefore analyzed the CHC profiles of the four phenotypes by gas chromatography (GC) and mass spectrometry (GC-MS) to determine whether and how ovarian activity and mating status are reflected in their cuticular hydrocarbon profiles.

Methods

Ant collection and rearing conditions

We excavated colonies of *Crematogaster smithi* in the summer of 2005 from their nests in soil in juniper–oak-pine forests in the Chiricahua Mountains in Southeast Arizona, USA (for details on the collecting site see Heinze et al., 1995, 1999). The nests consisted of a single queen, workers, intermorphs, and winged virgin queens and males. Virgin queens remain in the nest until the mating season starts in mid-August and then disperse to mate and found their own colony. Colonies were housed in boxes (20 x 13 x 5 cm^3) with moist paper in the Southwestern Research Station, Portal, AZ, and later in Regensburg in similar-sized boxes with a plaster floor at 25°C and 60% humidity and day/night time adjusted to Arizona time twice a month. The ants were fed with *Drosophila*, pieces of cockroaches, and sugar water three times per week. For the analysis we selected seven medium sized queenright colonies. All CHC extractions were completed within four months after transfer to the lab.

Identification of cuticular substances

For the separation and identification of CHCs by GC-MS, pooled extracts from 10 workers each were used. Hexane extracts were first fractionated with hexane and dichloromethane using SiOH cartridges (Macherey-Nagel, Düren, Germany). The nonpolar fraction was further fractionated on silica gel treated with 5% $AgNO_3$ to separate saturated from unsaturated hydrocarbons.

To confirm the identification of alkenes, alkadienes, and alkatrienes based on GC-MS data, unsaturated compounds were hydrogenated using palladium on carbon and hydrogen, and the resulting alkanes were identified. Dimethyldisulfide derivatisation was carried out to determine the position of double bonds following Dunkelblum *et al.* (1985).

GC-MS analysis was performed with an Agilent Technologies 6890N gas chromatograph coupled with an Agilent 5373 Mass Selective Detector (Agilent Technologies, Böblingen, Germany). The GC was equipped with a J & W DB-5 fused silica capillary column (30 m x 0.25 mm ID; df = 0.25 µm; J & W, Folsom, CA, USA). Temperature was programmed from 60°C to 310°C at 5°C/min and held for 10 min at 310°C. Helium was used as a carrier gas at a constant flow of 1 ml/min. Injection was carried out using a split/splitless injector at 250°C in the splitless mode for 60 sec. Electron impact mass spectra were obtained with an ionisation voltage of 70eV and a source temperature of 230°C. MSD ChemStation Software (Agilent Technologies, Böblingen, Germany) for Windows was used for data acquisition.

Solid phase micro extraction of cuticular compounds and GC analysis

Egg-laying queens (n = 7) from seven colonies, intermorphs (n = 16) and non-laying workers from five of these seven colonies (n = 18) were analyzed for differences in their CHC profiles. Egg-laying virgin queens (n = 5) taken from 3 colonies were analysed as a fourth group to determine how mating status influences the CHC profiles of queens. Because workers started attacking and killing virgin queens shortly after the end of the mating season we isolated them in 25ml glass tubes with moist cotton for 2 - 3 month until chemical analysis. All virgin queens shed their wings after a few days and started laying eggs immediately after separation from their natal nests. Their egg-laying rate appears to be similar to that of intermorphs: during 35 days in isolation, three virgin queens produced 18, 22, and 24 eggs, respectively. For the remaining two virgin queens only the presence of eggs was noted.

For cuticular extractions we used the non-destructive solid phase micro-extraction method (SPME; Belardi & Pawliszyn 1989) because colonies and therefore queens of this species are difficult to collect and we wanted to keep the ants alive for further studies. We extracted by rubbing a SPME fibre (7µm polydimethylsiloxane coating; SUPELCO, Deisenhofen, Germany) between

the head and the pronotum of live ants 100 times. The fibre was then inserted into the injection port of an Agilent 6890N gas chromatograph (Agilent Technologies, Böblingen, Germany) with split/splitless inlet and flame ionization detector and Rtx-5 capillary column (30m x 0.25 mm x 0.5 um, Restek, Bellafonte, PA, USA). Helium was used as a carrier gas with a constant flow of 1 ml/min. The temperature was kept at 100°C for 1min, increased to 180°C, at 30°C/min, from 180 to 300°C at 2°C/min, and kept at 300°C for 10min.

Verification of single profiles by extraction of multiple ants

SPME analysis of single workers gave ambiguous results about the presence of two minor peaks (1 and 19, see below). Because workers are small compared to queens and intermorphs we pooled extracts to verify that the non-detection of peaks 1 and 19 is not due to a qualitative difference among castes but results from the detection threshold of the GC. Ants were killed by freezing, stored individually at -20°C, and thawed for 3min before CHC extraction. Extracts from individuals from each group (queens: n = 3; intermorphs: n = 8; workers: n = 10) were pooled by rubbing their bodies with the same SPME fibre (9µm polydimethylsiloxane coating) and analysed as described above with slightly changed conditions (temperature was kept at 60°C for 1min, increased from 60°C to 180°C at 30°C/min, from 180°C to 300°C at 2°C/min, and kept constant at 300°C for 5 min). Profiles from pooled individuals were analyzed by eye with focus on those peaks that appeared to be absent in workers.

GC data statistic analysis

To overcome statistical problems associated with multicolinearity and non-normality of GC-derived data we applied a standard procedure including selection of peaks and normalization of peak areas. We included only those 19 peaks with a relative area of more than 1% present in at least 80% of the ants of a given group (Liebig et al., 2000). Peak areas were transformed according to Reyment (1989) $Z_{ij} = \log[X_{ij}/g(X_j)]$, with X_{ij} being the peak area *i* for ant j, $g(X_j)$ the geometric mean of all peaks of ant *j*, and Z_{ij} the transformed area of peak *i* for ant *j*. The standardized peak areas were reduced to five factors by a principle component analysis (PCA) (minimum eigenvalues of 0.8; method of factor rotation: varimax raw) and the resulting factors were analyzed by discriminant analysis (DA) (SPSS 13.0). Mahalanobis distances for between-group comparisons were obtained from Statistica 6.0.

In addition, we applied an alternative method to test for significant differences between groups. Using relative peak areas we computed an analysis of similarity (ANOSIM) based on comparing distance between groups with distance within groups. Calculations were performed with the Bray-Curtis similarity measure with 10000 replicates of group membership permutation (PAST 1.75b, Hammer *et al.* 2001).

To identify compounds that contributed most to the differences across queens, intermorphs and workers we compared the proportional peak areas with a non-parametric Mann-Whitney-U pairwise comparison of groups. P -values were corrected using the sequential Holm's Bonferroni method (Holm 1979).

Results

3.1. Identification of cuticular substances

GC and GC/MS analysis revealed 19 CHC peaks (Figure 1) that were consistently present across all groups (see below). These substances were subject to further analysis. A total of 15 compounds were identified as saturated and unsaturated hydrocarbons from their characteristic mass spectra (Table 1). All alkanes were determined by comparing retention times and mass spectra of the extracts with purchased alkanes. The corresponding alkenes were identified by their typical mass spectra, their DMDS derivatisation products and a comparison of their retention time and mass spectra with those of synthesised alkenes. To confirm the GC-MS data of alkenes, alkadienes and alkatrienes all unsaturated compounds were hydrogenated and the resulting alkanes were identified. The positions of double bonds for eight substances (peaks 6, 7, 8, 11, 13, 14, 15, 18) could not be identified due to the lack of DMDS derivatisation products.

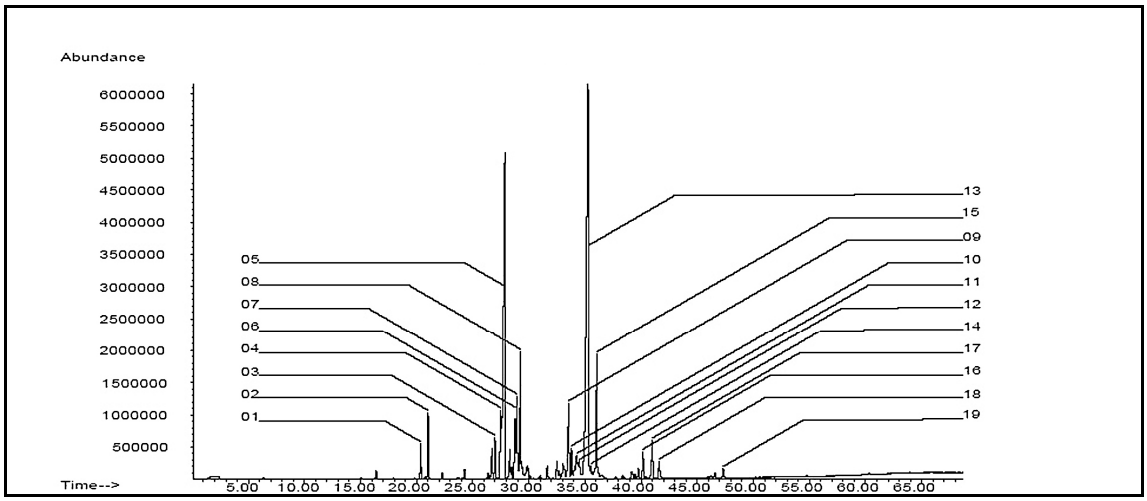

Figure 1. Chromatogram of pooled *C. smithi* queens (n = 3). Arrows denote peaks (1-19) used for the PCA, for details see Table 1.

GC – GC/MS analysis of single and multiple individuals

The 19 CHC peaks varied in their relative quantities. Two peaks were reliably detected only in pooled extracts: in chromatograms obtained by SPME from single individuals, peak 19 (n-Hentriacontane) appeared to be absent in workers and three of five virgin queens, but present in all queens and intermorphs. Similarly, peak 1 ((Z)-9-Tricosene) was present in all queens/intermorphs, but appeared to be absent in 17 of 18 workers (94.4%) and four of five virgin queens. Extracting multiple ants showed that these substances could indeed be recovered with SPME, although in very low quantities.

A pairwise comparison revealed significant differences among the four examined phenotypes (Mann-Whitney U-test, sequential Bonferroni correction). Four peaks (2, 4, 8) appeared to be characteristic of workers and were present in greater proportions than in all other groups (Figure 2, Table 1). Four peaks (4, 11, 12, 14) were present in greater proportion in intermorphs than in queens. Workers and intermorphs resemble each other in the relatively low proportion of peak 11, while two peaks (12, 14) were more pronounced only in intermorphs.

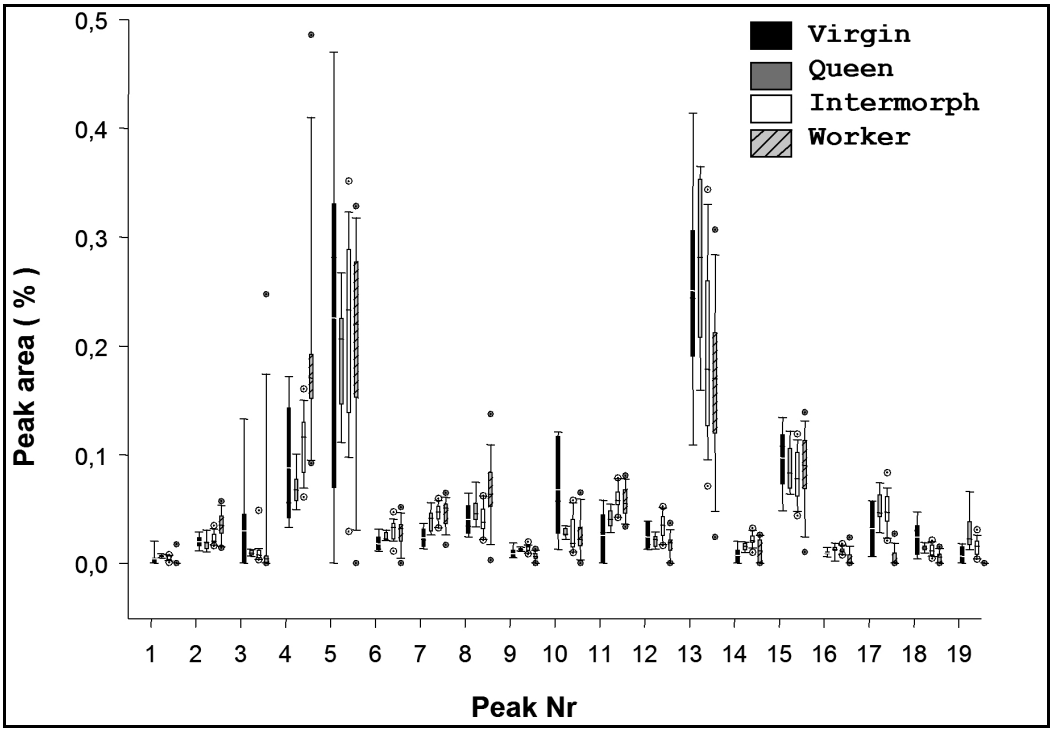

Figure 2. Proportion (%) of peak areas for workers, intermorphs, mated queens, and virgin queens. Box plots show median and 25% and 75% quartiles. Whiskers depict the range of 95% of all cases. Extreme outliers for workers and intermorphs are denoted by circles.

Fertile queens were characterized by peak 19, which was only present in low amounts in virgin queens and workers. Five substances had increased proportions both in queens and intermorphs (peaks 1, 9, 16, 17, 19).

A principle component analysis (PCA) resulted in five factors that combined explained 85.9 % of the variance. Discriminant analysis based on these factors showed significant differences between workers, female sexuals, and a single group consisting of both intermorphs and queens (Wilks' Lambda 0.051, $F= 13.6$, $p < 0.001$; Figure 3), which could not be separated ($p > 0.99$). All seven assigned queens ($n = 7$) and all intermorphs ($n = 16$) were classified as belonging to the same group. Three of five virgin queens were correctly classified, and one each grouped with "intermorphs/queens" and "workers," respectively. Seventeen of 18 workers (94.4%) were correctly recognized as "workers" and one (5.4%) as "intermorph/queen" (Table 2).

As the DA requires transformed proportional data we also tested for differences using relative peak areas with the less conservative ANOSIM. ANOSIM resulted in overall significant differences between groups ($R = 0.270$; $p < 0.001$) while pairwise ANOSIM posthoc comparison again did not detect differences between queens and intermorphs (Table 3).

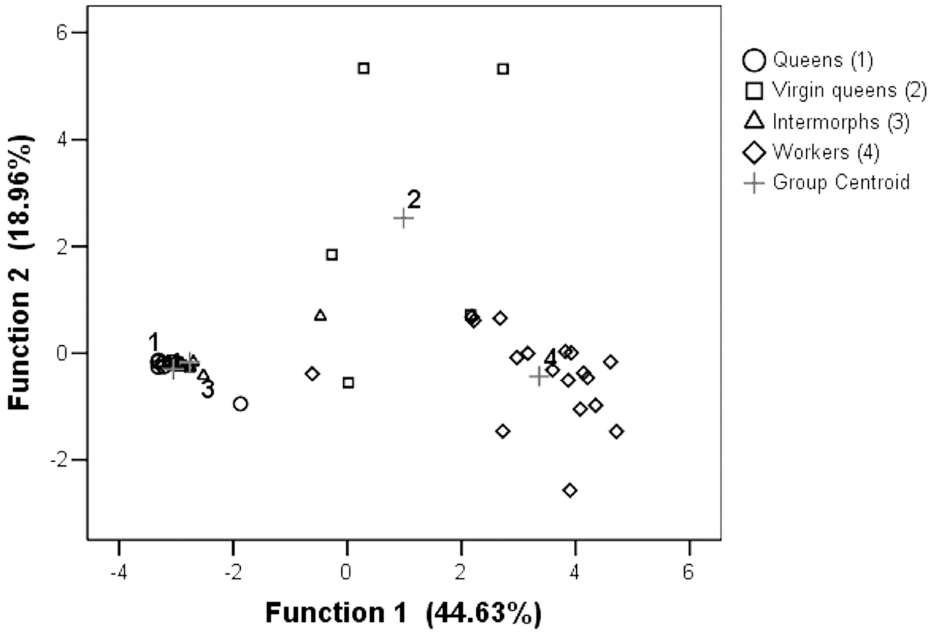

Figure 3. Discriminant analysis of the three morphs of *C. smithi:* workers (n = 18), intermorphs (n = 16), queens (n = 7) and virgin queens (n = 5).

Discussion

Our study documents that in the myrmicine ant *Crematogaster smithi*, different reproductive status is associated with variation in cuticular hydrocarbon profiles, but that mating status appears to be uncoupled from what has been interpreted as a fertility signature. The CHC bouquets of egg laying, inseminated queens and egg laying, uninseminated intermorphs were similar in an increased presence of n-nonacosan (peak 17) and could neither be distinguished by discriminant analysis nor by ANOSIM. Their bouquets were clearly distinct from those of non-reproductive workers.

In contrast, virgin queens and intermorphs/queens differed significantly in their chemical profiles, though at least virgin queens and intermorphs had similarly developed ovaries and crudely similar egg laying rates (J. Oettler, unpublished). Egg laying rates alone therefore cannot account for the observed differences. Instead, they might reflect either the absence of signalling in isolated queens, different age, or the special requirements of virgin queens concerning protection against desiccation etc. It has previously been shown in queens of the ant *Pachycondyla inversa* that egg-laying, founding queens as well as virgin queens produce only low quantities of a substance thought to signal fecundity in older queens before first workers have eclosed (D'Ettorre *et al.* 2004). The needlessness of signalling fecundity when in isolation might explain the difference between the cuticular profiles of similarly fertile virgin queens and intermorphs. In addition, virgin queens were collected in the late pupal stage and had eclosed just prior to the mating season (July/August), i.e., less than four months before analysis, while queens and intermorphs were collected as adults. Cuticular hydrocarbon profiles are known to change with age (Cuvillier-Hot *et al.* 2001, Kaib *et al.* 2000), probably due to physiological processes (metabolism and gland development), behaviour (exchange of substances) and interactions with the abiotic environment (Wagner *et al.* 1998, Kaib *et al.* 2000, Johnson & Gibbs, 2004, Hora *et al.* 2008). Furthermore, queens of *C. smithi* found their nests independently by digging in dry rocky soil and their specific CHC profile might protect them against desiccation.

The apparent similarity among the cuticular profiles of older queens and intermorphs suggests that CHCs differ with ovarian activity and not with mating status. Egg laying alone appears to be associated with a particular chemical profile, regardless of whether the eggs are fertilized or not. Previous studies, for example in orphaned colonies of *Pachycondyla inversa* (Heinze et al., 2002) and *Myrmecia gulosa* (Dietemann *et al.* 2005), in the parthenogenetic ant *Platythyrea punctata* (Hartmann *et al.* 2005), in the queenless ant *Diacamma ceylonense* (Cuvillier-Hot *et al.* 2001), and in queenright colonies of the honey bee *Apis mellifera* (Katzav-Gozansky *et al.* 2004), similarly documented that the cuticular profiles of egg laying, unmated workers may approach those of fertile queens or gamergates at least in quality, if not in quantity. In this study, n-nonacosan (Peak 17) appeared to be associated with fertility and to relate to the observed egg laying rates.

Multivariate analysis of 19 CHCs gave ambiguous results as it failed to discriminate between queens and intermorphs. Nevertheless, behavioural observations in *C. smithi* suggest that workers are more sensitive than our discriminant analysis and well capable of recognizing queens and treating them differently from intermorphs; i.e. queens are more frequently groomed than intermorphs (Heinze *et al.* 1995). This clearly documents one major weakness of multivariate analyses of cuticular hydrocarbon patterns: much of the similarity of cuticular hydrocarbon patterns of queens and intermorphs might be due to substances, which are not used in communication but serve other functions on the cuticula, and which in a discriminant analysis obliterate variation in those substances that indeed are perceived and used as signals. Furthermore, we did not investigate other cuticular substances, such as proteins, which might also be used as recognition cues (Turillazzi *et al.* 2006). To fully comprehend chemical communication and to decipher, which parts of the variation are meaningful in the context of communication, future research would require bio-tests with synthesized single compounds and compound mixtures.

Table 1.

Identification and mass spectra of the 19 analyzed substances revealed by GC/MS that were used for the Discriminant analysis. Numbers correspond to peak labels in Figure 1. For compounds that could not be fully identified, we list the ions present in the mass spectrum by mass-charge ratio (m/z). The relative peak areas that were significantly different between groups and contributed significantly to the discrimination of caste are displayed (MWU-test, Holm's sequential Bonferroni correction; NS = non-significant after sequential Bonferroni correction; p – values in brackets before Bonferroni correction).

Table 2

Classification matrix after discriminant analysis: Absolute numbers of individuals that were a posteriori recognized according to their a priori assigned group are shown. In total 78.3% of individuals were correctly classified. Note that all queens were classified as "intermorphs".

Table 3

Matrix of the Mahalanobis distances and ANOSIM distances among queens, female sexuals, intermorphs, and workers. ANOSIM results are given in the upper part and Mahalanobis distances in the lower. Note that values for queens and intermorphs in relation to female sexuals and workers are almost identical and that intermorphs and queens are very close.

Peak Nr.	RT (Peak)	RI (DB5)	Substance	Queen -intermorph	Queen - Worker	Intermorph – Worker	Virgin Queen - Queen	Virgin Queen - Intermorph	Virgin Queen - Worker
1	20.354	2279	(Z)-9-Tricosene		**< 0.001**	**< 0.001**			
2	21.020	2300	n-Tricosane		**0.005**	**0.007**			**NS (0.025)**
3	26.992	2478	(Z)-9-Pentacosene		**NS (0.046)**				
4	27.506	2493	(Z)-1-Pentacosene	**0.008**	**< 0.001**	**< 0.001**			**0.014**
5	27.845	2500	n-Pentacosane						
6	28.769	2531	Pentacosadiene *m/z* (%): 43 (95); 68 (100); 82 (65); 96 (51); 109 (19); 306 (2); 348 (6)					**NS (0.032)**	
7	28.950	2536	Pentacosadiene *m/z* (%): 43 (89); 68 (100); 82 (61); 96 (50); 109 (17); 306 (2); 348 (5)				**NS (0.042)**	**0.002**	**0.009**
8	29.254	2545	Pentacosadiene *m/z* (%): 43 (71); 68 (100); 82 (66); 96 (52); 110 (31); 123 (11); 138 (11); 348 (11)			**(0.004)**			
9	33.549	2663	(Z)-9-Heptacosene		**0.003**	**< 0.001**			
10	33.800	2680	(Z)-7-Heptacosene						

11	34.250	2694	Heptacosatriene *m/z* (%): 43 (100); 55 (85); 67 (94); 83 (56); 97 (53); 109 (30); 125 (10); 151 (13); 289 (1); 374 (3)	**0.005**	**0.025**			**0.008**	**0.025**
12	34.472	2700	n-Heptacosane	**< 0.001**		**0.001**			
13	35.261	2725	Heptacosatriene *m/z* (%): 43 (36); 55 (31); 67 (100); 83 (44); 95 (49); 109 (36); 151 (8); 191 (5) 289 (2); 374 (4)		**NS** **(0.015)**				
14	35.524	2733	Heptacosadiene *m/z* (%): 43 (92); 55 (53); 68 (100); 82 (67); 96 (49); 109 (18); 334 (4); 376 (6)	**0.011**		**0.014**		**0.005**	
15	36.067	2750	Heptacosadiene *m/z* (%): 43 (75); 55 (51); 67 (100); 81 (66); 96 (54); 110 (35); 124 (11); 138 (13); 376 (10)						
16	40.175	2875	Nonacosene		**0.021**	**< 0.001**			
17	40.993	2900	n-Nonacosane		**< 0.001**	**< 0.001**			**0.011**
18	41.619	2919	Nonacosatriene *m/z* (%): 43 (41); 55 (33); 67 (100); 81 (43); 95 (48); 109 (34); 121 (7); 151 (10); 191 (4); 402 (3)		**< 0.001**	**< 0.001**			**0.021**
19	47.322	3100	n-Hentriacontane		**< 0.001**	**< 0.001**	**0.019**		

Table 1

	Queens	Gynes	Intermorphs	Workers	% correct classified	Total Nr.
Queens	0	0	7	0	0	7
Gynes	0	3	1	1	60	5
Intermorphs	0	0	16	0	100	16
Workers	0	0	1	17	94.4	18

Table 2.

DA \ ANOSIM	Queen	Female Sexual	Intermorph	Worker
Queen		R = 0,33 p < 0,05	R = 0,07 p = 0,20	R = 0,33 p < 0,01
Female sexual	**26.63** F = 11,52 p < 0,001		R = 0,42 p < 0,01	R = 0,39 p < 0,05
Intermorph	**0.15** F = 0,12 p = 0,99	**23.36** F = 13,35 p < 0,001		R = 0,24 p < 0,001
Worker	**45.09** F = 36,19 p < 0,001	**15.83** F = 9,28 p < 0,001	**41.16** F = 59,35 p < 0,001	

Table 3

Chapter 5

One ant can make a difference: the adaptiveness of queen-worker intermorphs in the ant *Crematogaster smithi*.

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** JO conducted the experiments. JO and MBD performed the statistical analysis. JO, MBD and JH wrote the manuscript.

Workers of *C. smithi*. Photograph © Alex Wild 2005 (used with permission)

Abstract

We present a novel strategy how ants increase their inclusive fitness by allocation of the energy from worker-laid male-destined eggs into the production of new queens. Sex allocation in *Crematogaster smithi* is based on an adaptation that exploits an ancestral developmental pattern of *Crematogaster* (Orthocrema) species. Sex ratio and total sexual production depend on the presence of a third female caste intermediate between workers and queens in fertility, behavior, and morphology. Sex allocation becomes more female biased in larger colonies with growing numbers of intermorphs. Colonies without intermorphs instead produce a male-biased sex ratio. The main function of such intermorphs is the production of unfertilized, viable eggs, which constitute a major part of the diet of larvae and the queen. Genetic data show that only a few of the eggs laid by intermorphs develop into adult males. Less than half of the mature colonies in the field contain this specialized morph resulting in a population-wide sex ratio in agreement with queen and worker optima.

Introduction

In 1976, Trivers and Hare presented data on realized sex ratios of 20 ant species and derived a theory combining inclusive fitness theory (Hamilton 1964) with sex allocation theory. Corrected for weight differences, investment in new queens was found to be higher than into males, as predicted by inclusive fitness theory based on relatedness. Trivers and Hare reasoned that relatedness asymmetries lead to asymmetric individuals' social behavior, most pronounced by the queen-worker conflict. This theory has since then be thoroughly tested in species with varying inter-colony relatedness that might differ with number of queens heading a colony (Nonacs 1986) or number of males that mate with a single queen (Sundström 1994). In both cases, increasing numbers lead to lower relatedness of workers, which thus favor male over female production. Currently three mechanisms are known how queens and workers adjust the sex ratio to favor their interests. All fitness functions of sexuals being equal, (1) queens lay male or female eggs (primary sex ratio), (2) workers might selectively rear males or females (secondary sex ratio) and (3) workers themselves may produce male eggs in species where workers retained functional ovaries (worker reproduction). In colonies headed by a singly mated single queen theory predicts that workers favor a 3 : 1 male to female ratio, while the queen prefers a 1 :1 ratio. We here present an abbreviated new mechanism how workers alter the ratio by combining worker reproduction with selectively raising of female offspring. We examined this in natural populations of a species where nearly half the colonies contain a specialized worker that shares both queen and worker characteristics.

A key socio-ecological adaptation of ants is queen-worker polyphenism. Female larvae can develop into queens or workers, with both castes being morphologically and physiologically adapted to their future roles in the colony. Queens have wings and light-sensitive ocelli (both necessary for dispersal; Moser *et al.* 2004), a spermatheca for storing sperm, well-developed ovaries, and extensive fat reserves, which enable them to found new colonies and lay fertilized (worker- or queen-destined) eggs. Workers are smaller than queens, wingless, and have a reduced morphology that is thought to be adapted for foraging and brood care (Darwin 1872, Hölldobler & Wilson 1990). However, workers typically have ovaries and can lay unfertilized eggs, which due to the haplodiploid sex determination system common to all Hymenoptera can only develop into males (Hammond & Keller 2004, Wenseleers & Ratnieks 2006). Among ants, queen-worker polyphenism is only absent in ca. 100 species of the subfamily Ponerinae, in which the queen caste has been secondarily lost and replaced by mated workers, and in socially parasitic lineages which have secondarily lost the worker caste (Hölldobler & Wilson 1990, Heinze 1998; Peeters & Ito 2001).

In many ants species, adult females with an intermediate morphology between queens and workers can occur, typically as rare malformations with highly variable morphologies that almost never reproduce (so-called "intercastes") (Heinze 1998). However, in some species a female caste with an intermediate, relatively invariable, morphology occurs alongside normal queens and workers ("intermorphs": Heinze 1998; e.g. *Leptothorax ssp.*: Buschinger & Heinze 1992, *Ephebomyrmex imberbiculus*: Heinze *et al.* 1992). In these species, the wingless intermorphs co-exist with winged queens, but unlike the latter the intermorphs can only mate inside the nest and cannot found colonies independently. Wingless intermorphs usually display an alternative reproductive non-dispersing strategy that supplements the winged queen caste (*Technomyrmex vitiensis*: Oettler & Heinze this thesis, *Temnothorax rugatulus*: Rüppell *et al.* 2002).

The genus *Crematogaster* (Formicidae: Myrmicinae) comprises approximately 420 described species and is one of the most species-rich genera of ants (Wilson 2003, Moreau 2008). Wingless intermorphs commonly occur in at least ten Old and New World species of the subgenus *Orthocrema* (Santschi) (Table 1), suggesting that the presence of intermorphs is evolutionarily stable and possibly adaptive. Intermorphs have similar external morphologies in all *Crematogaster (Orthocrema)* species in which they have been found, but their reproductive physiology has so far only been studied in *Crematogaster smithi* (Heinze et al. 1995, 1998, 2000; Oettler *et al.* 2008). *C. smithi* intermorphs cannot mate because they lack a spermatheca and external genitalia, and hence can have sons but not daughters (workers, intermorphs, queens) (Heinze *et al.* 1995). Heinze *et al.*

(1998) showed that ca. 50% of colonies contain 1 – 10 intermorphs, which appear to peacefully coexist with the single mother queen. *C. smithi* intermorphs have never been observed to engage in colony defense or foraging (Heinze *et al.* 1995). *C. smithi* intermorphs are known to lay viable male-destined eggs in queenright as well as in orphaned colonies (Heinze *et al.* 1998). Both queens and intermorphs produce additional smaller trophic eggs, but in comparison, queens produce proportionally more trophic eggs. At least some of the intermorph-laid male-destined eggs are eaten by larvae and the queen, but the proportion that survive –and hence the contribution of intermorphs to the adult male brood– are unknown (Heinze *et al.* 2000).

The aim of this study is to determine why having intermorphs might be adaptive for *C. smithi* colonies, specifically testing the following mutually exclusive hypotheses: First, intermorphs might increase colony productivity -and thus the inclusive fitness of workers and queens- by maintaining the sons of intermorphs (eggs and/or young larvae) as living food stores (cf. Chapuisat *et al.* 1997) in the nest during the most favorable season (i.e., immediately after the summer monsoons, July-September), to be selectively cannibalized during the lean season (fall, spring). A critical prediction of this hypothesis is that colonies with intermorphs should raise more queen-derived sexuals and/or produce a sex allocation ratio that is more biased towards virgin queens (which are larger and thus more costly to produce than males) than colonies without intermorphs.

Second, having intermorphs in the colony might increase the inclusive fitness of workers if intermorphs function as additional efficient reproducers that specialize in producing males (Heinze 2000). In singly mated Hymenopteran insect societies, workers would increase their average relatedness to the male brood by replacing queen sons (brothers; average life-for-life relatedness $\bar{r} = 0.25$) by worker or intermorph sons (nephews; $\bar{r} = 0.375$) (Hamilton 1964, Trivers & Hare 1976). However, mainly or exclusively queens sons are produced in many singly and multiply mated species of eusocial Hymenoptera (Hammond & Keller 2004; Wenseleers & Ratnieks 2006), typically as a result of “self-restraint” (i.e., workers refraining reproduction in the presence of the queen) and/or “worker policing” (i.e., workers discriminating in favor of queen sons by eating worker-laid eggs and/or attacking reproductive worker sisters) (Ratnieks *et al.* 2006; D’Ettorre *et al.* 2004; Hartmann *et al.* 2004). Self-restraint and worker policing in singly mated species are thought to have evolved because worker reproduction reduces colony productivity (Cole 1986; Gobin *et al.* 2003; Endler *et al.* 2004; Wenseleers *et al.* 2004; Wenseleers & Ratnieks 2006; Hartmann *et al.* 2004; Dijkstra & Boomsma 2007). However, workers that tolerate male production by one or a few fecund intermorphs, rather than by many relatively infecund workers, would increase their relatedness to the male brood while minimizing the colony-level cost of inefficient worker reproduction. Selection might thus favor workers that raise one or a few sisters as intermorphs and discriminate against queen and worker sons, in favor of intermorph sons. Critical assumptions of this hypothesis are that workers are more related to nephews than to brothers (i.e., the single mother queen must have an effective mating frequency <2; Foster & Ratnieks 2001) and that a considerable proportion of adult males in colonies with intermorphs are intermorph sons.

Materials and Methods

Study site and collections

Crematogaster smithi (Creighton) is a ground-nesting ant from the arid mountains of Arizona (USA) and Sonora, Mexico. It exhibits a cryptic lifestyle with above-ground activity restricted to cooler temperatures during the late evening and night. Nests consist of a single tunnel (up to 40 cm deep) that connects up to 10 nest chambers.

Workers forage for small insects, ingest their prey outside of the nest and distribute food by regurgitation of liquids to other workers and intermorphs. Direct offerings of solid food items to the queen have not been observed. Instead, she and the larvae have been frequently observed to feed on eggs laid by workers and intermorphs. Mating flights occur in July-August immediately after the first summer monsoon rain. Mated queens shed their wings, dig a hole and start new nests independently (J Oettler pers. observation).

In July 2005 and 2006, we collected 56 complete colonies from the area around the Southwestern Research Station in the Chiricahua Mountains, Arizona. Once we located a nest entrance we carefully removed the soil and followed the nest tunnel to the bottom chamber; excavations were performed meticulously with a dinner knife and spoon. Excavation of one colony lasted up to two hours depending on the substrate. We confirmed that the colony had been completely excavated by digging for an additional 20 min after the last chamber had been opened. Immediately after collection, we counted all workers, intermorphs, queens and sexuals. For the sex ratio analysis we included only sexuals from 2005 that were collected a fortnight before the annual mating flight. Exclusively adult sexuals and no younger sexual brood were present at the time of colony collection. Immediately after colony excavation, we stored all males and a sample of workers in 100% ethanol for later genotyping. Colonies collected in 2005 were transported to Regensburg (Germany) and installed in the laboratory (24°C and 60% relative humidity, with seasonal changes in day/night time adjusted according to Arizona time), where they were fed three times weekly with chopped cockroaches and diluted honey. We also collected adult males produced in spring 2006 in these queenright laboratory colonies and stored them in 100% ethanol for genotyping.

Genotyping workers and males

From samples that had been preserved in the field in 2005, we haphazardly selected 149 workers (range 12 – 19 per colony) and 156 males (range 3 – 28 per colony) from nine queenright colonies for genotyping. We also genotyped 60 males (range 6 – 16 per colony) from five queenright laboratory colonies (see above). We extracted genomic DNA with the PureGene Kit (Gentar Systems, Germany) and amplified the microsatellite loci C20 and C9 (GenBank accession numbers: AF 167066 and AF 167064) following the protocols in Heinze et al. (2000). We ran the PCR products on an ABI 310, manually scoring peaks. These loci show considerable variability (C20: 4 alleles, expected heterozygosity H_e = 0.617; C9: 10 alleles, H_e = 0.820) (Heinze *et al.* 2000).

We calculated the average within-colony relatedness, the population-wide allele frequencies, and the inbreeding coefficient (F_{is}) with the program Relatedness version 4.2 (Queller and Goodnight 1989).We then deduced the most likely genotypes of the queens and their mate(s) for each colony with the program Matesoft Silver version 1.0 (Moilanen *et al.* 2004). We estimated the effective queen mating frequency (M_e) of each queen from the relative contributions of the detected mates to the worker offspring with formula 2 in Foster *et al.* (2000). Because only males that carry an allele that is not present in the queen can be unambiguously assigned as non-queen sons (i.e., intermorph or worker sons), we corrected for non-detection by dividing the number of detected non-queen sons by the probability of detection (P_d ; estimated with formula 2 in Foster *et al.* 2001). Our microsatellite loci allowed us to distinguish between queen sons and non-queen sons, but not between intermorph and worker sons. However, the relative contributions of intermorphs

and workers can be estimated by comparing the proportion of non-queen sons between colonies with and without intermorphs, assuming that the proportion of reproductive workers, their fecundity, and the survival of their sons are not affected by the presence of intermorphs in the same colony.

Estimating sexual production

In 2005, we haphazardly selected mature, flight-ready virgin queens (n = 8; from seven colonies) and males (n = 11; collected from the mating flight) for weighing. We dried them for at least 72 h at 50 - 55° C, and weighed them with 0.01 mg precision. We then estimated the ratio of the production and maintenance cost to the colony of a virgin queen versus a male with the frequently used formula (e.g. Boomsma *et al.* 1995; Bourke 2005; Dijkstra & Boomsma in press):

$$c = \left(\frac{\bar{f}}{\overline{m}} \right)^{0.7} \qquad \text{(Eqn. 1)}$$

In which c is the "sexual cost ratio", $\bar{f}$ and $\overline{m}$ are the average dry masses of adult virgin queens and males, respectively, and the power of 0.7 gives an overall correction for the lower respiration rate and higher fat content of virgin queens compared to males (Boomsma & Isaaks 1985). We estimated the variance (i.e., the uncertainty in the prediction) for *c* with formula 6 in Dijkstra & Boomsma (in press). For each colony, we estimated the "Relative sexual production" (i.e., the total energy invested in sexuals, expressed as the number of male equivalents) of each field colony from 2005 with (cf. Boomsma & Nachman 2002):

$$S_i = cF_i + M_i \qquad \text{(Eqn. 2)}$$

In which S_i is the relative sexual production in colony *i*, *c* is the sexual cost ratio, and F_i and M_i are the number of virgin queens and males in colony *i*, respectively.

Explaining between-colony variation in numerical sex ratio

To identify colony traits that are positively or negatively associated with a virgin queen-biased sex ratio, we performed a maximum likelihood logistic regression with sequential model simplification in SAS 9.1.3 for Windows XP Professional (SAS Institute Inc., Cary, NC, USA). The method is described in detail in Boomsma & Nachman (2002) and Dijkstra & Boomsma (in press). Briefly, we used "Colony-level numerical sex ratio" (i.e., the proportion of virgin queens among all the sexuals in the colony) as the dependent variable, "Number of intermorphs", "Number of workers", and "Sexual production" (see above) as covariates, and "Colony" as random factor, also testing all second order interaction terms (e.g. Boomsma & Nachman 2002, Ichinose *et al.* 2006, Dijkstra & Boomsma in press). We weighted the contribution of each colony to the model by the total number of sexuals in the colony (Dijkstra & Boomsma in press). We identified the least significant term in each step with a two-tailed Type II Wald χ^2 test and removed it from the model, until only significant terms remained (Dijkstra & Boomsma in press). We corrected for overdispersion (cf. Wilson & Hardy, 2002) of the colony-level numerical sex ratios by dividing all χ^2 values by the ratio of the residual deviance and the residual degrees of freedom (Dijkstra & Boomsma in press). We calculated a pseudo-r^2 value (i.e., an analogue of the r^2-value in a linear regression, expressed as the proportion of total deviance that is explained) for each step with formula 7 in Dijkstra & Boomsma (in press). Finally, we estimated the population-level numerical sex ratio (i.e., the weighted average of the colony-level numerical sex ratios, expressed as the proportion of virgin queens among the sexuals in the population) from the minimum adequate model, with all significant covariates set at their respective average values (Dijkstra & Boomsma in press).

Comparing operational and predicted population-level sex allocation ratios

It has been repeatedly shown that the inclusive fitness of workers is always maximized at a more virgin queen-biased population-level sex allocation ratio than is optimal for queens (e.g. Trivers & Hare 1976, Boomsma & Grafen 1991, Pamilo 1991, Chapuisat & Keller 1999). When mother queens have full, unconstrained power over the sex ratio in their colony ("queen control"), the stable equilibrium population-level sex allocation ratio is predicted to be 0.50 (the so-called "queen optimum" $\hat{Q}$; expressed as the proportion of energy invested in virgin queens rather than males across the population), irrespective of the proportion of non-queen sons in queenright colonies and the effective queen mating frequency (Table 1 in Pamilo 1991). The prediction for the queen optimum has a variance of zero (formula 4 in Dijkstra & Boomsma). Under full worker control over the sex ratio, the stable equilibrium population-level sex allocation ratio is predicted to be equal to the "worker optimum" $\hat{W}$ (expressed as the energy invested in virgin queens), which can be estimated with (cf. formula 9 in Pamilo 1991):

$$\hat{W} = \frac{\overline{g}_m}{\overline{g}_f \frac{v_f}{v_m} + \overline{g}_m} \qquad \text{Eqn. 3}$$

In which $\overline{g}_f$ and $\overline{g}_m$ are the average genetic relatednesses of workers to virgin queens versus males in the same colony, respectively, and v_f and v_m are the so-called "reproductive values" for mother queens and their mates, corresponding to the number of genome copies that queens and males are expected to contribute to the future gene pool, assuming that the population size is stable and that there is no selection, mutation, or drift (Pamilo 1991). The values of $\overline{g}_f$, $\overline{g}_m$, and $\frac{v_f}{v_m}$ can be estimated with (Pamilo 1991):

$$\frac{v_f}{v_m} = 2 - \psi \qquad \text{Eqn. 4}$$

$$\overline{g}_f = \frac{\frac{3}{4} + \frac{1}{4}\left(\overline{M}_e - 1\right)}{\overline{M}_e} \qquad \text{Eqn. 5}$$

$$\overline{g}_m = \frac{1}{2} + \frac{\psi}{4} \qquad \text{Eqn. 6}$$

In which ψ is the proportion of non-queen sons in queenright colonies and $\overline{M}_e$ is the average effective mating frequency of queens. Eqns. 3 – 6 assume that all colonies have a single queen, that reproduction by intermorphs and workers in orphaned colonies is negligible, and that there is no local resource competition, local resource enhancement, or local mate competition (Pamilo 1991; Chapuisat & Keller 1999; Dijkstra & Boomsma in press). To determine the likelihoods of queen versus worker control in *C. smithi* (cf. Bourke 2005; Dijkstra & Boomsma in press), we compared the predicted worker and queen optima to the operational population-level sex allocation ratio (*I*; expressed as the proportion energy invested in virgin queens), which we estimated with the standard formula (e.g. Boomsma *et al.* 1995, Bourke 2005):

$$I = \frac{cN}{(c-1)N + 1} \qquad \text{Eqn. 7}$$

In which *c* is the sexual cost ratio (see above) and *N* the operational population-level numerical sex ratio (expressed as the proportion of virgin queens; estimated from the minimum adequate model). We estimated the variances of $\hat{W}$ and *I* with formulas 3 and 9 in Dijkstra & Boomsma (in press).

Additional information on statistics

Unless otherwise indicated, all statistical tests were two-tailed for $\alpha = 0.05$, and were performed in SPSS version 13.0 for Windows XP. Results are given as mean ± SE.

Species	Description of morph	Region	Reference
C. minutissima	Intermorph	Florida, USA	J.O. unpub. (NMNH, Washington D.C.)
C. baduvi	Described by Forel as Macrogyne and Microgyne	Java	J.O. unpub.(MHN, Genève)
Crematogaster biroi (Mayr) var smythiesi (Forel)	Two types of Intermorphs: Macro- and Microintermorph. No description of queens	India	J.O. unpub.(MHN, Genève)
Crematogaster biroi (Mayr) var. aitkeni (Forel)	Two types of Intermorphs Macro- and Microintermorph. No description of queens	Ceylon	J.O. unpub. (MHN, Genève)
C. rasoherinae	Intermorph	Seychelles	J.O. unpub.(MHN, Genève)
C. cf. curvispinosa	Intermorph	Sonora, Mexico	Heinze et al. 1999
C. bryophilia, C. curvispinosa, C. nigropilosa	Intermorph	Costa Rica	JT Longino, pers com 2005
C. cf. baduvi	Intermorph	SE Asia	Buschinger et al. 2002

Table 1. *Crematogaster (Orthocrema)* species for which intermorphs are known. Note that the descriptions are based on external morphology and that the biology for none of these species is known. *C. minutissima* appears to be the sister species to *C. smithi*. The first five species in the list are descriptions from Museum specimen and intermorphs were recognized by developed ocelli and pronotal structures.

Results

Frequency of intermorphs

All of the collected colonies (n = 56) had a single queen, with worker numbers ranging from 4 – 1370 (median = 383.5). Intermorphs were never present in any colony with less than 59 workers (n = 7, 13 % of colonies), while 23 (41%) colonies with over 59 workers likewise did not contain intermorphs. The remaining 26 colonies (46%) contained between 1 and 16 intermorphs (median 3.5; 1st quartile = 2.0 and 3rd quartile = 6.0). We assumed that colonies with less than 60 workers were immature and presumably in their first year after colony founding (J. Oettler, unpublished data). There was a significant positive association between the number of intermorphs and the number of workers (Spearman rank test; n = 115; r_S = 0.384, p < 0.007). Colonies with intermorphs did not contain significantly more workers than colonies without intermorphs (Mann-Whitney U; n_1 = 26, n_2 = 23; U = 206.0, p = 0.062).

Effective queen mating frequency

F_{is} was not significantly different from zero for either microsatellite locus (C20: t-test; F_{is} = 0.345 ± 0.153, t = -2.26, p > 0.05. C9: t-test, : *Fis* = -0.075 ± 0.080, t = 0.94, p > 0.2) which indicates the absence of inbreeding in the population.

The average relatedness between workers from the same colony was $\bar{r}$ = 0.734 ± 0.056 (nine colonies; n = 149 workers), which does not differ from the predicted value of 0.75 for societies headed by a single, singly mated queen (t-test; t = 0.32; p > 0.5). However, our estimate for the average effective queen mating frequency was $\overline{M}_e = 1.14 \pm 0.12$ because the contribution of a second queen's mate to the worker brood was detected in two (22%) of the genotyped colonies. Because we used only two microsatellite loci of moderate heterozygosity, our estimate for $\overline{M}_e$ could be an underestimate if some queen mates were undetected. We observed a strong paternity skew in these two colonies ($c_{.obs}$ = 0.74) which indicates that males contribute unequal amounts of sperm or that sperm is incompletely mixed in the spermatheca.

Contribution of intermorphs and workers to the male brood

The average relatedness between males in field colonies was $\bar{r} = 0.517 \pm 0.121$ (nine field colonies; 156 males), which is not significantly different from the predicted relatedness of $r = 0.5$ between brothers (t-test; t = 0.14; p > 0.5). None of the genotyped males from field or heir being haploid. The average probability of detecting a non-queen son with the two available microsatellite loci was $\overline{P}_d = 0.41 \pm 0.05$.

We detected a total of four non-queen sons in the field laboratory colonies were heterozygous at either microsatellite locus, which is consistent with tcolonies, of which two (50%) occurred in one colony with a single intermorph, and two (50%) in one colony without intermorphs. After correction for non-detection, the estimated proportion of non-queen sons among the males was 0.071. The data obtained from field colonies are consistent with the data from queenright laboratory colonies in 2000 (Heinze *et al.* 2000) and 2006 (Fig 1). These results indicate that in queenright field and laboratory colonies, intermorphs (and workers) produce few adult sons.

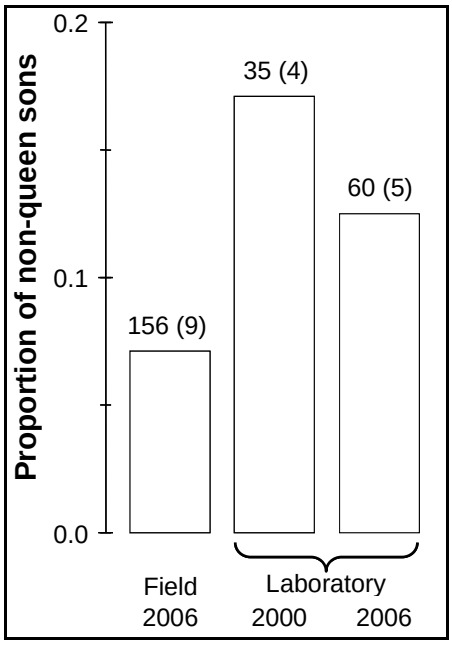


Figure 1. Results of microsatellite analysis of worker relatedness and putative worker reproduction. Given are data on colonies collected in (1) summer 2005 and a subset of colonies that produced males in the lab in (2) spring 2006. For a comprehensive analysis of all available date we included and reanalyzed the data from the (3) lab colonies studied by Heinze *et al*. (2000).

Intermorphs are associated with a gyne-biased colony-level numerical sex ratio

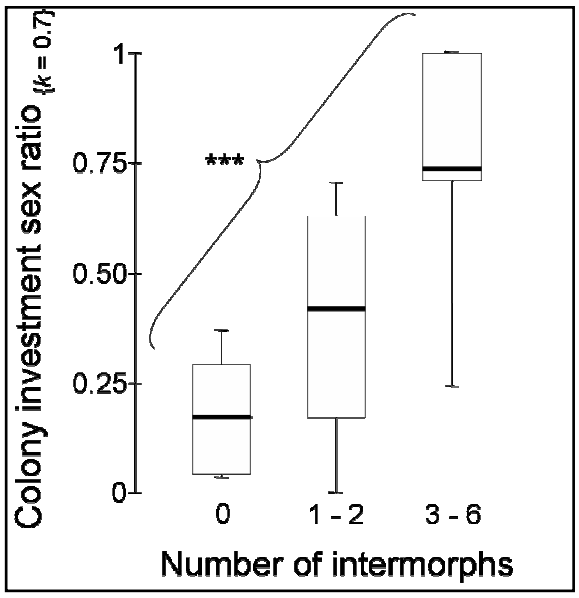


Figure 2. Box plots of the colony-level sex allocation ratio (female / male; assuming that the sexual cost ratio $k = 0.7$) are plotted against the number of intermorphs in the colony. "***" denotes significance at the 0.001 level for the positive effect of increasing numbers of intermorphs on the colony-level sex allocation ratio in the minimum adequate model.

The pseudo-r^2 of the minimum adequate model was 0.75, indicating a good fit between the minimum adequate model and the observations. The covariate "Number of intermorphs" was highly significant ($F_{1, 12} = 15.00$, $p = 0.002$), indicating that for a colony with an average number of workers, the colony-level numerical sex ratio became more biased towards virgin queens with increasing numbers of intermorphs per colony (Fig. 2). The covariate "Number of workers" was also significant ($F_{1,12} = 6.92$, $p = 0.048$), indicating that the colony numerical sex ratio became slightly more virgin queen-biased with an increasing number of workers when there is a single intermorph (i.e., the median number of intermorphs) in the colony. The interaction "Number of workers x Number of intermorphs" was likewise significant ($F_{1,11} = 7.88$, $p = 0.017$), which indicates that there was no further virgin queen-biasing effect of having additional intermorphs when the colony contained two or more intermorphs as well as more than ca. 700 workers. None of the other tested interactions were significant ($p \geq 0.10$). Importantly, the covariate "Sexual production" was not significant ($F_{1,10} = 0.01$, $p = 0.914$), which indicates that the total investment into sexuals per colony was independent of the level of bias towards virgin queens. This lack of correlation between the colony-level numerical sex ratio and the sexual production was consistent with our finding that sexual production did not differ between colonies with and without intermorphs (Mann-Whitney U, $z = -0.35$, $p = 0.724$).

The operational population-level numerical sex ratio and sex allocation ratio

The mean dry mass of adult sexuals was 1,41 ± 0.21 mg for virgin queens and 0.09 ± 0.01 for males, yielding an estimate of 6.97 ± 0.81 for the sexual cost ratio, assuming $c_{k = 0.7}$. Fig. 2a shows the estimated operational population-level numerical sex ratio (*N*), and Fig. 2b the corresponding operational population-level sex allocation ratio (*I*), relative to the predicted worker and queen optima. The operational population-level sex allocation ratio did not differ from the worker optimum (z = 0.69, p = 0.487) and likewise did not differ from the queen optimum (z = -1.40, p = 0.160), suggesting that both workers and the queen achieve partial control over the colony-level sex ratio.

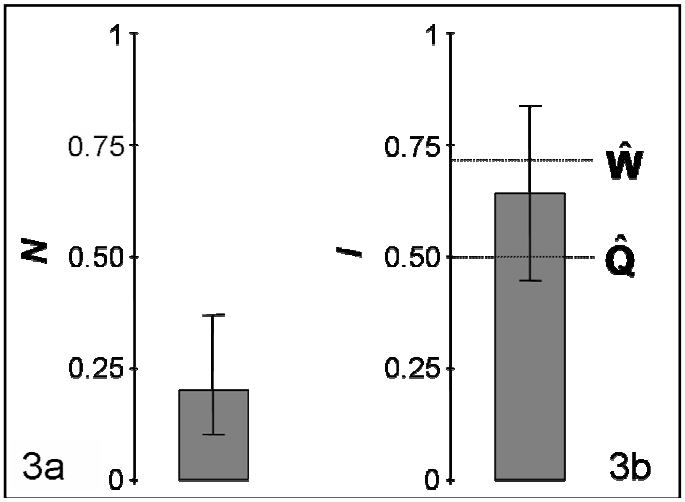


Figure 3. Estimates for the operational population-level numerical sex ratio obtained from the minimum adequate model (N; (3a) and the corresponding estimate for the operational population-level sex allocation ratio (I; 3b) are plotted against the vertical axis. The worker optimum $\hat{W}$ and tbe queen optimum $\hat{Q}$ for the population –level sex allocation ratio are indicated by horizontal lines. Error bars denote the 95% confidence intervals

Discussion

The role of intermorphs

Between-colony and between-species variation in sex ratio has been explained by variation in the genetic structure of colonies (review: Meunier *et al.* 2008), variation in resource availability (Nonacs 1986), and between-colony differences in the level of queen vs. worker control over sex allocation (Chapuisat & Keller 1999; Mehdiabadi *et al.* 2003; Dijkstra & Boomsma in press). Our study suggests that the colony-level sex ratio in the ant *C. smithi* depends strongly on the presence of intermorphs. Our data on male parentage allow us to reject our first hypothesis, namely that intermorphs might function as an "extended phenotype" (cf. Dawkins 1982) of their sister workers, by efficiently replacing queen sons with males to which workers are more related (intermorph sons). Contrary to this hypothesis, we found that most (93%) of adult males produced in queenright colonies of *C. smithi* are queen sons, while only 7% might be intermorph sons, worker sons, or a mixture of both. However, our results on sex allocation are at least consistent with our second hypothesis, namely that the presence of intermorphs enables a colony to invest relatively more in the more costly virgin queens.

Understanding how energy flows through the colony is fundamental for the interpretation of the relation of intermorphs and female reproduction. Smith (2007) showed that in the harvester ant *Pogonomyrmex badius* sex allocation is a function of resource availability, in that under optimal conditions resources are allocated towards queens. Much analogue to stored seeds *in P. badius*, in *C. smithi* the diet of the larvae and the queen appears to consist exclusively of eggs (Heinze *et al.* 1995). Although 19% of the workers (n = 1253 workers, J.O. unpub.) have developed ovaries with large oocytes, the fecundity of intermorphs is much higher. Intermorphs lay 20 times more eggs per mg weight than workers. The proportion of trophic eggs produced by intermorphs is 2% compared to 20% laid by the queen (Heinze *et al.* 1995). However, intermorphs produce eggs which would eventually develop into male larvae and are kept as living food stores ("fratricide" sensu Chapuisat *et al.* 1998). Having intermorphs that produce protein-rich "living food stores" in the form of viable male offspring might thus enable colonies to rear more individuals of the larger, more costly sex even under conditions in which proteinaceous food is scarce, such as during the dry season in the xerothermic forests in Southern Arizona. Therefore, colonies with intermorphs should produce a more female-biased sex ratio and/or a greater total sexual production. As predicted, the colony-level numerical sex ratio increased with an increasing number of intermorphs.

We found evidence that some eggs laid by either workers or intermorphs developed into adult males. All eggs present in a colony are laid or deposited onto a single pile and some worker-produced eggs might be missed by those workers that feed the larvae. Foster & Ratnieks (2001) argue that workers cannot discriminate the maternal origin of male larvae but that they can sense whether eggs are laid by workers or the queen. Because of the vast number of eggs and the similarity of the cuticular hydrocarbon profiles of intermorphs and queens (Oettler *et al.* 2008) we suppose that erroneous identification of eggs possibly occurs. Foster & Ratnieks (2001) also assume that the killing of eggs instead of larvae conserves investment of energy. In their model they incorporate a "novel benefit" in that these resources are reallocated to queen-derived males and females. Our observations are in agreement with the model by Foster & Ratnieks (2001) and indicate that the elimination of eggs does not necessarily result in the complete loss of energy.

The underlying mechanism of the development of intermorphs is not known, but the production of intermorphs and virgin queens are temporally decoupled. Five colonies collected in July 2005 produced intermorphs three month later in the lab under artificial day/night time regime adjusted to Arizona local time. The production of new queens, instead, was synchronized across all colonies in spring 2006. This suggests that intermorphs are not queens whose development was aborted; this is further supported by the observation that intermorphs commonly occur in various species of the subgenus *Crematogaster* (*Orthocrema*) (Table 1). What causes the variation in numbers of intermorphs across colonies is also unknown. Small, presumably immature, colonies do not possess intermorphs. It is likely that only larger, mature colonies, have enough resources to

invest into this particular female caste. Why some large colonies do not have intermorphs remains unclear, although the results show that colony size is unaffected by the presence or absence of intermorphs, but that, when present, the numbers of intermorphs increase with colony size.

Why having intermorphs might be adaptive

From the level of the colony, intermorphic egg layers may be explained as a valuable adaptation to food shortage. From the level of the individual, intermorphs might have first evolved as selfish individuals that attempt to increase their own inclusive fitness by producing males, to which the other workers react by policing through egg-eating (Ratnieks 1988), or in the case of *C. smithi*, by reinvesting worker-produced haploid eggs into the development of queen-destined larvae. Worker policing due to relatedness asymmetry is not expected in *C. smithi* because workers are on average more closely related to their nephews than brothers. On the other hand, policing can be favored if worker egg laying negatively effects the total reproductive output of the society, either because workers fight for egg laying rights or fertile workers engage less in non-reproductive tasks (Cole 1986). Intermorphs are highly efficient egg-layers and forcing them to take over non-reproductive tasks in the colony would be a waste of investment. Hence, once intermorphs have become mature, workers benefit the most by allowing them to lay eggs. While intermorphs might first have originated in evolution as rare malformations, or alternatively as caste conflict "cheaters" (cf. Wenseleers *et al.* 2005), they subsequently spread in the population due to a combination of individual, kin, and colony-level selection.

We hypothesize that intermorphs can inflict costs to the workers and the queen and that fitness returns of having intermorphs would be reversed. So, in order to evaluate the consequences for inclusive and direct fitness and hence the possible selective pressures we first have to consider the costs and benefits of having intermorphs. Table 2 depicts the theoretical interaction effects of an additional intermorph on reproductive and non-reproductive sisters and the queen. The cost of an intermorphs for the colony – and hence shared by all parties, by reducing the energy budget for sexual production – is the production cost of an intermorph. This cost seems to be small, considering the smaller size and lower weight compared to a virgin queen. Intermorphs seem to be associated with a more gyne-biased colony-level sex ratio. Note that the operational population-level sex allocation ratio seems to lie intermediate between the queen and worker optimum but that it is not significantly different from either. This suggests that there may only be weak selection on individual mother queens to achieve a colony-level sex ratio that is as male-biased as possible, and that there may be only weak selection on workers and intermorphs to achieve one that is as gyne-biased as possible.

Here may lay the key to explaining why not all colonies have intermorphs: at the current operational population-level sex allocation ratio, workers are mildly positively affected by or indifferent to intermorphs, and queens mildly negatively affected by or indifferent to intermorphs. As intermorphs become more common in the population, the population sex allocation ratio goes more towards virgin queens. Intermorphs then become strongly maladaptive for queens, and steadily less beneficial to workers and eventually neutral. If the operational population-level sex allocation ratio is at the worker optimum, there is no more selection on workers to achieve any particular colony-level sex ratio (i.e., workers have exactly equal fitness irrespective of the colony-level sex ratio, although of course the greater the sexual production, the higher their fitness). But at this point the population-level sex allocation ratio is far too gyne-biased for mother queens, so selection on queens is strong to achieve a colony-level sex ratio that is as male-biased as possible. Selection on workers will recommence to operate as soon as the population-level sex allocation ratio departs from the worker optimum, exactly because selection has been indifferent to the sex ratio. The same would be true for queens, when the population sex allocation ratio is equal to the queen optimum of 0.50. Hence, we predict an evolutionary tug-of-war between queens and workers over the population-level sex allocation ratio.

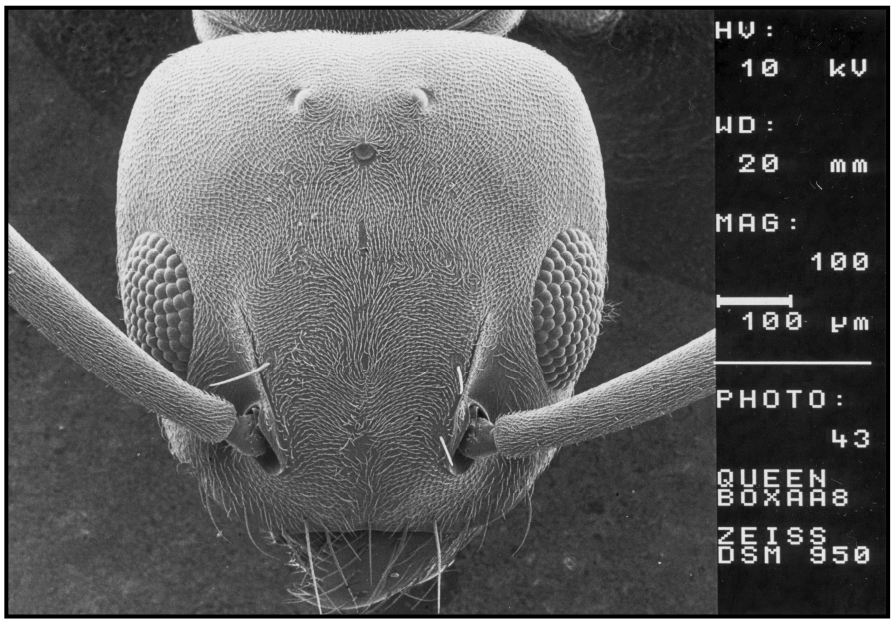
	Self (additional intermorph)	Reproductive sister[1,2]	Non-reproductive worker[2]	Mother queen
The production and maintenance of an intermorph reduces the budget for sexual production	$\Delta\varpi_t < 0$	$\Delta\varpi_t < 0$	$\Delta\varpi_t < 0$	$\Delta\varpi_t < 0$
Some queen sons get replaced by sons of an additional intermorph	$\Delta\varpi_t >> 0$	$\Delta\varpi_t > 0$	$\Delta\varpi_t > 0$	$\Delta\varpi_t < 0$
Some non-queen sons get replaced by sons of an additional intermorph	$\Delta\varpi_t >> 0$	$\Delta\varpi_t << 0$	$\Delta\varpi_t = 0$	$\Delta\varpi_t = 0$
The colony-level sex ratio becomes more biased towards virgin queens[3]	$I < \hat{W} \Rightarrow \Delta\varpi_t > 0$ $I \approx \hat{W} \Rightarrow \Delta\varpi_t = 0$ $I > \hat{W} \Rightarrow \Delta\varpi_t < 0$	$I < \hat{W} \Rightarrow \Delta\varpi_t > 0$ $I \approx \hat{W} \Rightarrow \Delta\varpi_t = 0$ $I > \hat{W} \Rightarrow \Delta\varpi_t < 0$	$I < \hat{W} \Rightarrow \Delta\varpi_t > 0$ $I \approx \hat{W} \Rightarrow \Delta\varpi_t = 0$ $I > \hat{W} \Rightarrow \Delta\varpi_t < 0$	$I < \hat{Q} \Rightarrow \Delta\varpi_t > 0$ $I \approx \hat{Q} \Rightarrow \Delta\varpi_t = 0$ $I > \hat{Q} \Rightarrow \Delta\varpi_t < 0$

Table 2. The sign and strength of the effects of an additional intermorph on the total inclusive fitness (ϖ_t) of various types of colony mates. *W* is the worker optimum for the population-level sex allocation ratio, *I* is the operational (i.e., the current) population-level sex allocation ratio, and *Q* is the queen optimum for the population-level sex allocation ratio. In our case, $Q < I < W$ (but *I* is not significant different from either $Q = 0.5$ or $W = 0.71$). “<< 0" and "< 0" denote a strong and a weak negative effect, respectively, and ">> 0" and "> 0" a strong and a weak positive effect.

Chapter 6

Polyphenism of female reproductives in the tramp ant *Technomyrmex vitiensis*

Jan Oettler and Jürgen Heinze



Winged queen of *T. vitiensis* SEM Photograph © B. Lautenschläger 2007

Abstract

Colony reproduction in the tramp ant *Technomyrmex vitiensis* is characterized by the co-occurrence of dispersing winged queens and local, wingless, intermorphic reproductives. A morphometric analysis of intermorphs and workers clearly separated the two castes. Intermorphs constitute a substantial proportion of the colony, but in our sample only a small fraction of intermorphs were inseminated despite the presences of winged males. Wingless males, which mate with intermorphs in other species, have as yet not been found in *T. vitiensis*. Our data support earlier findings from closely related *Technomyrmex* that reproduction is based on an opportunistic strategy enabling the species to rapidly colonize new habitat.

Introduction

The evolutionary and ecological success of ants is to a large extent founded on a pronounced female polyphenism, with queens and one or several types of workers functionally and morphologically specialized for their specific tasks. In addition, female reproductives of many ant species exhibit a similarly striking polyphenism tightly linked to alternative reproductive and dispersal tactics. In many species, founding queens disperse on the wing and start a new colony solitarily, using their internal fat reserves and digested flight muscles to rear their first offspring (Peeters & Ito 2001). Monogynous species with independently founding queens are usually characterized by a long reproductive period with high queen fecundity, but the queens' death invariably leads to the decline of the colony. Independent founding is associated with increased gene flow and reduced sibling competition, but an individual queen's probability of surviving the initial phase of colony foundation is extremely low (Johnson 2006). In contrast, colonies of species with dependently founding queens may recruit their own female sexuals to guarantee colony survival beyond the longevity of a single queen. Dependently founding queens are often characterized by a short reproductive period and low fecundity but a high probability of surviving until maturity (Hölldobler & Wilson 1990). They are often smaller in size and pose lower investment costs to the colony than independent queens, which are provisioned with large body resources. Whenever dispersal abilities are limited and mating occurs among nestmates, strategies must have evolved to minimize the constraints resulting from inbreeding and intercolonial competition.

In some cases, dependently founding queens have lost their wings and other adaptations for flight and may resemble the non-reproductive worker castes in their external morphology and size (Heinze 2008). Such queens are thus almost as cheap and fast to produce as workers. As numerous terms have been proposed for wingless reproductives, we here adopt the function-based terminology proposed by Buschinger & Winter (1976) and refer to them as "intermorphic queens" (further abbreviated with "IM") to distinguish them from irregularly occurring, pathological intermediates without special function (Heinze 1998).

In this study we focus on the genus *Technomyrmex*, which is remarkable in two ways. First, it contains many widely distributed, opportunistic tramp species, with *T. brunneus* Forel, 1895 (previously referred to as "the white-footed ant", *T. albipes* (Smith, 1861), Bolton 2007) probably the most prominent. *Technomyrmex* is therefore of considerable interest to conservation biologists (e.g., Warner & Scheffrahn 2004, 2005). Second, a large number of species within *Technomyrmex* have evolved a peculiar mode of colony reproduction that combines dispersal by winged queens and colony propagation by wingless, intermorphic replacement reproductives. Individuals, which are morphologically intermediate between queens and workers, are presently known from 25 of the 43 described species of the *T. albipes* group (Bolton 2007). They lack the pronounced mesonotum associated with the wing apparatus of female reproductives that disperse by flight and thus superficially resemble normal workers.

The reproductive biology of intermorphs has been described in detail for *T. brunneus* from Japan (referred to as *T. albipes*, Yamauchi *et al.* 1991, Ogata *et al.* 1996, Tsuji *et al.* 1991). Winged males and females are produced once a year and disperse synchronously in the summer. After mating, winged queens found new colonies independently. Once the colonies grow, IMs are produced, which mate in the nest with wingless males and supplement and finally replace the queen. About half of all individuals in larger colonies may be inseminated IMs. Such colonies grow quickly and spread through fission, i.e., groups of IMs and workers leave the nest and initiate new colonies nearby. *T. pallipes* (Smith, 1876)(Bolton 2007) and *T. difficilis* Forel, 1892 appear to show a similar life history (Deyrup 1991, Warner 2005). Here we provide more detailed data on the caste system and life history of still another species, *T. vitiensis* Mann, 1921.

Materials and Methods

Collection

T. vitiensis is a species presumably of Southeast Asian origin, which has been introduced into European and US greenhouses of botanical gardens where it forms large supercolonies. We found *T. vitiensis* in the botanical garden of Bonn University, Germany. It inhabits the warm house in large numbers, being an annoying pest to the gardeners. Like *T. brunneus* (Tsuji *et al.* 1991), *T. vitiensis* forms polydomous nests. The ants nest opportunistically in cavities in plants and do not construct special nest structures. Removal of the outer layers of palm trees almost always revealed workers, intermorphs, and winged males together with brood. Groups of workers, intermorphs, and brood were also found on the inner side of large leaves and other highly instable nest sites, either indicating that optimal nest sites have become limited in the greenhouse or that predation pressure is low. Despite intensive search we could not find winged or dealate female sexuals or wingless males.

Housing conditions

Colony fragments were transferred into 20cm x 20cm x 10cm plastic boxes with plaster flooring and small cavities in the plaster provided as nest sites. We kept the ants at a 12-12h light-dark regime at 26°C and 60% humidity and fed chopped cockroaches and diluted honey twice a week.

Plaster nests unlikely match the preferred nest site and consequently the ants tried to escape. Though the walls of the plastic boxes were regularly coated with Fluon®, the ants escaped from one box and for a few months inhabited hidden cavities in the walls of the climate chamber or the tubing system. Foragers from this nest were occasionally seen at night and during the lesser frequented weekends also during the day, and groups of workers were sometimes found at water bottles and other moist areas on the work benches. Here, we collected three winged females together with workers, but neither brood nor males were present. The outbreak was eventually stopped using filter papers soaked with a sugar solution containing 2% boric acid (suggested by A. Suarez; see also Klotz & Moss 1996, Klotz *et al.* 1997, 1998, 2000). Boric acid is less toxic than other commercially available pest control agents. It affects the nervous system and, because it acts slowly, is readily spread by trophallaxis throughout the whole colony. Filter paper baits were renewed every evening over a period of two weeks. Dead ants were regularly found, but not necessarily in the vicinity of the bait. After two weeks of applying baits no new *T. vitiensis* were observed.

Morphometry

For the morphological analysis we chose 68 ants roughly covering the whole range from small to large individuals. To assess, which morphological characters discriminate workers from IMs, we measured five continuous characters under a binocular to the nearest 0.01 mm at 20x magnification [Head width (HW), head length (HL), scape length (SL), pronotal width (PW), mesosoma length (ML)]. We also investigated three discrete characters (presence/absence of ocelli; presence/absence of spermatheca, number of ovarioles). When the typical iridescence of the spermatheca indicated the presence of sperm, the spermatheca was also studied at higher magnification under a binocular microscope. To control for observer bias we examined internal and external characters separately and later combined the data.

Because continuous morphometric external variables deviated from normality data were log-transformed. To overcome conflicts associated with collinearity of morphometric data we reduced the number of characters by a principal component analysis (eigenvalues > 0.80). We compared the extracted factors using a t-test with ‘caste’ as grouping variable based on the presence

or absence of ocelli ('worker', n = 46; 'IM', n = 22). Missing data was substituted by the corresponding mean.

To test whether external morphology was a good predictor of caste we performed a discriminant analysis based on the PCA using Statistica 13.0. Again, individuals were *a priori* grouped as "worker" or "IM" based on the presence or absence of ocelli and the characters were entered together.

To further discriminate workers and IMs from winged queens we counted the ommatidia of one compound eye each of three winged queens, eight IMs, and eight workers using SEM visualization. Because the number of queens was limited we do not have data from their ovaries.

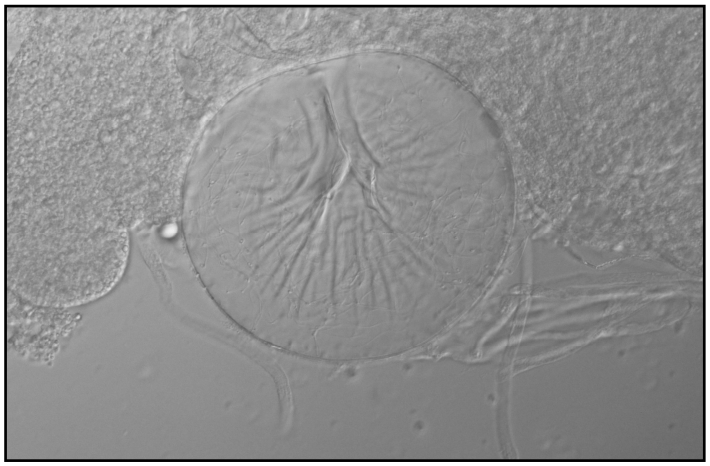


Figure 2. Spermatheca of an inseminated intermorph of the ant *Technomyrmex vitiensis*. Only few spermatozoa were present (see text). Pict ure taken at 100x with a ZEISS Axiophot microscope.

Results

Morphometry

Our study revealed a clear association between ovarian anatomy and external morphology. Ocelli (appearance ranged from traces to a full set of three ocelli) were present in 22 of 68 examined individuals (32%), all of which had ovaries consisting of 10 to 24 ovarioles (median 16.5; quartiles 15 and 19.75) and a spermatheca. Individuals without ocelli did not have a spermatheca and always four ovarioles.

The factor analysis reduced the five morphological characters to two factors (PC1 and PC2) that combined explained 78.4% of the variance (PC1 = 60.7%; PC2 = 17.7%). The single contributions (factor loadings) of the characters cumulated to the PC1 by HW (-0.78) + HL (-0.87) + SL (-0.53) + PW (-0.80) + ML (-0.87) and to PC2 by HW (0.03) + HL (-0.01) + SL (0.82) + PW (-0.46) + ML (-0.05). The low factor loading of SL in PC1 together with its positive contribution to PC2 is caused by an opposite trend, as all other characters were larger in intermorphic queens. IMs therefore are clearly distinct from workers in external morphology (t-test, df = 66; PC1 ($t = -6.21$), $p < 0.001$; PC2 ($t = -6.56$), $p < 0.001$).

In the discriminate analysis based on PC1 and PC2 all but three individuals were correctly classified to groups *a priori* defined based on the absence or presence of ocelli (one "worker" without ocelli and with 4 ovarioles was classified as IM; two IMs with ocelli and 16, respectively 17 ovarioles were classified as workers, Fig 1). It is noteworthy that the latter IM was one of the two inseminated specimens. Nevertheless, IMs and workers differed significantly in continuous morphological traits (Wilks-Lambda 0.24, $F = 104.95$, $p < 0.001$) in addition to ovarian anatomy and the development of ocelli. The examination of the compound eyes revealed further differences between the different types of females. Number of ommatidia (mean ± SE) differed significantly between winged queens (107.00 ± 1.42), IMs (70.13 ± 1.19) and workers (54.76 ± 1.42) (one-way ANOVA, Fishers' post hoc test; df = 16; $p < 0.001$).A closer examination of the ovaries of IMs revealed the presence of sperm in one spermatheca (Fig. 2) and of traces of sperm in a second.

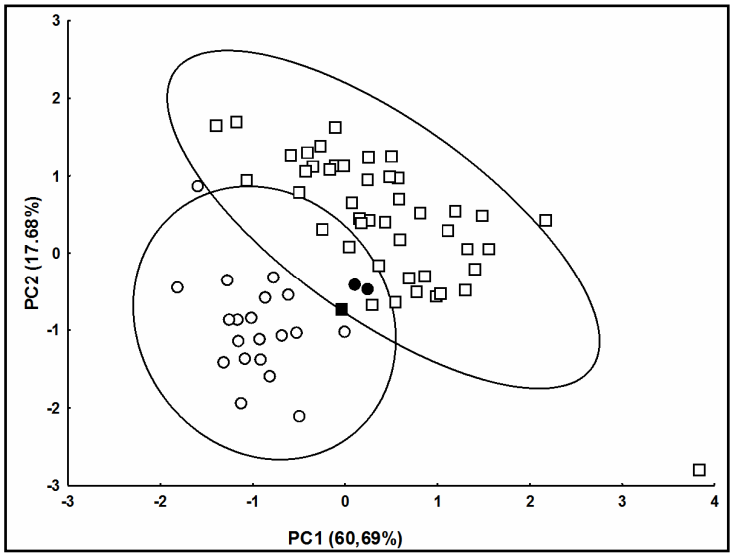


Figure 1. Discriminant function of intermorphs (circles) and workers (squares) based on two principle components. Prediction interval ellipses for each caste are based on 95% probabilities. Closed symbols indicate individuals that were incorrectly classified in the DA.

Discussion

Like other species of the *Technomyrmex albipes* - group, *T. vitiensis* shows two distinct types of female reproductives: winged queens, which are apparently produced only under certain environmental conditions and presumably disperse and colonize new habitat patches, and wingless replacement reproductives, which mate in the nest and locally increase colony size. Intermorphic reproductives can reliably be distinguished from workers by the presence of ocelli and ovarian anatomy. They are also distinct in external morphology, although both castes show some degree of size variation in all measured external characters. As in *T. brunneus* and *T. difficilis*, our sample of *T. vitiensis* indicates that IMs make up for a considerable proportion (32%) of the colony.

Despite of the presence of numerous winged males, only one IM had a completely filled spermatheca and we also could not observe mating attempts (unpublished). In *T. brunneus*, IMs usually mate with wingless, ergatoid males (Yamauchi *et al.* 1991) and the copulatory organs of winged and wingless individuals differ considerable in size (Ogata et al. 1996). Ergatoid males have been found in six species of the *T. albipes* group. One possible explanation for the low number of inseminated IMs might therefore be that winged males of *T. vitiensis* are similarly specialized for mating exclusively with winged queens, while IMs mate with ergatoid males. However, we neither found winged queens in the greenhouse nor ergatoid males under any condition. IMs might therefore occasionally be capable of mating with winged males. Nevertheless, their paucity in our colony fragments raises the question about the origin of female brood. The possibility of thelytokous parthenogenesis, i.e., the production of female brood from unfertilized eggs, has as yet not been excluded. Morphological queens were absent in the greenhouse and in lab colonies but developed somewhere in the climate chamber after the accidental escape of colony fragments from our nest boxes. This indicates a switch of reproductive strategy from colony growth to dispersal in response to changed environmental conditions, similar to that observed in *Cardiocondyla* (Cremer & Heinze 2003).

Like other species of the *Technomyrmex albipes*-group, *T. vitiensis* is an invasive ant species, which is widely distributed throughout greenhouses and botanical gardens, though it is unclear whether it negatively affects the plants (e.g., Bolton 2007) or on the contrary prays on herbivores and plant parasites (Gaume *et al.* 2005). We managed to eradicate an escaped colony fragment that had moved into cavities in our climate chamber by providing baits soaked with a sugar solution containing 2% boric acid. Crucial for the success of this treatment is that food is exchanged among individuals by trophallaxis. The exchange of liquids among adults has never been observed in *T. brunneus* and instead trophic eggs appear to play a crucial role in nutrition (Yamauchi *et al.* 1991). A boric acid treatment relying on active food exchange might therefore be less effective in controlling this species. In contrast, we frequently observed trophallaxis in *T. vitiensis* (unpublished), allowing for the toxic to spread throughout the colony.

Chapter 7

First recorded mating flight of the hypogeic ant *Acropyga epedana* with its obligate mutualist mealybug *Rhizoecus colombiensis*

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Figure 1. *Acropyga epedana* queen with mealybug between her mandibles and i copula with male. Photograph © Alex Wild 2005 (used with permission).

Abstract

On 26-July, 2005 a mating aggregation of *Acropyga epedana* Snelling (Hymenoptera: Formicidae) was observed in the Chiricahua Mountains in south-eastern Arizona. This is the first record of a mating flight of *A. epedana*, the only nearctic member of this pantropical genus. Mating behavior was observed, newly mated queens were collected, and a complete colony was excavated. New information is reported on the natural history and mating behavior of the species. The identity of a mealybug mutualist, *Rhizoecus colombiensis* (Homoptera: Rhizoecinae) is confirmed. Reproductive females participating in flights all carried mealybugs between their mandibles, indicating a vertical transfer of mealybugs with their ant hosts. No captured foundresses survived long in captivity, most likely due to the death of their mealybugs. The colony excavated had a single queen, though polygyny is common in the genus. Nearly all workers within the nest were heavily parasitized by mites, although males or gynes were not parasitized. These natural history observations are discussed with regard to this poorly understood mutualistic relationship between *Acropyga* ants and their mealybug partners.

Introduction

The entirely hypogaeic and pan-tropical ant genus *Acropyga* Roger (Hymenoptera: Formicidae) lives in obligate symbiosis with mealybugs (Homopotera: Sterrnorhyncha: Coccoidea: Rhizoecinae). The ants feed on the sugar-rich feces of mealybugs which in turn feed on root-sap (Johnson *et al.* 2001), an association described as trophobiosis (Delabie 2001). Female mealybugs are carried by virgin queens during their nuptial flights (Johnson et al. 2001), a behavior called trophophoresy (LaPolla *et al.* 2002). Though workers, males, and queens of *A. epedana* Snelling have been previously collected and described in the Chiricahua Mountains in Arizona (LaPolla 2002), they seem rare, though this may be due to their underground lifestyle, and little is known about the species. This is the only species in the genus reported for the nearctic (LaPolla 2004), and it has been found only in southeastern Arizona. Here, we describe a mating flight of the species along with observations of colony founding, worker behavior, and parasitization of workers by mites.

Results and Discussion

The mating aggregation and the colony that was sampled occurred in a shallow ditch alongside a primitive asphalt road, bordered by short grass, just 200m west of the Southwestern Research Station (1645 m N31°53.028’ W109°12.378’), near Portal, Arizona. Beyond the ditch was a steep incline without vegetation and the ditch and road received direct sunlight for most of the day. The surrounding habitat was dominated by an open oak-pine-juniper forest. Station (1645m N31°53.028’ W109°12.378’), near Portal, Arizona. Beyond the ditch is a steep incline without vegetation and the ditch and road receive direct sunlight for most of the day. The surrounding habitat was dominated by an open oak-pine-juniper forest.

The mating flight occurred 2 days after the first heavy summer monsoon rains (26 July 2005), although there was very little precipitation on the day of the flight. The aggregation was first seen at ~16:30h. Most of the males were hovering or swarming above small clumps of grass and rocks. Females entered the aggregation, after which multiple males simultaneously attempted to mate with them. Males outnumbered females by ~10:1. After mating most females immediately shed their wings and searched for suitable nest sites; only a few females flew from the aggregation. All females carried a mealybug in their mandibles. On 6 August 2005 at the same site, a single alate female carrying a mealybug was found actively mating, but no swarm could be found and only a single male was present (Figure 1).

Nine dealated queens were collected immediately after the nuptial flight on 26 July, and an additional 15 queens the next day under rocks at the same site. We transferred them into standard water tubes used for raising a colony from a foundress (a 20ml culture tube with ~15ml water in the bottom stoppered with cotton, and sealed at the top with additional cotton). The 9 foundresses collected on the day of the nuptial flight lived for 36 ± 2 days (mean ± 1 SE). Of the 15 foundresses collected the day after the flight, only 4 were still alive by 31 July 2005 when they were transferred to a set of water tubes with live roots. Two remained alive through 6 August, and the final two died on the 8th and 30th of September. Another *A. epedana* dealate queen was collected under a rock on 2 August 2005, 4 km away from the original site (1839 m, N31°52.682’; W109°14.165’), and died on 10 September. None of the foundresses ever produced eggs.

A complete nest was excavated on 31 July 2005, a few meters from where the mating aggregation occurred. It was excavated to a depth of ~30cm. The nest had a diameter of ~30cm, and was partially beneath the asphalt road. To ensure the completeness of the excavation the hole was expanded in all directions for at least 5 cm after ants were last found. The nest consisted of a diffuse connection of small chambers and tunnels. At 20 cm depth elongate chambers were found along

small roots of unknown species of plants. Mealybugs were on top of these roots and ants were nearby in the chamber. No ants or mealybugs were found in the upper grass roots. One *Acropyga* queen was collected from the nest at mid depth.

The colony contained 234 workers, 1 queen, 48 mealybugs and 100 males. The presence of males indicates that the mating season was still in progress. Neither larvae nor female sexual offspring were found in the colony; only a single pupa was found, but it was too damaged to ascertain its caste. Upon arrival in the lab, the queen was attacked by several workers that repeatedly bit her and had a firm grasp of her legs. For observation, the colony was transferred into a plaster nest with grass roots inserted into chambers. The colony did not move into the protected chambers but stayed with the small pile of dirt they were introduced with, piling the mealybugs together on the dirt.

The majority of workers were found dead upon arrival at the lab. Workers exposed to microscope light immediately became agitated and quivered. Along with this photophobic behaviour the ants appeared to desiccate quickly. Approximately 95% of the workers were parasitized by small mites. The mites infested most body parts, but seemed to prefer the meso- and metathorax, with many workers having mites symmetrically positioned on either side of the thorax (Figure 2). Mites were also common on the underside of the head. Though most workers had 1–3 mites and some had up to 6, they did not seem to be physically impaired. After the death of an ant the mites immediately (within 1min) detached and crawled away.

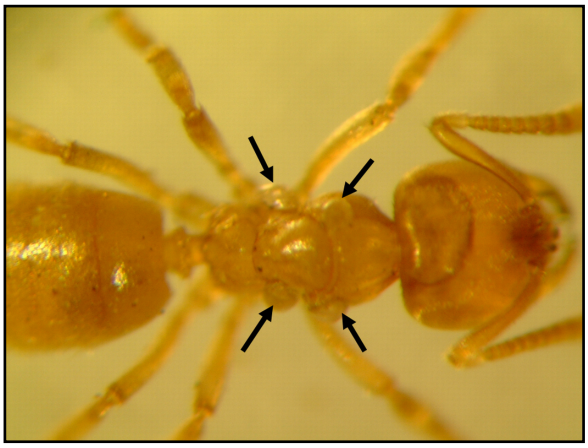


Figure 2. Acropyga epedana worker with mites, arrows point to mites. Photograph by C.R. Smith and J. Oettler.

Three *Hypoponera inexorata* (Formicidae, Ponerinae) queen ants were found associated with *A. epedana* colonies. One *H. inexorata* queen with workers was found under the rock at the surface of the colony excavated on 31 July. Another was found under a rock with a founding *A. epedana* queen the day after the nuptial flight on 26 July. A third was found with *A. epedana* workers under a separate rock on the same day. The *H. inexorata* queen collected near the *A. epedana* colony was transferred to the plaster nest along with the colony. Though no aggression was noticed between the *Hypoponera* queen and *A. epedana* workers, the queen was found dead the following day.

Mealybugs collected with queens during flights and found within the colony were all *Rhizoecus colombiensis* (Homoptera: Rhizoecinae). Williams & LaPolla (2004) also collected this species from *A. epedana*, although this mealybug was previously only known from Colombia. The obligate mutualism between *Acropyga* ants and mealybugs is an excellent system for the study of co-evolution, including vertical transmission of both partners through time. Although an obligate association is apparent for both partners, across the single genus *Acropyga* there are several genera of mealybug (LaPolla 2002), indicating the possibility of multiple “domestication” events.

These observations on the mating flights and mating behavior of *A. epedana* are consistent with reports for other *Acropyga* species (Eberhard 1978; Buschinger *et al.* 1987; Williams 1998; Johnson *et al.* 2001). On the day that the mating swarm *of A. epedana* was observed, many other ant species also flew, likely cued by the beginning of the monsoon rains. The finding of *H. inexorata* queens in close association with *A. epedana* is most likely due to their similarity in flight time and preferential habitat under rocks.

The structure of the excavated nest was consistent with that reported previously for *A. epedana*, and for other *Acropyga* species. The size of the colony excavated, however, was an order of magnitude smaller than that reported for neotropical species (LaPolla *et al.* 2002). Only one queen was found in the nest though polygyny is suspected for this and other species, possibly as an adaptation to increase the genetic diversity of both the worker and mealybug populations within the nest (LaPolla *et al.* 2002).

The high parasitization rate of workers by mites is consistent with previous collections of *A. epedana* (LaPolla, personal communication). The cuticle of workers is very thin and light in color (LaPolla 2004) most likely a result of their subterranean lifestyle, which may increase their susceptibility to ectoparasites. On the other hand, the males and reproductive females that emerge from underground for nuptial flights were not parasitized, and survived in captivity for up to 6 weeks. This contrast may be due to physiological differences between the worker and reproductive castes, and the adaptations necessary for dispersal in this species. The eyes of workers were highly reduced in size relative to queens, which may affect their ability to migrate above ground.

Acknowledgements

We thank D. Voegtlin (UIUC) and D. Miller (Systematic Entomology Laboratory, Agriculture Research Service, US Dept. of Agriculture) for help with mealybug identification, and J. LaPolla for helpful discussion. Ant specimens were vouchered with S. Cover (Musuem of Comparative Zoology, Harvard University, Cambridge, MA) and J. Trager (Missouri Botanical Garden, St. Louis, MO). Mealybug specimens were identified by and vouchered with D. Miller (Systematic Entomology Lab., Agricultural Research Service, USDA, Beltsville, MD).

Chapter 8

Phylogeny of the ant genus *Cardiocondyla*: Evolution of male morphology and life history strategies

Jan Oettler and Jürgen Heinze

Fighting *Cardiocondyla obscurior* males. Photograph © JO.

Abstract

The expression of alternative male phenotypes as a result of sexual selection is widespread in the animal kingdom. A striking example is found in the ant genus *Cardiocondyla* that displays a male dimorphism of wingless and winged males. *Cardiocondyla* has become an important model system for evolutionary biology. We reconstruct the phylogeny of *Cardiocondyla* using sequences from three nuclear and one mitochondrial gene for 20 species of *Cardiocondyla*. We find a strong correlation of this phylogeny with male morphology and life history traits. The common ancestor to the free-living extant *Cardiocondyla* (Formicidae: Myrmicinae) most likely possessed both regular winged males and wingless males. The novel wingless phenotype is specialized for intranidal mating, i.e. mating with mainly related queens. Except for a derived monogyne (single-queen) clade, *Cardiocondyla* have multiple breeding queens (polygyny). Local mate competition, which leads to a female-biased sex ratio, is widespread and appears to be not associated with male morphology.

Introduction

Differential expression of the same genotype resulting in alternative discrete morphology is common throughout the animal kingdom. Polyphenisms are adaptations to natural selection (such as predation, camouflage or feeding preference) and sexual selection. Sexual conflict in particular accounts for much of the observed polyphenism in males which have different life history strategies than females. In order to understand the nature of derived traits displayed by extant species, it is fundamental to assess the phylogenetic relationships. Here, we focus on males of the ant genus *Cardiocondyla*, our model system for the study of sexual conflict, immunity, behaviour, chemical communication and genomics.

The life history strategies of male ants have received much less attention than their female counterparts. After all, workers and queens have the more prominent roles in haplo-diploid social insect colonies. In general, hymenopteran males have a short lifespan, they do not contribute to the maintenance of the colony and their behavior during mating flights (their sole purpose) is difficult to study. Males are considered to be mating machines; once mating is accomplished, they die. However, it is obvious that the male genome plays an important role in the evolution of haplo-diploid species by the process of haploid "genetic cleansing" of adaptive and non-adaptive mutations (Wilfert *et al.* 2007). The haploid male genome is subject to strong selection compared to the female genome due to the lack of balancing copies of genes (Smith and Shaw 1980) where deleterious alleles are unlikely to get vertically passed on, and are thus more likely to be sorted out. The parthenogenetic mode of male determination can reduce the costs of mating with low-fitness conspecific males or males of the wrong species which appears to be common in some sympatric living closely related species (Feldhaar *et al.* 2008).

In contrast to the general view of male passivity in ants, the males of some species have evolved alternative strategies that entail a more active role in social life. Almost exclusively these life history strategies are directly linked to reproduction. At present only two species are described where males substantially contribute to the work-force of the colony. *Camponotus herculeanus* adult males engage in trophic exchange and male larvae of *Oecophylla longinoda* produce silk which is used for nest construction (Hölldobler & Wilson 1990).

Universally, loss of wings is one of the most dramatic adaptations in the social hymenoptera and its evolution a precursor to novel life history strategies. Winglessness in ants is obligatory for non-dispersing workers that in general have a reduced morphology compared to their mother queens. Size difference is usually accompanied by physiological constraints resulting in more or less sterile helpers which are crucial for the apparent ecological dominance of extant ants. While there is no question concerning the adaptive value of wingless workers (a key concept of eusociality), winglessness in queens constraints dispersal. Dispersal can serve to both decrease inbreeding and reduce competition among relatives for new nest sites. Despite these disadvantages, numerous species representing most ant subfamilies have evolved wingless queens as either the exclusive morph or as an additional low cost reproductive morph in addition to normal winged queens that guarantee gene flow (Oettler this thesis, Tsuji *et al.* 1991, Heinze & Tsuji 1995). Abouheif and Wray (2002) showed that the underlying patterns of the development of wings are extremely plastic. On the other hand, wingless males are rare throughout the ants. In addition to some social parasites with highly derived life history strategies (*Anergates* (Heinze & Buschinger 2007), *Plagiolepis* (Passera 1964), *Formixocenus* (Francoeur *et al.* 1985, Buschinger *et al.* 1994)), three unrelated genera have evolved wingless males in combination with wingless queens (Ponerinae: *Hypoponera* (Hamilton 1979), Dolichoderinae: *Technomyrmex* (Ogata *et al.* 1996), Myrmicinae: *Pogonomyrmex* (RA Johnson pers com)). *Hypoponera* wingless males mate with queens in their pupal stage (Foitzik *et al.* 2002) and fight with rival males (Hamilton 1979). In *Technomyrmex* the occurrence of wingless females and males is correlated with an alternative dispersal strategy of budding (a group of intra-nest mated wingless queens and workers leave the nest) and independent nest foundation (a winged queen leaves the nest to mate with winged males from different nests). Male winglessness is phylogenetically isolated; those species with wingless males likely suffer from the costs of inbreeding (i.e. diploid male load).

The ant genus *Cardiocondyla* (Formicidae: Myrmicinae) is unique among the Formicidae because winglessness of males is suggested to be ancestral for the genus (Heinze et al 2005) and this trait is not correlated with winglessness of females. *Cardiocondyla* is an inconspicuous genus of very minute comprising an estimated 100 old-world species though only 50 species are currently described (Seifert 2003). The wingless male-morphology is present in all *Cardiocondyla* species for which males are known (Seifert 2003, Heinze et al 2005, JH collection records). In addition some species contain winged males (Kugler 1983), to either supplement or replace the wingless mating strategy under specific environmental conditions (Cremer & Heinze 2003). The variety and extent to which male polymorphism and competitive behaviors are realized resemble Hamiltons descriptions of Fig wasps (Hamilton 1979). Winged and wingless males differ sharply in their reproductive tactics: as predicted, winged males tend to be dispersers and attempt to mate with unrelated female sexuals away from their natal nests, while the wingless males stay and mate with closely related female sexuals. Wingless males are long-lived, feed, mate many times and interact with other males. Some males may even show territoriality within nests (Froschhammer pers,). A remarkable aspect of wingless males is that spermatogenesis continues throughout their adult life but is terminated in winged males at an early age (Heinze & Hölldobler 1993), with the exception of the unusual brachypteran winged males in *C. kagutsuchi* (Yamauchi *et al.* 2005, see below).

In addition to the dichotomy of intra- and extranidal mating strategies, wingless *Cardiocondyla* males generally display two distinct species-specific morphologies which are accompanied by different behavior: Males with “saber-shaped” mandibles fight over reproduction by attempting to kill adult rivals. Their curved and elongated mandibles are adapted for seizing other males. A dominant male immobilizes a rival and applies a substance from the hindgut to which fellow workers respond and engage in the killing of the subordinate male. In contrast, males with worker-like “shear-shaped” mandibles directly kill developing rivals, when the cuticle is still soft, defeating males are literally cut into pieces.

The study of the wingless, so called ergatoid (worker-like), males in comparison to their winged relatives provides insight into the role of the often neglected sex. At present we have just begun to understand the evolution of *Cardiocondyla*. The aim of this study was to derive a comprehensive phylogenetic tree to study the evolution of morphology and behavioral strategies, especially of males. The results of a previous phylogeny left room for speculations about the origin of male morphology (Heinze *et al.* 2005). For this study we combined information from three nuclear genes with a mtDNA fragment resulting in 2.5 kb sequence data. We tested following predictions: (1) the wingless male morph has evolved once and is ancestral to the genus, (2) *Cardiocondyla* comprise two lineages with associated male morphology that diverged early in time, and (3) the winged male morph has independently and repeatedly regained.

Materials and Methods

Taxon and gene sampling

Our analysis of *Cardiocondyla* (Formicidae: Myrmicinae: Formicoxenini) included 20 species, one undescribed species and samples of at least three species from an undescribed species complex (Seifert 2003 and pers com). These samples represent all lineages within *Cardiocondyla* for which males are known. Because of morphological and/or nucleotide variation in 4 species groups (*C. wroughtonii* group, *C. emeryi* group, *C. nuda* group and the undescribed *C.* n. sp group) the final data set comprised 50 *Cardiocondyla* samples. For the outgroup we used seven closely related Formicoxenini species, five distantly related myrmicine species and one species from outside the Myrmicinae. In total we included 61 specimens (Table 1). Sequences for most of the outgroup were obtained from three previous studies (Brady *et al.* 2006, Moreau *et al.* 2006, Moreau 2008).

DNA extraction, amplification and purification

We extracted DNA from single workers using a chelex method (Sambrook et al 1989). For the nuclear protein-coding genes PCR amplification consisted usually of 35 cycles of 30-60s at 95°C, 54-58°C (annealing) and 72°C, preceded by 3min at 95°C and followed by 7min at 72°C. PCR was carried out in 25ul volume reactions with 1ul of template and 24ul master mix (10.25ul H2O, 5ul Enhancer, 2.5ul 10x Buffer, 2.5ul 25mM MgCl2, 0.25ul 100mM dNTPs, 1.5ul 10mM Primer each, 1ul (= 1U) Taq). For the amplification of the COI/COII fragment and primer sequences see Heinze et al (2005). The PCR products were gel-purified with the NucleoSpin Extract kit (Macherey-Nagel). All products were sequenced in both directions (ABI Prism 310) and sequences verified by analysis of two nestmates for each taxon.

We amplified three nuclear genes (a 346bp fragment of *wingless*, a 524bp fragment of *LwRhod* and a 272bp fragment of *Histone 3*) that recently proved to contain substantial information for the analysis of phylogenetic relations within subfamilies (Moreau et al 2006, Brady et al 2006) as well as genus levels (Ward & Downie 2005, Moreau 2008). For *Histone 3* we obtained sequences only from species representatives, i.e. one sample of the *C.* n. sp.-complex, four samples of the *C. wroughtonii* group and two *C. kagutsuchi* samples. From initial universal primers we developed specific primer pairs for *wingless* and *Histone 3* (Table 2). In addition we extended an existing mtDNA data set (1414bp of the region *COI/COII*, including *tLeu*) to generate information for the relationships of more closely related taxa.

Alignment

We aligned the sequences with BioEdit (Hall 1999) using the Clustal W algorithm and then manually corrected ambiguous regions. For the protein-coding genes we used the amino acid compositions to verify the alignment based on the nucleotide sequences. *LwRhod* contains an intron which has been excluded in previous studies (Moreau *et al.* 2006, Ward & Downie 2005, Brady *et al.* 2006) because the alignment across distantly related genera/families is questionable and almost impossible. For the purpose of this study a 93bp fragment of the intron was used for the analysis of the ingroup (for the alignment see Fig. 1). The 41bp non-coding region between the 3' end of *COI* and *tRNALeu* instead was included in all analyses. Running an initial Bayesian analysis with and without this region did not result in any changes of posterior probabilities.

Phylogenetic reconstruction

A preliminary analysis of *Histone 3* on a subset of the data revealed relationships much different from the topology derived by Heinze *et al.* (2005) based on mtDNA. Although *Histone 3* has recently been used by several authors for a variety of phylogenetic studies on Articulata (sensu Nielsen 2001) (e.g. Colgan *et al.* 1998, Jorgensen *et al.* 2008, Arango & Wheeler 2007, Bergsten & Miller 2007, Cryan 2005, Ogden & Whiting 2005) and especially the 'hyperdiverse' ant genus *Pheidole* (Moreau 2008), the possibility of pseudogenes cannot be excluded (without cloning the PCR products). Hence, for this study we employed several separate analyses of the entire data set and the ingroup only, once with *Histone 3* and once without. To verify the results of the concatenated data set and to explore the contribution of the single genes to the topology we also analyzed all genes separately.

For this study we refrained from using distance-based and parsimony-based methodology. Instead our analysis relied entirely on model-based methods. Distance-based methods (such as Neighbor-Joining) use sequence similarity to evaluate pairwise evolutionary distance between taxa to reconstruct a phylogeny. Parsimony-based methods estimate the topology that minimizes the number of inferred evolutionary changes. Although there is an almost philosophical debate over the applicability of model-based methods, a large and growing body of studies shows their accuracy (cf. Zwickl 2006, for a short review on potential sources of error of Bayesian inference see Jones 2008) and suggests that using alternative methods might be redundant. Hence, phylogenetic relationships were based solely on the model-based methods of a maximum likelihood algorithm and a Bayesian approach. We performed Bayesian analyses with MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001) and verified the most important analysis with maximum likelihood using the GARLI algorithm (Zwickl 2006).

Bayesian analysis

We did not want to assume specific priors to generate an artificial conservative estimate. Thus, we tested alternative data sets and underlying models. Several separate MrBayes analyses were performed to generate a comprehensive understanding of the possible phylogenetic relationships (Tab 3). The most restrictive data set with specified models was divided into 13 or 16 partitions depending on whether *Histone 3* was included. One partition for each of 1st, 2nd or 3rd codon positions of the respective gene fragment and one partition for the region of *tLeu* between *COI* and *COII*. For the analysis of the ingroup we added one additional partition for the intron in *LwRhod*. With MrModeltest 2.2 (Nylander 2004) in conjuncture with PAUP 4.0b10 (Swofford 2001) we assigned the evolutionary model and likelihood settings using the Akaike information criterion for each partition and also for the entire undivided dataset. Table 4 shows the estimated evolutionary models for the single partitions. An initial run based on 10000000 generations did not generate a different topology compared to a single GTR + Γ + I nucleotide substitution model; hence we opted for estimation of parameters during each run. Relaxing the assumptions, we also tested whether the topology would be altered by assigning a single GTR + Γ + I model to the entire sequence block.

We performed the analysis with the default number of four Markov chains (three heated, one cold) and the default heating parameter set at 0.2. Each analysis of multiple genes was performed with an MCMC length of 10000000 generations and we compared the results of an initial run for the entire concatenated data set with an independent second run of 30000000 generations. Bayesian posterior probabilities were similar and we continued with using 10000000 generations. Analyses of the single genes were conducted using 5000000 generations. The chain was sampled every 100 generations after discarding the first 100 000 generations (the burn-in). We assessed the burn-in and the run convergence by comparing the mean log likelihoods by eye and by using the program Tracer v1.4 (available at http://tree.bio.ed.ac.uk/software/tracer/). The consensus tree is based on the proportion of clade probabilities, which could potentially bias the results (Jones 2008). The tree file was read into and edited with FigTree 1.1.1 (available at http://tree.bio.ed.ac.uk/).

Maximum likelihood

Because Bayesian inference can overestimate the clade probabilities, we analyzed the entire concatenated data set (including and excluding *Histone 3*) and the ingroup-only data set using GARLI with model parameters estimated during the run. The default model in GARLI is a GTR + I + G which matches the results of MrModeltest for the entire data set. In contrast to other programs GARLI can terminate the analysis before the maximum number of generations is reached after a defined number of generations without topological changes. These setting were definined as *genthreshfortopoterm = 10000*; *scorethreshforterm = 0.05*; *significanttopochange = 0.01*; *stopgen = 5000000*; *stoptime = 5000000*. To ensure that likelihood values converged consistently we used a repeated measure of 100 pseudoreplicates defined as *bootstrapreps = 100*, that we subsequently analyzed with PAUP 4.0b10 (Swofford 2001) to calculate a consensus tree.

Results and Discussion

1) General validity of the concatenated data set

Basic findings

The entire data set contained 2452 sites and 61 taxa (A = 0.2663, C = 0.2234, G = 0.1653 and T = 0.3450). By employing sequences of three nuclear genes to an extended mtDNA dataset we reconstructed the phylogeny of *Cardiocondyla* with high support by both the Bayesian and the maximum likelihood analysis. We corroborated our predictions for early male evolution in that the wingless male morph is ancestral and that an early split resulted in two distinct clades within *Cardiocondyla* that are in agreement with male morphology. One clade represents the saber-shaped male mandible type (*C.* n. sp group and *C. wroughtonii* group) and the second clade is characterized by males with shear-shaped mandibles (the remaining *Cardiocondyla*). Using a mixed model in which each gene was assigned its own GTR + Γ + I model in comparison to a single model for the entire sequence did not result in a different topology and posterior probabilities remained very similar (results not shown). Our analyses recovered an appropriate fit of the outgroup + ingroup relationship and strong support for the relations within the ingroup (Fig 2, Fig 4).

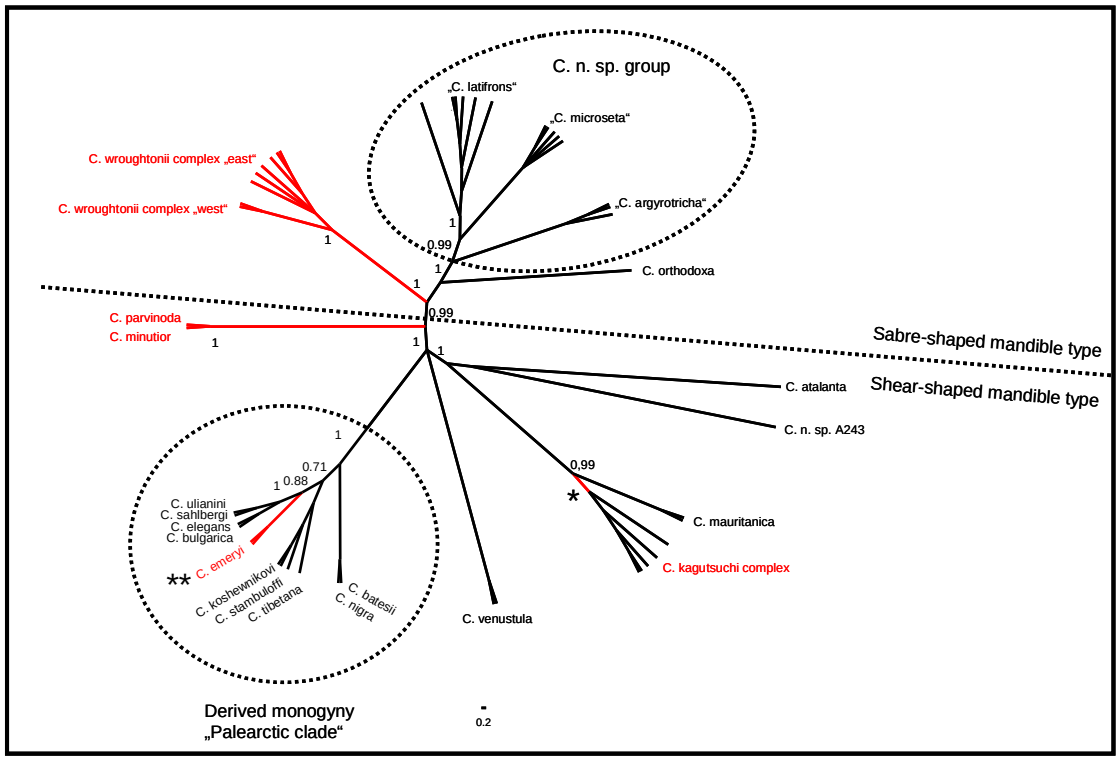


Fig 4. Unrooted Bayesian tree with branch length. For simplicity we deleted redundant taxon names. Preliminary names for taxa of the "*C.* n. sp. *group*" are given. Red lines indicate taxa which have winged and wingless males. The remaining taxa only have wingless males.

* No DNA was available for the *C. kagutsuchi* sample that is reported to have both male morphs. Thus, we graphically marked the base of the *C. kagutsuchi* complex.

** The position of *C. emeryi* is questionable (see text).

Dataset conflicts

Conspicuous is the long branch of *C. emeryi*, nested within the palearctic clade (see below, Fig 2). By removing the 272bp fragment of *Histone 3* we revealed a strong effect of this marker on the position of *C. emeryi*. After excluding *Histone 3* the analysis reconstructed a basal divergence of *C. emeryi* (Fig 3) but basal nodes lost support below significance. The clade consisting of the *C.* n. sp group and *C. wroughtonii* group is not supported (bpp = 0.65, ML = 64%). Within the second clade a monophylum of *C. emeryi* + the remaining *Cardiocondyla* is also not supported (bpp = 0.65; ML = 65%) although male morphology supports its affiliation to the clade and we favor the most parsimonious explanation of a single origin of the shear-shaped mandible character over convergent evolution. After excluding *C. emeryi* from the analysis we again reconstruct a similar topology as the initial data set including *Histone 3* and *C. emeryi*.

C. emeryi is distributed in Africa from coast to coast, the Near East and, being a successful tramp species (Heinze et al 2006), numerous Atlantic, Carribean and Pacific islands. The species shows a higher degree of worker polymorphism in microsculpture than the remaining *Cardiocondyla* (Seifert 2003). To answer the question whether *C. emeryi* really diverged early in time and to solve the deep DNA-sequence based phylogenetic relationships of *C. emeryi* and the remaining *Cardiocondyla* future work needs to employ additional species from the *C. emeryi* group (*C. weserka*, *C. neferka* and *C. yemeni*) and more data from nuclear gene fragments (in progress, M. Suefuji diploma thesis).

Topologies of single gene analysis

In order to test the validity of the single gene fragments we employed separate Bayesian analyses of single gene alignments, rooted to the respective most distant taxon for which sequences were available (*LwRhod*, *wingless*, *COI/COII*: *Acropyga epedana* (Formicinae); *Histone 3*: *Pogonomyrmex maricopa* (Myrmicinae)). Except for *Histone 3*, all genes recovered the monophylum *Cardiocondyla* with high support (data not shown). Overall all single-gene analyses recovered similar ingroup topologies. Again, an ingroup-only analysis of *Histone 3* also showed diverging patterns (data not shown), which indicates that our sequences of *Histone 3* - one of the five main genes involved in the structure of chromatin - may contain multiple pseudogenes across samples. However, in order to avoid arbitrary choosiness (Mattern 2003), we included the data, assuming that the short size of the sequence would only contribute to a small proportion of the overall error. Major inconsistencies within the remaining genes were found for *C. emeryi* and the sister species *C. minutior* and *C. parvinoda* and are discussed in detail below.

The outgroup relationships

Basically, the relationships within the outgroup are not resolved. Assuming that *Histone 3* accounted for much of the unclear relationships (see above), a subsequent exclusion of *Histone 3* did not result in any clear picture. When rooted for the most distant taxa (*Acropyga epedana*) all analyses recovered *Temnothorax* as a sister taxon to *Wasmannia* (thus placed outside of the Formicoxenini) which contradicts earlier results of molecular phylogenetic (Brady *et al.* 2006, Moreau *et al.* 2006) and morphological analyses (Bolton 2005). *Dilobocondyla sp.* is recovered as the outgroup to the remaining taxa with high support (bpp = 1, ML = 99%). Our samples of the genera *Atta*, *Pogonomyrmex*, *Messor* and *Aphaenogaster* are grouped together, but are nested within the formicoxenine genera *Podomyrma*, *Atopomyrmex*, *Xenomyrmex* and *Teratanter*. Here, terminal resolution appears appropriate in that *Aphaenogaster* and *Messor* as well as *Myrmica* and *Pogonomyrmex* stand in sister relationship. Most likely uncertainties in this study are due to the lack of a more comprehensive data set (in terms of taxa and genetic markers). However, basal relationships have been extensively studied (Brady *et al.* 2006, Moreau *et al.* 2006) and the purpose of including these myrmicine species was to test for the monophyly of *Cardiocondyla* and an appropriate fit of the analysis.

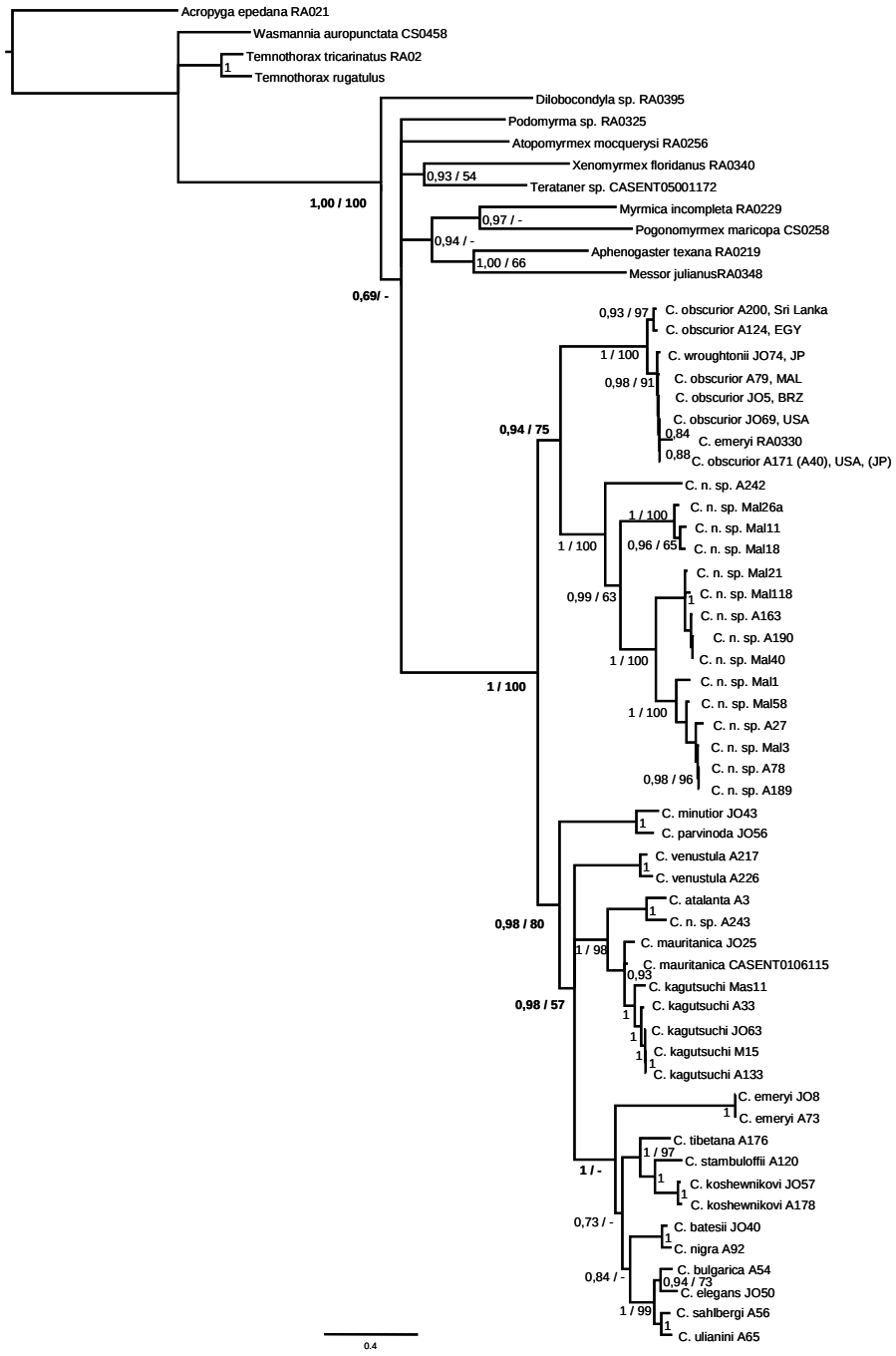


Figure 2. Bayesian tree with branch length based on the entire data set. Given are values (bayesian posterior clade probabilities / ML bootstrap support) above 0.9 bpp. Missing ML values denote abbreviated topologies from the GARLI analysis.

2) Evolution of Cardiocondyla

The species of the saber-shaped male mandible morph

The sequences of *C. emeryi* RA0330 (Moreau *et al.* 2006) is grouped within the *C. wroughtonii* clade in all analysis, indicating wrong taxonomic identification. Within the *C. wroughtonii* group the *C. obscurior* samples from Egypt and Sri Lanka (A200, A124. Both are tramp species of unknown origin) form the sister taxon to the samples from Malaysia, Japan and the USA. Our sample of *C. wroughtonii* is nested within this clade. This position is puzzling because nesting behavior and morphometry of workers, queens and males indicate clearly two separate entities of *obscurior* and *wroughtonii* (Seifert 2003). We propose an early divergence of east and west populations and a separation of *C. wroughtonii* from the Japanese *C. obscurior*. Both species from East Asia have invaded the western hemisphere probably via commercial traffic and founder events likely led to high variability. *C. obscurior* from Florida and Okinawa differ in number of chromosomes (Yamauchi unpub, cf. Seifert 2003), but our sequences were identical. Thus, one sample had to be removed from the analysis, but is shown in brackets in Fig 2. *C. obscurior* from Florida produces only very few alate males compared to samples from Brazil and Okinawa. Although more samples from western (African, West-Asian) populations are needed, based on our extensive sample size we propose to either reintegrate *C. wroughtonii* into *C. obscurior*, or, alternatively, a radical revision of this species group.

The second clade within the saber-shaped mandibled males consists of two separate entities: An undescribed species of the *C. paradoxa* group (*C.* n. sp. A 242) is recovered as the sister species to the *C.* n. sp. group. The male morph is unknown but according to the position in the tree we predict the species to contain ergatoid males with saber-shaped mandibles. If, in addition, winged males would occur, the derived ergatoid male monomorphy would be limited solely to the *C.* n. sp. group. The not yet described *C.* n. sp group still remains mysterious. Preliminary species groups erected on the basis of morphometry (Seifert pers com) are in part supported (see Fig 4). Our analysis indicates three discrete taxa. Yamauchi *et al.* (pers com) succeeded in crossing queens and males of presumably different species with different morphology. Further samples, morphology-based alpha-taxonomy and behavioral studies, are needed to shed light on this complex.

The species of the shear-shaped male mandible morph

The evolution of the second large clade within *Cardiocondyla* remains unresolved due to the above described difficulties. Although the nodes of particular interest are supported it is clear that the positions of the polygyne tramp species *C. minutior + C. parvinoda, C. venustula* and *C. emeryi* need deeper research. *C. minutior* and *C. emeryi* both contain wingless and winged males and wingless males appear to be the ancestral condition for the remaining species. Again, this hypothesis remains to be investigated. Nevertheless, the analysis reconstructed a polytomy characterized by multiple queens per colony and a derived clade with a common monogyne colony structure.

The polygyne species

C. minutior and *C. parvinoda* form the sistergroup to the remaining *Cardiocondyla*, but this position is only weakly supported (bpp = 0.88, ML = 80%). The separate analysis of the single gene fragments shows that only *wingless* supports a basal divergence. The undescribed *C.* n. sp. (A243) is reconstructed as the sister species to *C. atalanta*. These latter species form one polygyne clade of Asian origin together with *C. mauritanica* and *C. kagutsuchi*. Both species are successful tramp species. *C. kagutsuchi* has been collected throughout the indo-pacific regions and is a complex species group with broad morphological variation of the wingless males (Froschhammer pers com). In addition, *C. kagutsuchi* samples may contain ergatoid males or both male morphs and one Japanese population contains males with a morphology intermediate between ‘true’ ergatoid and winged males. This indicates either speciation in process or cryptic species. In any case, further

examination of morphological and sequence divergence as well as the degree of reproductive isolation is needed. It is noteworthy to point out that the extent to which male polymorphism is realized in the *C. kagutsuchi* complex is homologous to the one of the distantly related *C. wroughtonii* group.

The monogyne species

The analysis supports the earlier finding based on mtDNA that single-queen (monogyne) palearctic species are derived from ancestral multiple-queen (polygyne) species (Heinze *et al.* 2005, Schrempf *et al.* 2006) and that queen number might be a legitimate character in the context of this phylogeny. Due to the uncertainties of the position of *C. emeryi* the phylogeny within the monogyne species remains unclear. The cosmopolitan polygyne tramp species *C. emeryi* is grouped within the palearctic 'monogyne clade' by the ML analysis with weak support but forms the outgroup in the Bayesian analysis. Again, the long branch makes its position questionable. The relation of the *C. elegans* group and the *C. bulgarica* groups differs from the morphology-based taxonomy, which placed *C sahlbergi* close to *C. bulgarica* (cf. Heinze *et al.* 2005).

3) General discussion

Evolution of male morphology

From the tree we derive two alternative scenarios for the evolution of the morphology of the wingless males (Fig 4).

1) The wingless males possessed saber-shaped mandibles. Thus, the *C. wroughtonii* group represents the ancestral state. Due to the odd mandible form, males of species with saber-shaped mandibles rely on the help of workers as they are limited to seizing males, but cannot kill them. The sister group to the saber-shaped clade evolved shear-shaped mandibles. Males of species with shear-shaped mandibles are specialized for injuring and killing male pupae and adults by simple biting (cf. male fighting in Fig wasps, Hamilton 1979). The evolution of shear-shaped mandibles would have been a result of sexual selection under local mate competition. Local mate competition is common in *Cardiocondyla* and has been described for the 'shear-shaped' *C. batesii* (Schrempf *et al.* 2005), *C. kagutsuchi* (Yamauchi *et al.* 2005), *C. minutior* (Heinze *et al.* 2004) but also for the 'saber-shaped' *C. obscurior* (Cremer & Heinze 2002, de Menten *et al.* 2005).

2) The common ancestor to *Cardiocondyla* possessed shear-shaped mandibles. *C. emeryi* or *C. minutior / C. parvinoda* would resemble the ancestral state with both male morphs and shear-shaped mandibles of the ergatoid male.

Both of the above two scenarios are equally likely in respect to the recovered phylogeny. However, considering the similar morphology of the mandibles of winged and saber-shaped wingless males we favor the first scenario: the saber-shaped mandible appears to be a perpetuation of an ancestral trait while the shear-shaped mandible appears to be a derived and advanced condition.

Common to both scenarios is the interpretation that the ancestor of *Cardiocondyla* must have possessed both male morphs. Likewise, under both scenarios the winged males were lost convergently; once within the saber-shaped mandible clade (*C.* n. sp. group) and once within the remaining *Cardiocondyla* with shear-shaped mandibles (assuming that *C. emeryi* and *C. minutior* have diverged early). The winged male morph was regained in one Japanese population of the *C. kagutsuchi* complex (no sample was available, but see Figure 4) and once in *C. emeryi* (nested within the monogyne palearctic clade). *C. kagutsuchi* samples from Japan usually contain ergatoid males and have been reported to have 1n = 28 chromosomes. In addition, a sample with almost identical morphology from the most southern Japanese island occurs which contains two male morphs and is characterized by 1n = 27 chromosomes. One parsimonious interpretation is that the mechanism underlying male polyphenism in *Cardiocondyla* is not hardwired but instead a pleiotropic network of pathways where few genomic changes can alter the expression of a trait

resulting in the development of an alternative phenotype. This assumption is in agreement with our current understanding of the elaborate female caste developmental patterns in the myrmicine ants *Pheidole morrisi* and *Crematogaster lineolata*. Abouheif and Wray (2002) concluded from their pioneering study that the underlying genetic mechanisms for female polyphenism (the wing network) in general is conserved for the Formicidae, but that switch points are labile and differentially realized in different species. The erroneous occurrence of gynandromorphs, i.e. ants that possess both male and female characteristics in *C. batesii* and *C. emeryi* (Heinze & Trenkle 1997, Santschi 1907) and transitional stages between winged and wingless males (Cremer *et al.* 2002), as well as totipotent early larval stages (Schrempf & Heinze 2006), suggests that differentiation of male morphology follows the same or similar developmental processes responsible for the caste differentiation of females.

Male numbers and behavior

Males of both 'saber-shaped' and the 'shear-shaped' form share a unique behavior in that males call their sisters for help in eliminating the subordinate. This trait has been described for the saber-shaped *C. obscurior* (Yamauchi & Kawase 1992) but has been also observed in *C. n. sp* and in the shear-shaped *C. venustula* (Froschhammer pers com). Intranidal male competition is ancestral to *Cardiocondyla* and may have promoted the evolution of the wingless male morph, although this is a classical "Hen and Egg" problem. Male-killing is likely to be very costly in species with small colony sizes, such as *Cardiocondyla.* The 'early male strategy' displayed by competing queens (Yamauchi *et al.* 2006, Suefuij *et al.* in prep) likely reduces overall fitness. Here, queens attempt to produce the first male that will have a head start in competition with rival males. Hence, the production of males is favored over the production of workers at the cost of establishing a safe environment which is normally achieved by early allocation of resources into many workers.

The costs of male competition could have led to the peaceful male strategy displayed by species of the 'monogyne clade' in that queens avoid direct competition and males tolerate each other. This strategy, too, is probably accompanied by costs because the initial survival of obligate independent colony founding queens is lower than in multiple-queen associations (Johnson 2003). A second strategy to minimize the costs of male-killing is displayed by *C. venustula* and *C. kagutsuchi.* In colonies with multiple breeding queens, males avoid each other by territorial behavior. Males stay close to one colony-fragment while queens and workers move freely between fragments (S. Froschhammer pers com).

Colonies of the saber-shaped clade and the shear-shaped *C. emeryi* usually have a single dominant male (although large colonies may contain up to three males). The remaining *Cardiocondyla* contain two or more males and reach high numbers in the polygyne *C. venustula* (1 - 10 males) and in the monogyne clade (*C. ulianini*, *C. stambuloffii* 10 – 20 males (Marikovsky & Yakushkin 1974, Seifert 2003), hence male number is not necessarily associated with monogyny. Marikovsky and Yakushkin instead suggest that monogyny is associated with the lack of aggression among workers of neighboring nests.

Inbreeding

To prevent inbreeding effects that normally lead to dead-end development of infertile diploid males, novel strategies must have evolved in the highly inbred *Cardiocondyla* (Schrempf et al 2006). Inbreeding is common and gene flow is limited in all species that entirely lack winged males and is bound to queen dispersal because wingless males in general do not leave the nest (except *C. elegans, C. ulianini*). Males, irrespective of the number of males, may monopolize all available females of a given colony within their lifetime, limiting horizontal gene transfer. Indeed continuous spermatogenesis enables impressive sexual stamina: Males of *C. minutior* mate up to 86 times (Heinze *et al.* 1998) indicating that a single male is sufficient to fertilize all available virgin queens of larger colonies. In the monogyne *C. elegans* the queen produces up to five males which show no aggression and only occasionally leave the nest to enter a neighboring nest. Here, gene-flow is guaranteed by young queens, although already mated by brothers in the natal nest, which are

carried to neighboring nest where they mate additional times (Lenoir et al 2006). In addition, like in *C. ulianini* (Marikovsky & Yakushkin 1974), *C. elegans* males may occasional leave the mother nest in search of new females.

Further studies are needed to examine the frequency of diploid males and to decipher the underlying genetic mechanism of sex-determination in *Cardiocondyla*. It has been shown in *C. obscurior* that colonies may suffer from outbreeding depression but not from inbreeding, as predicted by single-locus sex determination. One hypothesis to be tested is that a multi-locus sex determination system has arisen in the common ancestor to *Cardiocondyla*, analogous to the one that has been suggested for *C. obscurior* (Schrempf *et al.* 2006) and several social and solitary hymenoptera (Wilgenburg *et al.* 2006).

Table 1.

List of samples used in this phylogeny. For simplicity we will reference corresponding gene bank accession numbers at the final stage of this manuscript.

Table 2.

Primer sequences for amplification and sequencing of the nuclear DNA fragments used for this study.

Table 3.

Data sets and respective models used for phylogenetic inference by the MrBayes analysis. For prior settings see text.

Table 4.

Model parameters as estimate by MrModeltest.

Figure 1.

Alignment of the 94bp fragment of the *longwave rhodopsin* intron used for the analysis of the ingroup.

Figure 3.

Bayesian tree with branch length based on *LwRhod*, *COI/COII* and *wingless*; *Histone 3* excluded. Note that *C. emeryi* is here reconstructed at the terminal node of *Cardiocondyla*. A polytomy of *C. minutior* / *C. parvinoda*, *C. venustula* and the “monognye” species is not resolved.

Table 1

Subfamily/Tribe	Genus	Species	Collection Accession Nos	Location (year)
Formicinae	Acropyga	epedana	RA021	
Myrmicinae	Wasmannia	auropunctata	CS0458	
Myrmicinae	Temnothorax	tricarinatus	RA02???	
Myrmicinae	Dilobocondyla	sp.	RA0395	
Myrmicinae	Myrmica	incompleta	RA0229	
Myrmicinae	Aphenogaster	texana	RA0219	
Myrmicinae	Messor	julianus	RA0348	
Myrmicinae	Podomyrma	sp.	RA0325	
Myrmicinae	Atopomyrmex	mocquerysi	RA0256	
Myrmicinae	Xenomyrmex	floridanus	RA0340	
Myrmicinae	Terataner	sp.	CASENT05001172	
Myrmicinae	Temnothorax	rugatulus	EF013650 (Brady et al2006)	
Myrmicinae	Temnothorax	rugatulus	JH collection	Portal, USA
Myrmicinae	Cardiocondyla	n. sp.	A 189	MAL: Borneo (2007)
Myrmicinae	Cardiocondyla	n. sp.	Mal40	MAL: Borneo (2007)
Myrmicinae	Cardiocondyla	n. sp.	Mal1	MAL: Borneo (2007)
Myrmicinae	Cardiocondyla	n. sp.	Mal 11	MAL: Borneo (2007)
Myrmicinae	Cardiocondyla	n. sp.	Mal 26a	MAL: Borneo (2007)
Myrmicinae	Cardiocondyla	n. sp.	Mal 18	MAL: Borneo (2007)
Myrmicinae	Cardiocondyla	n. sp.	Mal 21	MAL: Borneo (2007)
Myrmicinae	Cardiocondyla	n. sp.	Mal 118	MAL: Borneo (2007)
Myrmicinae	Cardiocondyla	n. sp.	Mal 58	MAL: Borneo (2007)
Myrmicinae	Cardiocondyla	n. sp.	Mal3	MAL: Borneo (2007)
Myrmicinae	Cardiocondyla	n. sp.	A 27	INDO: Jawa (1999)
Myrmicinae	Cardiocondyla	n. sp.	A 190	MAL: Ulu Gombak (2005)
Myrmicinae	Cardiocondyla	n. sp.	A 163	MAL: Tionman (2005)
Myrmicinae	Cardiocondyla	n. sp.	A 78	MAL: Ulu Gombak (2002)
Myrmicinae	Cardiocondyla	atalanta	3 (A)	AUS: Flinders Range (1999)
Myrmicinae	Cardiocondyla	batesii	JO 40	ESP: Padule (2008)
Myrmicinae	Cardiocondyla	bulgarica	A 54	Adama-Saimbeyli Yardibi Köyü TR (2001)

Myrmicinae	Cardiocondyla	elegans	JO 50	FRA: Loire (2006)
Myrmicinae	Cardiocondyla	“emery”	RA0330	
Myrmicinae	Cardiocondyla	emeryi	JO 8	Kap Verde: Tarrafal
Myrmicinae	Cardiocondyla	emeryi	A73	Funchal, Madeira (2002)
Myrmicinae	Cardiocondyla	kagutsuchi	JO 63	MAL: Ulu Gombak (2005)
Myrmicinae	Cardiocondyla	kagutsuchi	Mas 11	JP: Okinawa (2006)
Myrmicinae	Cardiocondyla	kagutsuchi	A 133	THAI: Ko Chsang (2004)
Myrmicinae	Cardiocondyla	kagutsuchi	A 33	JP: Okinawa (2001)
Myrmicinae	Cardiocondyla	kagutsuchi	M 15(1) seifert	MAL: Ulu Gombak (2002)
Myrmicinae	Cardiocondyla	koshewnikovi	JO 57	MON: 47.45.09N;92.02.28E (2003)
Myrmicinae	Cardiocondyla	koshewnikovi	A 178	MON: Ingbazar (2004)
Myrmicinae	Cardiocondyla	mauritanica	JO 25	ESP: Cerro Gorda
Myrmicinae	Cardiocondyla	mauritanica	CASENT0106115	Genebank
Myrmicinae	Cardiocondyla	minutior	JO 43	life colony
Myrmicinae	Cardiocondyla	n. sp.	A 243	PNG
Myrmicinae	Cardiocondyla	nigra	A 92	ZYP:
Myrmicinae	Cardiocondyla	obscurior	JO 5	BRA: (2006)
Myrmicinae	Cardiocondyla	obscurior	JO 69	USA: Florida
Myrmicinae	Cardiocondyla	wroughtonii	JO 74	JP: Okinawa (2006)
Myrmicinae	Cardiocondyla	obscurior	A 171	USA: Lake Alfred (2004)
Myrmicinae	Cardiocondyla	obscurior	A 200	Sri Lanka: in Psidum Fruits
Myrmicinae	Cardiocondyla	obscurior	A 124	EGY: Talka, Elmansoga
Myrmicinae	Cardiocondyla	obscurior	A 79	MAL: Ulu Gombak (2002)
Myrmicinae	Cardiocondyla	n.sp	A 242	PNG
Myrmicinae	Cardiocondyla	parvinoda	JO 56	THAI: (2001)
Myrmicinae	Cardiocondyla	sahlbergi	A 56	TURK: Kayseri-Yahyali (2001)
Myrmicinae	Cardiocondyla	stambuloffii	A 120	Slantsher Brjag, BG (2003)
Myrmicinae	Cardiocondyla	tibetana	A 176	MON: Ingbazar 2004
Myrmicinae	Cardiocondyla	ulianini	A 65	Burulday, KYR (2000)
Myrmicinae	Cardiocondyla	venustula	A 217	USA: Hawaii 2005
Myrmicinae	Cardiocondyla	venustula	A 226	Ethiopia 2007

Table 2

Primer location	Primer name	Sequence
*LwRhod*opsin	LR143F	(Moreau 2008)
	LR639ER	(Moreau 2008)
Wingless	Wg-F JO	5’ GAA CTT CCG CGT GGT CGG CGA
	Wg-R JO	5’ GGT GCA GGA GCA CCT CTC GA
Histone 3	H3-F JO	5’ GGC AAG GC(AT) CCC CGA AA
	H3-R JO	5’ ATA TCC TTC GGC ATC ATC GTG AC

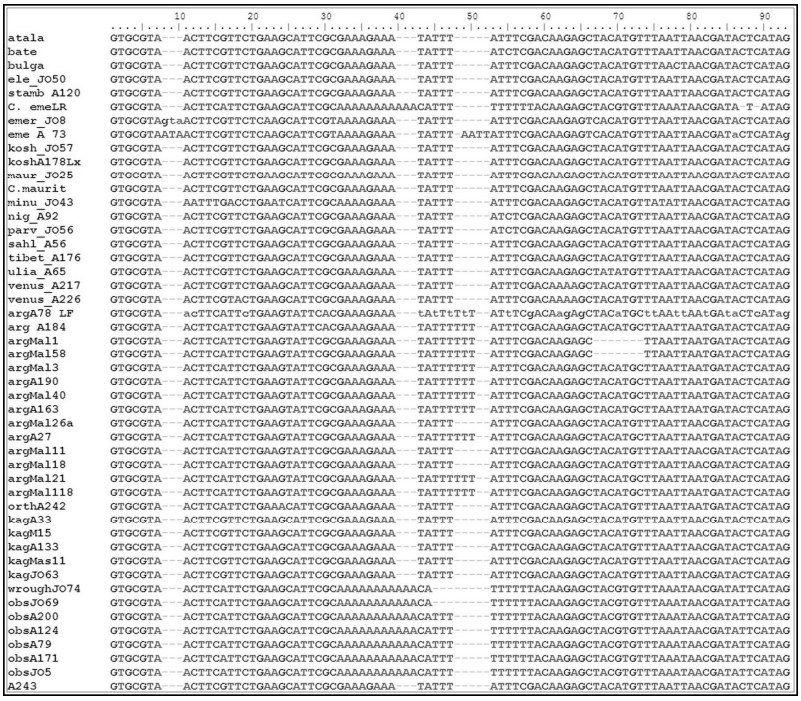
Table 3

Data set	Model	All genes without H3	All genes with H3	Single genes
Ingroup + Outgroup	Each gene separate GTR + Γ +I	LW (without Intron) + COI/COII + WG	LW (without Intron) + COI/COII + WG + H3	LW, COI/COII, WG, H3, tLeu (Intron)
Ingroup + Outgroup	One partition, single GTR + Γ +I	LW (without Intron) + COI/COII + WG	LW (without Intron) + COI/COII + WG + H3	
Ingroup	Each gene separate GTR + Γ +I	LW (with Intron) + COI/COII + WG	LW (with Intron) + COI/COII + WG + H3	
Ingroup (without *C. emeryi*) + Outgroup	Each gene separate GTR + Γ +I	LW (without Intron) + COI/COII + WG	LW (without Intron) + COI/COII + WG + H3	

Table 4

Gene fragment	Partition	Charset	Model	Likelihood settings
LWRhod	1	Gene 1 codon1	F81+G	Lset nst=1 rates=gamma
	2	Gene 1 codon 2	GTR+I	Lset nst=6 rates=propinv;
	3	Gene 1 codon 3	HKY	Lset nst=2 rates=equal
	4	Gene 2	HKY	Lset nst=2 rates=equal;
	5	Gene 3 codon1	HKY+I+G	Lset nst=2 rates=gamma
	6	Gene 3 codon 2	HKY+I	Lset nst=2 rates=propinv
	7	Gene 3 codon 3	GTR+I	Lset nst=6 rates=propinv
COI / COII	8	Gene 4 codon1	GTR+I+G	Lset nst=6 rates=gamma;
	9	Gene 4 codon 2	GTR+I+G	Lset nst=6 rates=invgamma
	10	Gene 4 codon 3	GTR+G	Lset nst=6 rates=gamma
	11	Gene 5	GTR+G	Lset nst=6 rates=gamma
	12	Gene 6 codon1	GTR+G	Lset nst=6 rates=gamma
	13	Gene 6 codon 2	GTR+I	Lset nst=6 rates=propinv
	14	Gene 6 codon 3	GTR+G	Lset nst=6 rates=gamma
wingless	15	Gene 7 codon1	F81	Lset nst=1 rates=equal
	16	Gene 7 codon 2	GTR+G	Lset nst=6 rates=gamma;
	17	Gene 7 codon 3	GTR+I	Lset nst=6 rates=propinv

Figure 1



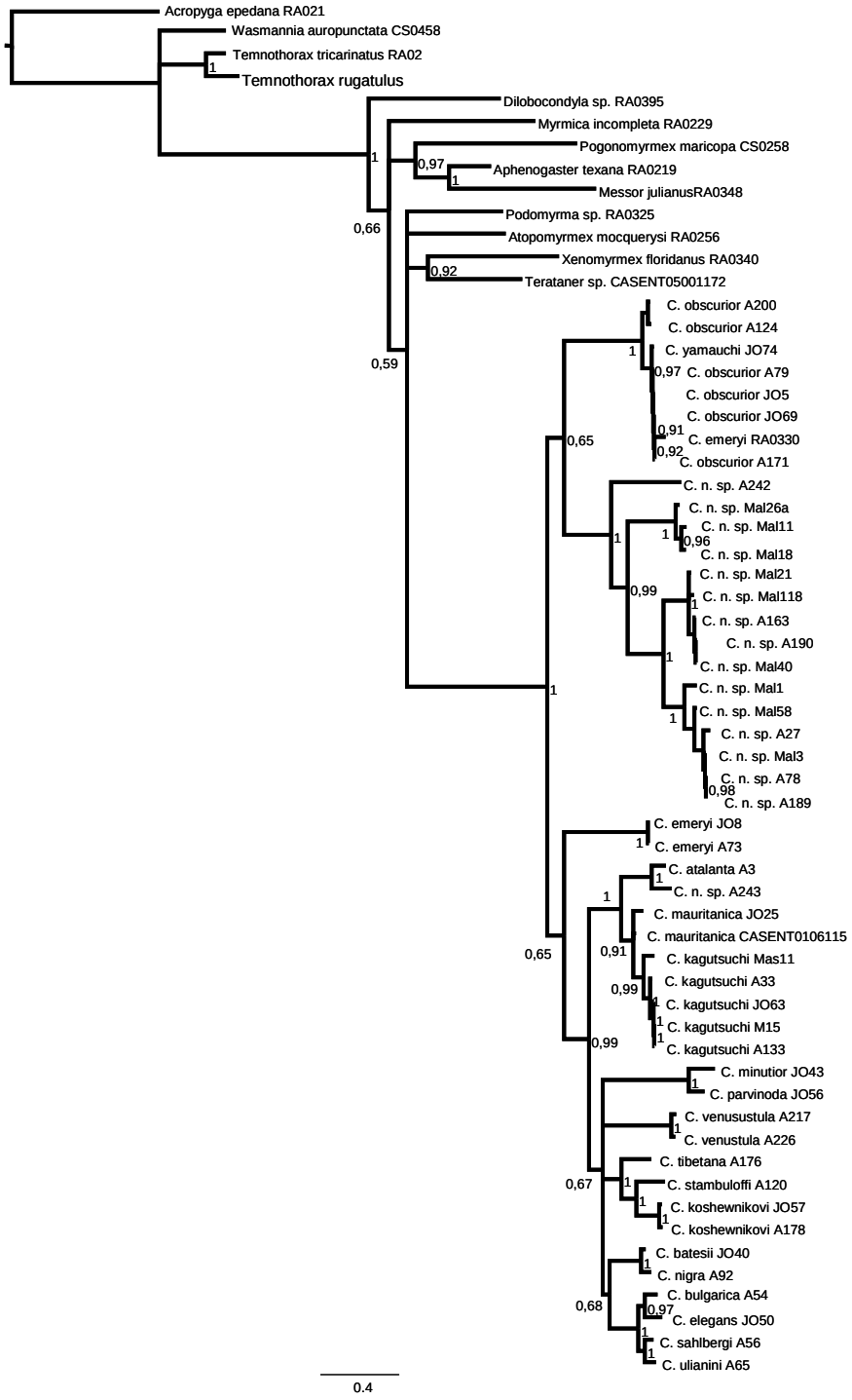

Acropyga epedana RA021
Wasmannia auropunctata CS0458
Temnothorax tricarinatus RA02
Temnothorax rugatulus
Dilobocondyla sp. RA0395
Myrmica incompleta RA0229
Pogonomyrmex maricopa CS0258
Aphenogaster texana RA0219
Messor julianusRA0348
Podomyrma sp. RA0325
Atopomyrmex mocquerysi RA0256
Xenomyrmex floridanus RA0340
Terataner sp. CASENT05001172
C. obscurior A200
C. obscurior A124
C. yamauchi JO74
C. obscurior A79
C. obscurior JO5
C. obscurior JO69
C. emeryi RA0330
C. obscurior A171
C. n. sp. A242
C. n. sp. Mal26a
C. n. sp. Mal11
C. n. sp. Mal18
C. n. sp. Mal21
C. n. sp. Mal118
C. n. sp. A163
C. n. sp. A190
C. n. sp. Mal40
C. n. sp. Mal1
C. n. sp. Mal58
C. n. sp. A27
C. n. sp. Mal3
C. n. sp. A78
C. n. sp. A189
C. emeryi JO8
C. emeryi A73
C. atalanta A3
C. n. sp. A243
C. mauritanica JO25
C. mauritanica CASENT0106115
C. kagutsuchi Mas11
C. kagutsuchi A33
C. kagutsuchi JO63
C. kagutsuchi M15
C. kagutsuchi A133
C. minutior JO43
C. parvinoda JO56
C. venusustula A217
C. venustula A226
C. tibetana A176
C. stambuloffi A120
C. koshewnikovi JO57
C. koshewnikovi A178
C. batesii JO40
C. nigra A92
C. bulgarica A54
C. elegans JO50
C. sahlbergi A56
C. ulianini A65
0.4

Figure2

Chapter 9

Sexual cooperation: Genomic response to sex in *Cardiocondyla obscurior*

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Note:

This chapter describes the current status of a main project of my thesis. Gene expression analysis using DNA microarrays have become invaluable in biological and especially clinical research for the simultaneous screening of large numbers of genes. Today, the quantitative reliability of commercially available oligo-based microarrays is superb. However, methodological uncertainties associated with custom-made spotted microarrays for non-model organisms (such as ants), especially from cross-hybridization of sequences with high similarity, requires that we confirm some of the major findings of this study by using an alternative technique, e.g. quantitative PCR on representative candidate genes in the near future. Moreover the complex experimental design requires the application of rather new statistical methods, which I am still learning. Nevertheless, this study reveals a fairly deep insight into the physiological polyphenic genomic expression of a single genotype, however I can only present my initial analyses.

Introduction

The ultimate goal of every biological entity is the successful vertical transfer of copies of the individual genome, combining the two essential traits that are subject to selection: Reproduction and survival. As a common rule, reproduction from the female's perspective is accompanied by a trade off between fecundity and costs to the reproductive such as increased rate of senescence. In breeding species males and females may differ in their reproductive interests. Naturally, the puzzling occurrence of two sexes contributing to one single object to selection (the species) and the interaction of the sexes have attracted broad interest (cf Arnqvist & Rowe 2005 and references therein). It has been shown that mating partners may exploit each other for selfish interests, resulting in a sexual arms race. In organisms with two sexes, the act of sex is the tempo-spatial point where the two sexes obligatorily come together and have to interact. For example, a male may enhance his own fitness by increasing the females short-term reproductive output at the cost of her survival in later life. Additionally, male-male competition might also physically harm the female and decrease total fitness. Logically, egoistic gender interests (which are shaped by sexual selection) would conflict with species survival (natural selection) thus the ESS should evolve away from extreme forms of mating exploitation that is costly for one sex from the population point of view.

Instead of one-sided exploitation, one sex (in most cases the female) might benefit from the mating partner who eventually bears the costs of this action, a situation described as sexual cooperation (Schrempf *et al.* 2005). In this section we focus on the interaction of male and female ants that persists for a long time after one partner (the male) has died. The striking evolutionary novelty of perennial eusocial insect (bees and ants) reproduction is the contribution of the short-lived males, which remain virtually present throughout the queen's life as sperm stored in her spermatheca, resulting in a de facto obligate lifelong pair-bond. Queens may lay thousands of eggs that in most studied species are fertilized by a single male, thus representing the most extreme form of obligate lifelong partner-commitment throughout the sexually active animal kingdom. One might speculate that this interaction contributed to the ecological success of ants.

The ecological success of ants relies on (1) dominance by the worker force, which (2) enable the colony to produce vast numbers of sexuals. Mated females (queens) undergo dramatic behavioural and physiological changes in response to sex. In general, ant queens that have left the safe environment of the mother nest (before or after mating) will eventually shed their wings, find a suitable nest site and start to transform the voluminous wing muscles into available energy. This energy is allocated into a first batch of brood that eventually develops and grows into a large 'somatic' body. After successful colony establishment the queen in most species will again remain in the safe environment of the colony, hidden from the outside world, and will restrict her actions to laying large numbers of eggs. It may take a few years before an ant colony grows to maturity and starts to produce new sexuals (males and females). Hence, in contrast to perennial solitary animals, males are predicted not to push the short-term fecundity of queens, in acceptance of fitness costs to the female, but to enhance the lifespan of the queen to benefit from lifelong reproduction.

Queens in ants (but also bees and termites) are famous for their long lifespan. In sharp contrast to other invertebrates, ant queens show an opposite pattern in the trade-off between senescence and reproduction: Reproductive individuals live significantly longer than non-reproductives (Keller & Genoud 1997, Heinze & Schrempf 2008) even when corrected for morphological differences and mating status (Tsuji *et al.* 1996). Because queens and workers share the same genotype, this pattern has attracted the interest of aging (sensu Baudisch 2008) research.

The two currently most studied pathways influencing queen longevity and therefore lifetime fecundity are: 1) resistance to oxidative damage and 2) the conserved insulin/insulin-like (I/Ils) signalling pathway. Oxidative damage theoretically should increase with lifespan as free radicals accumulate over time as a result of mainly mitochondrial metabolism. This hypothesis has been tested in *Lasius niger* by Parker *et al.* (2004) who showed that in contrast to the hypothesis a candidate antioxidant enzyme (superoxide dismutase) is expressed higher in workers than in

queens. However, one potential criticism is that their analysis did not take body size (as a proxy for metabolic rate) into account, just overall relative expression values obtained by northern blot. The conserved I/Ils signalling pathway appears to be related to aging in yeast, *C. elegans*, *D. melanogaster* and possibly mice (cf. Hughes & Reynolds 2005). In general, the I/Ils pathway is involved in regulating a variety of physiological processes. Weak mutations can enhance oxidative stress resistance and increase lifespan in *C. elegans* (Guarente & Kenyon 2000). Likewise a heritable mutation (in the gene *insulin-like receptor*) dramatically increases lifespan in *D. melanogaster*. Note, that the I/Ils pathway is currently just one candidate involved with aging: there are many more conserved genes/pathways that potentially play an ancestral role across taxa that need verification, i.e. *methusala* and *indy* from *D. melanogaster*.

Because the I/Ils pathway is upstream of the production of the egg yolk precursor vitellogenin in the social hymenoptera and vitellogenin (expressed lower in workers compared to queens) appears to function as an anti-oxidant (Seehuus *et al.* 2006) the I/Ils pathway is a good candidate in comparative aging research. Studies focusing on the differential expression of genes in workers versus queens (*Lasius niger*, Gräff *et al.* 2007) and reproductive versus non-reproductive queens (*Solenopsis invicta*, Tian *et al.* 2004) found – not surprisingly - differentially expressed levels of vitellogenin-homologues. Vitellogenin production in honeybee queens is negatively correlated with juvenile hormone (JH), hence JH is targeted as being correlated with senescence (Seehuss *et al.* 2006).Keller & Jemielity (2006) state that JH is a prime candidate as it is suggested to regulate age-related plasticity as well as trade-offs between longevity and fecundity in *Drosophila* and other insects. JH has also been shown to correlated with behaviour and immunity. A JH-mediated shutdown of Vitellogenine affects the immune system in honeybees and switching to foraging is associated with an increase in JH and in turn a cessation of vitellogenine synthesis.

In a previous study Schrempf *et al.* (2005) demonstrated that it is not only fecundity per se, that enables *Cardiocondyla obscurior* ant queens to delay death. Schrempf *et al.* dissected the role of egg-laying rate and longevity by subjecting queens to three different treatments: 1) Queens were allowed to mate with a fertile male, 2) queens were mated with sterilized males and 3) queens remained virgin. From here on we will refer to these treatments as mated, dummy mated and virgin, respectively. This treatment resulted in fully functional mated queens with high egg production and low-fecundity dummy-mated and virgin queens that produced only few haploid eggs. Schrempf *et al.* (2005) demonstrated that survival in both mated treatments was similar but that virgin queens had a 50% shorter lifespan. Hence, Schrempf et al. concluded that mating itself increases longevity, regardless of the transfer of viable sperm. In a follow-up study Schrempf and Heinze (2008) recently tested whether the two male morphs that occur in *C. obscurior* (see below: wingless and winged) have different effects on queen lifespan in response to sex. Queens mated with winged males lived significantly longer. Although correlative, they found that winged males have larger accessory glands than wingless males, thus a larger quantity of transferred sex peptides might enhance the queens lifespan.

One proximate factor by which the queen's lifespan might be prolonged is a potential physiological reaction associated with the transfer of substances from the male. Sex peptides (SP) that bind to sperm tails (Peng *et al.* 2005) have been shown to increase fecundity in *Drosophila* and decrease receptivity (Chapman *et al.* 2003). Wigby and Chapman (2005) instead found that *Drosophila* females which were continuously exposed to SP-deficit males had higher fitness as measured by female survival, even though they mate more often than queens mated with wild type males, thus displaying a SP-induced postmating decrease of receptivity.

In this study we aimed to detect the proximate causes for the observed aging patterns. Following a similar experimental setup as Schrempf *et al.* (2005), we profiled the gene expression of queens at two time points using a microarray (Wang *et al.* 2007) developed for the closely related fire ant *Solenopsis invicta* (Brady *et al.* 2006). Specifically, we tested for the immediate short-term response to sex and how sex affected the expression patterns at a mid-age. This study has been preceded by studies on sex in *Drosophila* and, recently, on the honey bee (Kocher *et al.* 2008) which allows us to interpret our results within the context of a broader taxonomic framework.

Materials and Methods

The study organism

Cardiocondyla obscurior is an ideal organism for the study of the underlying genomic mechanisms of various aspects of ant biology such as social behaviour, mating and aging. *C. obscurior* has a short generation time (6-8 weeks), produces sexuals year-round and – being opportunistic and undemanding – can be fairly easily reared under laboratory conditions. *C. obscurior* is a minute ant (queen size ~2mm) and common tramp species (Heinze *et al.* 2006) which in nature nests in high densities in small preformed cavities in decaying plant material. Colonies consists of single or multiple queens, 10-50 workers and, if present, one wingless male. Usually males in ants are winged dispersers which die soon after mating. In *C. obscurior* a second, long-lived, wingless male morph has evolved that replaces the dispersing winged male morph under optimal conditions (Heinze & Delabie 2005, cf. this thesis). These ergatoid (worker-like) males with life-long spermatogenesis remain in the nest and mate with their sisters. They engage in lethal fights with other males over the privilege of monopolizing the virgin queens (Kinomura & Yamauchi 1987). Once mated, queens shed their wings and stay in the natal nest or leave, accompanied by workers, to found a new nest (Heinze & Delabie 2005). Mated queens on average live 26 weeks but may reach a lifespan of up to one year in extreme cases (Schrempf *et al.* 2005).

The Solenopsis invicta *microarray*

The microarrays were made from 22,560 independent cDNAs generated from a fire ant expressed sequence tag project and are estimated to represent 11,864 different genes (Wang *et al.* 2007). Only the 18,438 spots yielding a single PCR product (representing 9,722 putative genes) were considered in the analyses.

Experimental design and sampling

General design

We sampled one and eight week old queens that have been subject to one of three mating status treatments. Queens have either remained virgin (‘Virgin’), or have been mated with a fertile (‘Mated’) or sterilized male (‘Dummy mated’). Although we aimed to detect the long-term effects of sex, experimental constraints (i.e. the average mortality of queens) limited us to rear mid-aged queens with a predictable survival probability. We assume that any detected effect on mid-aged queens will most likely continue to affect the organism at an older age as well.

Experimental setup

We performed 7 and 9 biological replicates for each treatment (mated, dummy mated and virgin) and time point (one and eight week old queens). To reduce inter-individual variation within replicate, we pooled two queens of each treatment at each time point. Thus, in total, we sampled 54 one-week-old and 42 eight-week-old queens resulting in a total of 96 experimental colonies.

Each colony was provided with 20 adult workers, 10 worker pupae, brood (8 larvae of each of the three stages, cf. Schrempf & Heinze 2006) and a single not-yet sclerotized queen pupae. Colonies were housed in petri-dishes (∅ = 10cm) with plaster-flooring in which small cavities have been carved to serve as nest sites. By adding male pupae that would eclose a few days prior to the queen in the two mating treatments we ensured that males were mature and functionally capable of mating when the queen reached sexual maturity. For the treatment with sterilized males we collected males one to two days after eclosion and exposed them, individually placed in 100ul cups, to x-ray (minimum 60 Gy; 0.12 Gy/min; ca 60min), a dose that induces sterility in similar-sized braconidae wasps (Grosch and Sullivan 1954). After irradiation we immediately returned the males

to their colony. The treatment resulted in increased mortality, enhanced cuticular pigmentation, initial disorientation, spontaneous spastic jerking of body parts (JO observations) and sperm degradation (Schrempf *et al.* 2005) compared to non-treated males. Nevertheless, surviving males after 24h always showed typical male activity inside the nest and observations of attempted matings confirm the sexual functionality of treated males. Colonies in which males (of both mated and dummy mated treatment) did not survive for more than 10 days were excluded. Colonies with males in which the queens did not shed their wings after three weeks in response to sexual activity, indicating either infertile queens or mating with non-functional males, were also excluded.

Colonies were fed three times a week with chopped cockroaches, *Drosophila* and diluted honey. We observed each colony three times a week for new male and queen pupae which were discarded. Brood and worker pupae were provided from stock donor colonies when necessary and both were always accepted by the workers. Fecundity was confirmed by noting the presence of newly laid eggs. Queens were collected individually in 1.5ml microcentrifuge tubes, snap-frozen in liquid N_2 and stored at -80°C.

Experimental design

To assess differences and similarities across samples for each time point we conducted a one-way loop design and inter-connected the loops by a comparison of mated young and mid-aged queens (Fig 1). To bridge across samples (i.e. to compare all possible combinations of mated, dummy mated and virgin 1 week old and 8 week old samples) we connected the 1 week and 8 week loops by hybridizing probe generated from mated 1 week old queens with mated 8 week old queens. This comparison was performed using a dye-swap design which also serves as a control for our unidirectional Cy3 / Cy5 loop design. It has been argued that the fluorescent dye binds to the probes in different quantity, thus positive differences might be caused by a dye bias. As we only had low amounts of aRNA available we decided against using a full factorial dye swap design, but performed a dye-swap for a subset of the samples to determine if such an effect could potentially bias the experiment globally.

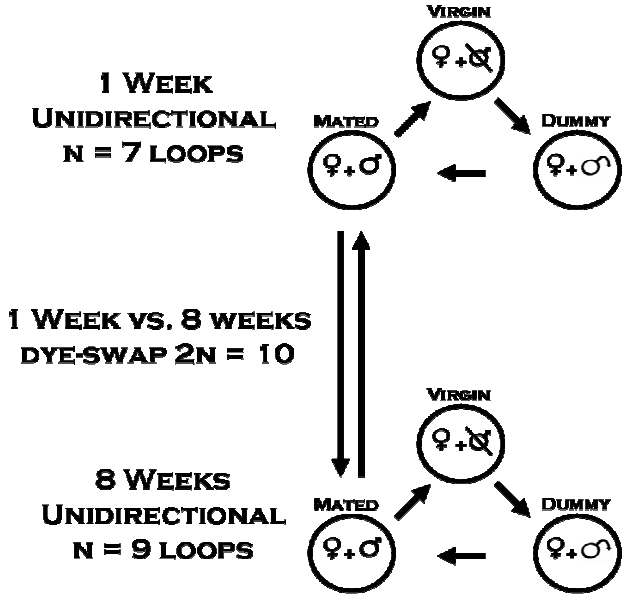


Figure 1 Experimental design

mRNA extraction and cDNA amplification

We extracted total RNA from whole queens with the Trizol Reagent (Invitrogen, Carlsbad, CA), following the manufacturers protocol. Tissue was homogenized with ceramic beads (Quackenbush) using a Fastprep bead shaker. Extraction of two queens resulted in a total RNA yield of 25 – 40µg after DNAse treatment (Ambion DNA-free, Austin, TX) and subsequent Microcon purification (Millipore, Billerica, MA). Extracted RNA was dried at 50°C in a vacuum centrifuge and re-dissolved in 12µl H_2O. 10µl of total RNA was used for amplification (MessageAmp II aRNA Amplification Kit, Ambion, Austin, TX) yielding 110-1625µg of amplified RNA (aRNA) for each sample.

Labeling and hybridization

Indirect labelling of aRNA of each sample was achieved by incorporating aminoallyl-dUTP and subsequent coupling to Cy3 / Cy5 fluorescent esters. Briefly, 9µg aRNA was mixed with random 9mers (2 µg/µl), 1 µl of Alien mRNA Spike mix (Stratagene), and water for a final volume of 36µl. This RNA/primer mix was incubated at 70°C for 10min, then chilled on ice for 5min. Reverse transcription was performed for 2hrs at 50°C after adding 12µl 5x first-strand buffer, 6 µl 0.1 M DTT, 1.2 µl 50x aa-dNTP mix (25 mM dATP, 25 mM dCTP, 25 mM dGTP, 15 mM dTTP, 10 mM aminoallyl-dUTP), 1 µl RNAse inhibitor (15 U/µl, Invitrogene) and 4 µl SuperScript II reverse transcriptase (200 U/µl). The RNA was then hydrolyzed with 30 µl of 0.1 M NaOH for 10min at 70°C and then neutralized with 30 µl 0.1 M HCl. The resulting labelled cDNA was finally purified with a Qiaquick PCR Purification Kit (QIAGEN) and dried in a speed vacuum centrifuge.

After adding 10 µl 0.1 M Na_2CO_3 buffer (pH 9), each cDNA probe was split in two samples and coupled with 4.5 µl Cy3 or Cy5, respectively. According to the experimental design two probes were then combined and unincorporated Cy-dye esters removed (Qiaquick PCR Purification kit, Qiagen) and eluted in 32 µl elution buffer. After adding 7.5 µl 20x SSC, 1.5 µl yeast tRNA (2 µg/µl), 1.5 µl polyA DNA (2µg/µl, Sigma), 0.9 µl 10% SDS and 8.5 µl elution buffer the mix was denatured for 1min at 95°C.

After cooling the final probe mixture to room temperature, 45ul was loaded onto the microarray. The arrays were placed in a hybridization chamber and the samples were then competitively hybridized for 20h at 64°C. Hybridization and all subsequent post-hybridization manipulations were performed in an ozone-free environment. After hybridization the slides were washed in six consecutive steps (2x5 min in 2x SSC, 0.1% SDS; 2x1 min in 0.2xSSC,; 1x1 min in 0.1x SSC; 1x5 min in 0.1x SSC, 0.1% Triton) at room temperature, dried by centrifugation (2min, 800rpm) and scanned with an Agilent DNA Microarray Scanner (Agilent Technologies, Santa Clara, CA).

Spot intensity reading and quality control

We quantified in the spot intensities with GenePix Pro 6.0 using the default settings with manual correction and/or flagging of ambiguous spots. False spot reads can result from mechanical damage, high background noise, and other aberrations. The background-subtracted median foreground values were used in the subsequent analysis. We assessed the quality of the arrays with RACE (Remote Analysis Computation for gene Expression data, Psarros *et al.* 2005). RACE is a program written using the statistical language R and outputs extensive quality checks and provides data visualization tools to assay array quality (Psarros *et al.* 2005). Arrays with higher than average spatial heterogeneous intensities due to technical problems (mechanical damage, air bubbles between array and cover slide, dryness) were considered as bad and subsequently we repeated the hybridization step.

General outline of the statistical analysis

635/532 nm wavelength ratios were pre-processed using the limma package from the Bioconductor (Gentleman *et al.* 2004) package in R (Ihaka & Gentleman 1996). Normalization of the spot data within arrays was computed with the print-tip loess function and the normalization was then scaled between arrays in order to reduce possible array effects.

To assess differences across treatments the resulting corrected spot intensities were subsequently subjected to a Bayesian analysis (BAGEL, Meiklejohn & Townsend 2005) and a ANOVA using limma. Statistical analysis of microarray data always has to correct for two types of errors: First, spot effects, caused by correlations between signal intensities of the two dyes on one spot and signal intensity variation from spot to spot within a dye. And second spot saturation effects, which is when the entire available probe has bound to the target on the spot. Several studies that compared inferences of BAGEL with classical linear models (ie ANOVA) and combinations of linear models with Bayesian inference (ie limma) showed the reliability of the analysis in regard to spot effects and spot saturation, hence methodological-derived false identification of negatives and

positives is unlikely (Meiklejohn & Townsend 2005). The results obtained after correcting for false positives and negatives at a significance threshold cut-off at $p < 0.001$ for both BAGEL and limma, respectively, were compared.

To generate more detailed contrasts we analyzed the data using limma. For the purpose of this chapter we focused on addressing one question: One of the main hypotheses of senescence predicts that oxidative stressors accumulate over time. Hence we tested whether a bias in metabolism related genes are overrepresented in young queens. Mating and the subsequent physiological response itself would reduce this bias. Thus we assessed the differential expression associated with physiological changes over time, by analyzing 'mated' 1 week and 'mated' 8 week old queens.

The limma output data was subjected to a cluster analysis (TIGR MultiExperimentViewer, available at http://www.tm4.org/mev.html) to assess similarities across treatments.

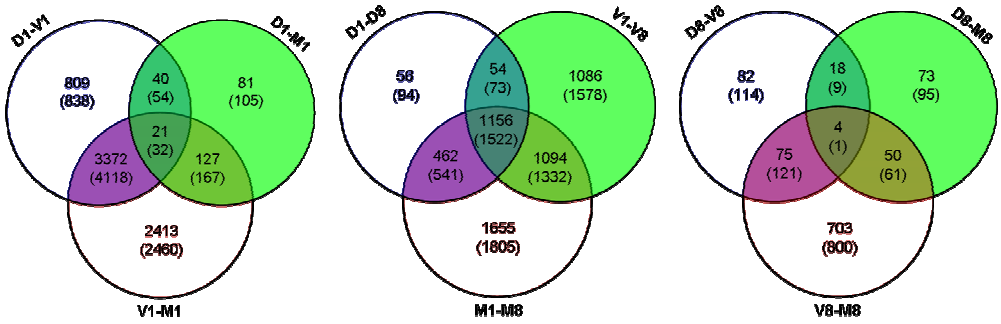
To assess difference across samples we computed a discriminant analysis on the top 1000 genes across all samples (Statistica 6.0).

Results and discussion

Validity of the analysis

We analyzed intensity data obtained from 58 hybridizations. These data were subjected to analyses using two different methods, a linear model-based procedure (limma) and a bayesian estimate (BAGEL).

The results obtained from limma overlap with those from BAGEL (Fig. 2). Limma appears to generate a more conservative estimate but also identifies significant differently expressed ESTs other than BAGEL. For example, comparing the results for M1-M8 (Fig. 2b) of 4,367 ESTs detected by limma, 193 were not identified by BAGEL (of a total of 5,200 ESTs).



Figures 2a,b,c. Clones that were differentially expressed between hybridizations. Overlapping regions depict ESTs that are shared between treatments. Given are results from limma and BAGEL (in parenthesis) after FDR correction at $p < 0.001$.

A discriminant analysis of the first 1000 most differentially expressed ESTs reliable separated the samples according to treatment. We first extracted three principal components (PC) that together explained 73.5 % of the total variance (PC1: 58.3 %, PC2: 10.7 %, PC3: 4.5 %). These were subjected to a stepwise discriminant analysis that showed significant differences across a priori assigned grouping (Wilks' Lambda: 0.008, F (21,138) = 28.40965, $p < 0.000$). The only arrays that the DA could not reliably distinguish were M1-D1 from V8–D8 ($p = 0.48$) and V8-M8 from M8-D8 ($p = 0.17$). [Note that these results are just an estimate for the final analysis which would consider all data. So far I have not been able to perform a PCA with an appropriate program (i.e. R). Statistic software such as Statistica or SPSS are limited in the numbers of input variables. An alternative visualization of these results - based on all spot intensities - is shown in Figure 4 and indicates that the differences between 1 week-old and 8 week-old samples are more pronounced than indicated by our DA.]

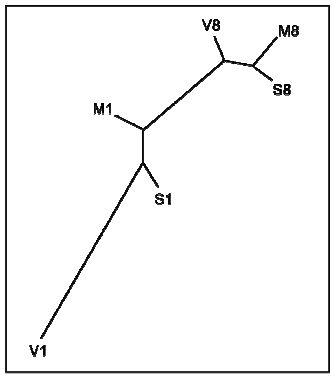


Figure 4: Phylipp neighbor-joining tree, based on distances calculated by BAGEL for 23616 spot intensities.

Within groups the results are less clear (Fig. 5, Tab. 1). The differences are most pronounced in M1-M8, V1-M1, V1-D1 and V8-M8 (see also Fig. 3). This indicates that the differences are caused (1) within the 1 week samples by the immediate response to sex and (2) after 8 weeks by physiological changes associated with the fully functional mated queen.

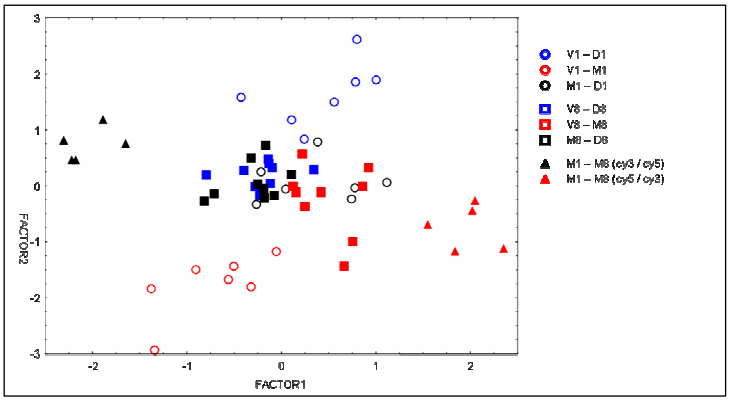


Figure 5. Discriminant function of all samples based on two principle factors extracted from the top 1000 differentially expressed genes. Factor 1 explained 58.3% and Factor 2 10.6% of the variance in the data matrix.

	Percent correct	V1-D1	V1-M1	M1-D1	V8-D8	V8-M8	M8-D8	M1-M8	M8-M1
V1-D1	85.7	6				1			
V1-M1	100		7						
M1-D1	28.6			2	3		2		
V8-D8	55.6			3	5		1		
V8-M8	77.8			1		7	1		
M8-D8	55.6			1		3	5		
M1-M8	100							5	
M8-M1	100								5
Total	72.4	6	7	7	8	11	9	5	5

Table 1: Discriminant analysis of top 1000 differentially expressed ESTs over all samples. Classification of cases to a priori assigned groups. Displayed in grey are the group-pairs that could not be discriminated.

Although we need to resolve the statistical issues, the results clearly show that mating by itself has profound effects on RNA expression patterns of queens. This indicates that the transfer of seminal substances or an alternative unknown response to copulation likely fools the uninseminated queen. As an immediate response to mating (Fig 2a) out of 23,532 ESTs 5,933 and 4242 showed significant differences for V1 versus M1 and V1 versus D1, respectively.

Overall, eight weeks after mating the differences across treatments are much lower. V8 versus D8 and D8 versus M8 differ by only few ESTs (82 and 73 unique ESTs, respectively) whereas the differences between M8 and V8 are still very pronounced (703). Dummy mated queens appear to be intermediate.

While these numbers inform us of the magnitude of the numbers of differentially expressed genes, the functional significance of these gene expression changes requires that we identify the genes behind the ESTs, sort them by function and expression intensity and finally analyze the lists for the shared and unique genes across treatments. For example, it is mysterious that the comparison of V1 and D1 reveals 809 unique ESTs, which are not found to be affected in M1. We expected the same genes to be differentially expressed across treatments assuming that they are related to reproduction. Unique genes that are differentially expressed may indicate an association with behavioral differences of these queens. We observed, but did not quantify, that virgin queens may leave the nest for short exploration trips. They also seem to attract workers less compared to mated queens which appear to receive more care. Like mated queens, dummy mated queens always stay inside the nest but appear to be more active than mated queens.

Gene annotation

Fire ant gene	M1 / M8 expression ratio	Putative gene product of best match	E-value	Putative function
SI.CL.6.cl.669.Contig2	2.65	myofilin protein (zeelin)	1.00E-128	Muscle: A band
SiJWG01BAR.scf	2.16	(ActC2)Actin C2.[Drosophila virilis]	1.00E-130	Muscle
SI.CL.18.cl.1809.Contig1	2.05	Q8WRN4_SOLIN Troponin C.[Solenopsis invicta]	1.00E-79	Muscle: Ca-binding
SI.CL.22.cl.2220.Contig1	2.01	Q3B715_APIME (TpnT)Troponin T isoform 2.[Apis mellifera]	1.00E-20	Muscle: Tropomyosin-binding
SI.CL.24.cl.2406.Contig1	1.99	Tropomyosin 1.[Lonomia obliqua]	1.00E-128	Muscle: Protein
SI.CL.22.cl.2220.Contig1	1.88	Q6T2Y4_DROSU (up)Troponin T-1 (Fragment).[Drosophila s..	1.00E-20	Muscle: Tropomyosin-binding
SI.CL.7.cl.744.Contig1	0.88	Aconitase, mitochondrial	1.00E-123	Mitochondrion: Citric acid cycle
SI.CL.5.cl.562.Contig1	0.86	Lipid storage droplets surface-binding protein 1 [in drosophila for providing lipids to oocyte]	1.00E-139	
SI.CL.12.cl.1209.Contig1	0.74	Isocitrate dehydrogenase	1.00E-135	Mitochondrion: Citric acid cycle
SI.CL.18.cl.1868.Contig1	0.74	Mitochondrial processing peptidase beta	0	Mitochondrion
SI.CL.4.cl.464.Contig2	0.67	(cytb)Cytochrome b.[Formica lugubris]	1.00E-130	Mitochondrion
SI.CL.3.cl.345.Contig1	0.67	(SdhB)Succinate dehydrogenase iron sulfur subunit B.[Lysiphlebus testaceipes]	1.00E-135	Mitochondrion
SiJWH09AAW.scf	0.64	Soluble guanylyl cyclase beta 1 subunit.[Apis mellifera]	1.00E-116	Intracellular, NO receptor.
SI.CL.5.cl.511.Contig1	0.62	Putative ATP synthase beta subunit.[Acyrthosiphon pisum]	1.00E-161	Mitochondrion
SI.CL.17.cl.1730.Contig1	0.61	Peroxiredoxin.[Gryllotalpa orientalis]	1.00E-111	Antoxidant enzyme
SI.CL.18.cl.1871.Contig1	0.58	Enolase	1.00E-109	Glycolysis
SI.CL.29.cl.2902.Contig1	0.58	Putative glyceraldehyde-3-phosphate dehydrogenase.[Oncometopia nigricans]	1.00E-166	Glycolysis
SI.CL.0.cl.030.Contig1	0.54	Mitochondrial porin	1.00E-128	Mitochondrion outer membrane
SI.CL.0.cl.041.Contig1	0.51	(COI)Cytochrome c oxidase subunit I (Fragment).[Myrmica ruginodis]	1.00E-175	Mitochondrion
SI.CL.6.cl.651.Contig1	-2.78	Yellow-g-like protein.[Solenopsis invicta].	0	Development: Royal jelly proteins
SI.CL.3.cl.356.Contig2	-0.89	Vitellogenin-2.[Solenopsis invicta]	0	Egg-yolk precursor
SI.CL.28.cl.2897.Contig1	-0.88	(PAH)Phenylalanine hydroxylase.[Papilio xuthus]	1.00E-142	Degrades excess Phenylalanine
SI.CL.17.cl.1720.Contig1	-0.67	Receptor for activated protein kinase C RACK isoform 1.[Bombyx mori]	0	
SI.CL.10.cl.1069.Contig1	-0.67	(RpS3)Ribosomal protein S3 (Fragment).[Lysiphlebus testaceipes]	1.00E-124	Translation
SI.CL.39.cl.3927.Contig1	-0.55	Nucleoporin NUP85	1.00E-138	Nucleolemma
SI.CL.2.cl.227.Contig1	-0.55	DNA-directed RNA polymerase II	1.00E-111	Transcription
SI.CL.18.cl.1802.Contig1	-0.52	(RpL10/Qm)Ribosomal protein L10/QM-like protein.[Lysiphlebus testaceipes]	1.00E-125	Translation
SI.CL.20.cl.2033.Contig1	-0.39	Eukaryotic translation initiation factor 3	1.00E-122	Translation
SI.CL.13.cl.1359.Contig1	-0.43	Cyclin	1.00E-132	

Table 2. Genes differentially expressed between M1 and M8 that significantly match annotated genes in public databases. Positive M1/M8 expression ratios depict over expression in M1.

At present we are unable to annotate most of the genes that showed significant different expression patterns across treatments. This is due to the computational challenge of finding homologues in the public database. The lack of data is probably primarily due to the evolutionary distance between *Drosophila* and the Formicidae. However, for a first quick analysis we were able to identify the putative orthologues for 153 out of 4,367 significant differentially expressed ESTs between mated one-week-old (M1) and mated eight-week-old (M8) queens. Of these, 69 ESTs were

down regulated in M8 (median fold change = 0.61, 26 ESTs > 1-fold change) and 84 ESTs were up regulated in M1 (median fold change 0.43, 64 ESTs > 1-fold change). Table 2 depicts a subset of genes that were differentially expressed between M1 and M8 by at least 1-fold change. From this we infer that in M1 glycolysis, metabolic citrate-cycle-related genes and other mitochondrial genes are more active. Tian *et al.* (2004) found that cytochrome c oxidase subunit 2 and STARS-like genes showed higher expression in dealate queens, genes that might be involved in flight muscle programmed cell death (Jones *et al.* 1978). We also find cytochrome c oxidase subunit 1 higher expressed in one week-old mated queens. In addition, muscle-related genes are over represented in M1. This could indicate that these genes are still active as a result of flight activity, although all M1-queens had lost their wings prior to sampling.

Genes encoding "yellow-g-like protein" and vitellogenin instead are over represented in M8, probably in association with ovarian activity. "cyclin" is a cell cycle regulator and the development of oocytes is associated with high cell division rate. In addition, some transcription and translation factors are higher expressed suggesting higher protein synthesis. The production of eggs requires the transfer of maternal transcripts into the developing oocytes

Conclusion

This is the first study that assesses genome-wide response to sex and the effects of fertility on ants. But this is just the tip of the iceberg. At the same time we can dissect sex and fertility and study age-related patterns. So far the data are hidden and we will have to finds ways to extract the valuable information, a problem of great concern shared by the community.

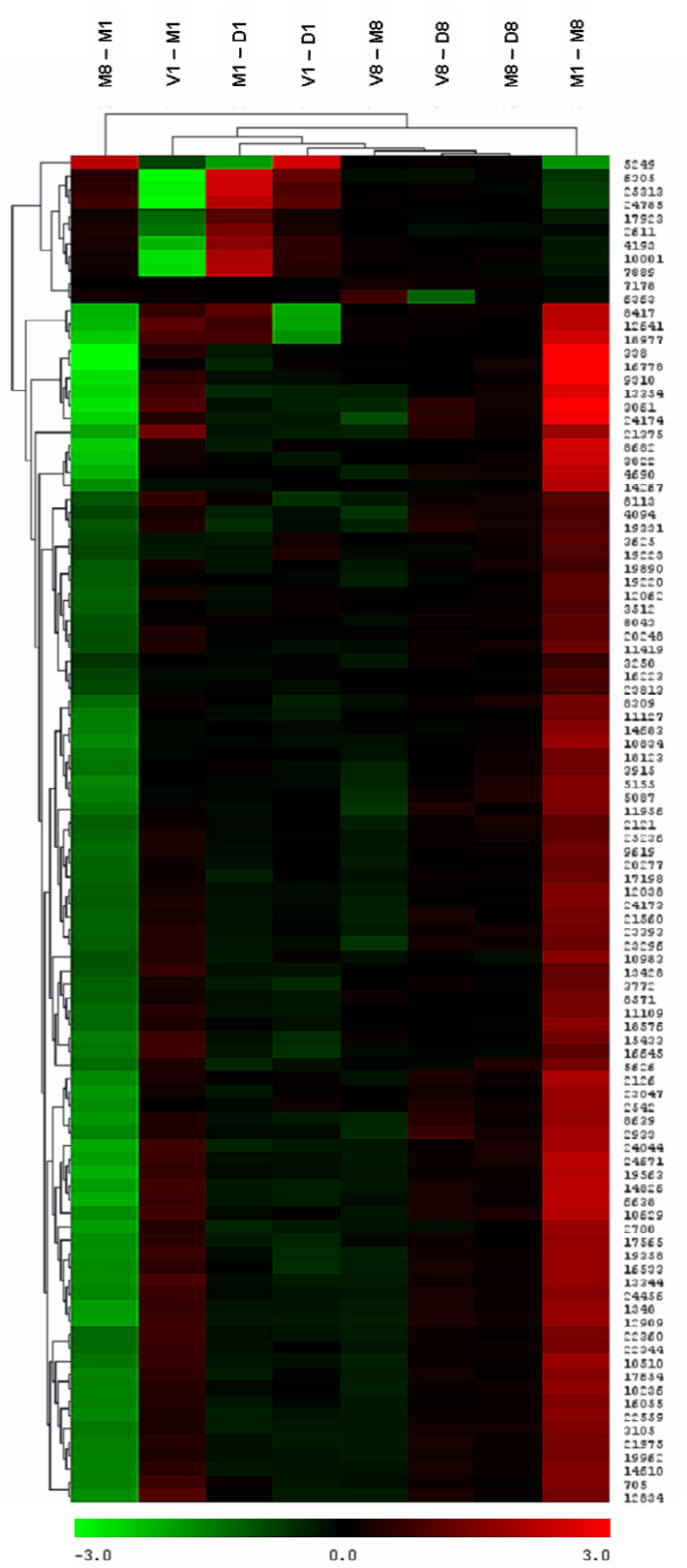
Nevertheless, we can demonstrate the accuracy of the experiment and show first results. Although not surprising the reults confirm some recent findings. Tian *et al.* (2004) studied changes in response to mating of *Solenopsis* queens but their sampling does not allow for a direct comparison with our study. They compared alates from laboratory colonies and mated queens from a mating aggregation in the field, hence both samples had experienced very different environmental conditions. In addition the mated queens were extracted between 1-5 days after they have been brought back to the laboratory and differences might be either caused by changes over time or post-mating (C. Coates pers. com.).

As a first preliminary result we can confirm that metabolic activity is higher in young queens and seems to be down regulated in older queens. To find out what the higher muscle-related gene-activity in M1 versus M8 means we need to compare with V1 which still had wings when we sampled them.

A recent study on the honeybee addressed post-mating changes and revealed similarities to *Drosophila*, showing that conserved pathways are involved in genomic response to sex. Kocher *et al.* (2008) studied multiple mated egg-laying queens, virgin queens and queens that mated once but did not start egg-laying. Their youngest samples were 9 days old queens, five days after mating which compares to our one-week samples. In contrast to our study the intermediate queens they studied had initiated ovary development but had not produced oocytes yet. However, this is a similar condition to *Cardiocondyla* dummy mated queens, which produce haploid eggs, but at the same rate of virgin queens. Kocher *et al.* reduced their data to a 'top 50 predicted genes' resulting from a class prediction function in GeneSpring to correlate expression with behavior and physiology. Whether these genes contain sufficient biological information is questionable. But we should aim to compare their sequences to ours in order to assess conserved similarities within the Apocrita for short term genomic response to sex.

However, our study is unique in that we can assess expressions at a second time-point. In the future we aim to annotate those genes that are specifically unique for dummy mated and mated queens and which may be involved in the prolongation of their lifespan.

Figure 3. MeV clusteranalysis of the top 100 (limma) differentially expressed ESTs across all samples. For the clusteranalysis we used the median coefficient for a given group. Depicted on the left and right column are the dye swap hybridizations for M1-M8. Depicted are normalized (log) ratios for Cy3 / Cy5.



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Summary

This thesis is concerned with phenotypic plasticity in ants. It presents an evolutionary series of studies that focus on the expression of plastic traits. The most powerful and probably the most successful strategy to alter the fitness of a species by means of adaptation without mutational changes is the flexibility of the genotype to be expressed in different phenotypes. Ant societies are by definition a result of plasticity. Without plasticity there would be neither worker caste nor any other derived phenotype. Without plasticity I imagine the world to be more uniform and much less exciting.

This thesis is organized as follows. As my studies are centered on plasticity, Chapter 1 summarizes both, the state of research and the main findings of this thesis. Chapter 2 briefly describes the evolution of this thesis, focusing on the constraints and opportunities I faced. The third chapter discusses the methodology. The remaining chapters present the six studies that I conducted during the course of my Ph.D., addressing natural history, sex ratio theory, chemical ecology, DNA based phylogeny and RNA expression patterns.

The species I studied represent four genera of three subfamilies within the Formicidae. *Crematogaster smithi* (Myrmicinae) contains a peculiar morph specialized for the production of haploid eggs. I describe (Chapter 5) a novel association of the allocation of resources and sex ratio in natural populations; i.e. haploid worker-produced eggs lead to a female biased sex ratio in favor of the workers. In Chapter 4 I describe the chemical hydrocarbon signature exhibited by the female morphs.

While collecting *C. smithi* in the Chiricahua Mountains, Arizona, USA during the annual monsoon season we discovered a mating aggregation of *Acropyga epedana* (Formicinae), an ant with a bizarre life history living in obligate mutualistic association with mealybugs. We studied (Chapter 7) the mating flight, behavior of workers and provide demographic data of an excavated colony.

From the botanical garden of Bonn, Germany I collected the tramp ant *Technomyrmex vitiensis* (Dolichoderinae) and we described (Chapter 6) the morphology of the wingless reproductive morph that displays an alternative life history strategy compared to winged queens.

I updated (Chapter eight) the phylogeny of the ant genus *Cardiocondyla* (Myrmicinae) incorporating nuclear markers and more taxa and reviewed the topology in respect to the evolution of the male polyphenism exhibited by this genus. For comparative evolutionary studies on Cardiocondyla, a reliable phylogeny is indispensable.

And finally, I studied (Chapter 9) the genome-wide response to sex of *Cardiocondyla obscurior* queens at two time points, using the fire ant EST library and present first insights on the complex changes associated with mating.

Zusammenfassung

Die vorliegende Arbeit handelt von phenotypischer Plastizität bei Ameisen und besteht aus verschiedenen Studien die sich mit der Ausprägung plastischer Merkmale beschäftigen. Die adaptive Flexibilität des Genoms, das sich in verschiedenen Phenotypen exprimieren kann, ist die wahrscheinlich erfolgreichste Möglichkeit die Fitneß einer Art zu verändern, ohne das es zu Mutationen kommen muß. Ameisen sind definiert als ein Resultat dieser Plastizität; ohne Plastizität gäbe es weder eine Außendienstarbeiterinnenkaste noch sonstige abgeleitete Phenotypen. Ohne Plastizität wäre die Welt wohl sehr viel uniformer und wahrscheinlich um einiges weniger spannend.

Die Arbeit unterteilt sich folgendermaßen: Da sich meine Projekte alle mit Plastizität befassen versuche ich in Kapitel 1 in mehreren Abschnitten in das Thema einzuleiten und präsentiere jeweils meine Ergebnisse. In Kapitel 2 beschreibe ich, wie die Arbeit entstanden ist und Kapitel 3 ist eine Zusammenfassung der Methodik. Die restlichen sechs Kapitel stellen meine Studien der letzten drei Jahre dar, die sich mit Naturgeschichte, Geschlechterverhältnis, chemischer Ökologie, DNS-basierter Phylogenie und RNS Expressionsmustern beschäftigen.

Die Arten, die ich untersucht habe, repräsentieren vier Gattungen dreier Unterfamilien innerhalb der Formicidae. *Crematogaster smithi* (Myrmicinae) zeichnet sich durch besondere Morphe aus, die auf die Produktion von haploiden Eiern spezialisiert sind. Ich beschreibe einen neuen Zusammenhang zwischen der Allokation von Ressourcen und dem Geschlechterverhältnis von Populationen im Feld; d.h. haploide Eier, die von diesen Morphen gelegt werden, führen zu einem Geschlechterverhältnis zu Gunsten der Arbeiterinnen. Im vierten Kapitel beschreibe ich zudem die kutikulären Kohlenwasserstoffsignaturen der weiblichen Morphen.

Zu der Zeit als ich *C. smithi* in den Chiricahua Mountains in den USA gesammelt habe, beobachteten wir ein Lek von sich Paarenden und Umwerbenden Geschlechtstieren von *Acropyga epedana* (Formicidae). Diese Art zeichnet sich durch einen obligaten Mutualismus mit einer Wurzellaus aus. Wir zeichnen in Kapitel 7 ein Bild des Paarungsverhaltens und des Verhaltens der Arbeiterinnen, zudem liefern wir demographische Daten einer freigelegten Kolonie.

Im Botanischen Garten in Bonn habe ich die invasive Ameisenart *Technomyrmex vitiensis* (Dolichoderinae) gesammelt und beschreibe in Kapitel 6 die Morphologie von flügellosen Königinnen die, verglichen mit regulären geflügelten Königinnen der Art, eine alternative Reproduktionsstrategie inne haben.

Mit Hilfe von nuklearen Markern, und unter Einbeziehung zusätzlicher Proben, habe ich die Phylogenie der Gattung *Cardiocondyla* (Myrmicinae) auf den neusten Stand versucht zu bringen (Kapitel 8). Ich diskutiere die Ergebnisse im Hinblick auf die Evolution des Männchen-Polyphaenismus. Da *Cardiocondyla* ein Modelorganismus für vergleichende evolutionäre Fragestellung ist, ist eine solide Phylogenie unabdingbar.

Abschließend beschäftige ich mich im neunten Kapitel mit den Folgen von Sex auf RNS Ebene bei *Cardiocondyla*-Königinnen über die Zeit. Die Arbeit basiert auf den für *Solenopsis invicta* entwickelten ESTs und beschreibt die komplexen Veränderungen welche durch Paarung ausgelöst werden.