Comparative analysis of reproductive tactics in the genus *Cardiocondyla*

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES DER
NATURWISSENSCHAFTEN (DR. RER. NAT.)
DER FAKULTÄT FÜR BIOLOGIE UND VORKLINISCHE MEDIZIN
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vorgelegt von
Sabine Frohschammer

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Chapter 1

Cardiocondyla pirata sp. n.–a new Philippine ant with enigmatic pigmentation pattern (Hymenoptera, Formicidae).

Seifert, B., & Frohschammer, S. (2013**).**

ZooKeys, (301), 13.

Personal contribution:

Discovery of the species; collection of one colony and their maintenance in laboratory.

Chapter 2

Male fighting and "territoriality" within colonies of the ant _Cardiocondyla venustula_.

Frohschammer, S., & Heinze, J. (2009).

Naturwissenschaften, *96*(1), 159-163.

Personal contribution:

Discovery of territoriality and observation of nests; planning of experiments together with JH; conduction of experiments; analysis of data together with JH; writing of the manuscript with correction of JH.

Chapter 3

In-and Outbreeding in _Cardiocondyla kagutsuchi_

Frohschammer, S., Schrempf, A., Bernadou, A.,& Heinze, J. (2014).

Manuscript

Personal contribution:

Planning of experiments together with JH; conduction of experiments; analysis of data together with AS,AB and JH; writing of the manuscript with correction of JH.

Chapter 4

Queen life-span and total reproductive success are positively associated in the ant *Cardiocondyla cf. kagutsuchi.*

Heinze, J., Frohschammer, S., & Bernadou, A. (2013).

Behavioral Ecology and Sociobiology, *67*(10), 1555-1562.

Personal contribution:

Planning of experiments together with JH; conduction of experiments; collection and preparation of data.

Chapter 5

Virgin ant queens mate with their own sons to avoid failure at colony foundation.

Schmidt, C. V., Frohschammer, S., Schrempf, A., & Heinze, J. (2014).

Naturwissenschaften, *101*(1), 69-72.

Personal contribution:

Discovery of mother – son mating; planning of experiments with JH; conduction of experiments with CS.

Chapter 6

A heritable component in sex ratio and caste determination in a *Cardiocondyla* ant.

Frohschammer, S., & Heinze, J. (2009).

Frontiers in Zoology, *6*(1), 27-33.

Personal contribution:

Discovery of hint towards heritability of sex ratio; planning of experiments with JH; conduction of experiments; analysis of data together with JH; writing of the manuscript together with JH.

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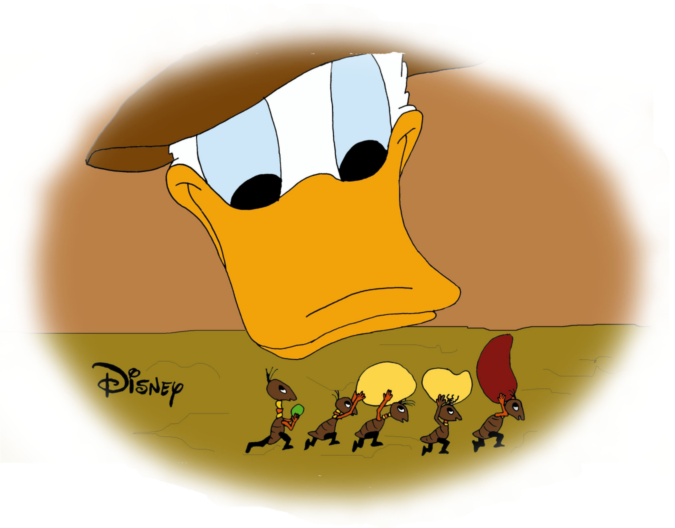
General Introduction



"The difference between fascination and rejection usually lays in the view of the spectator"

Fascination about ants

In Donald Duck's case his fascination for the strength and the skill of a single ant quickly switched into annoyance when he realized that its nestmates take away his food.

"Ants are everywhere". This sentence gets a new meaning when you start a picnic and after a while you are in good company of many of those tiny insects that want to share your food. Even if you are willing you cannot ignore them, they know to call your attention through spraying formic acid or using their small stings, when you squeeze them inside your sleeves.

Myrmecologists in contrast are fascinated by this omnipresence of ants. With exception of Antarctica, ants can be found on all continents and in nearly all terrestrial habitats above the permafrost base and show an enormous variety in shape and in their ways of living.

Ants were not always that successful. The first ants appeared more than 100 m years ago in the early Cretaceous and preyed on other insects. Their radiation started with the appearance of flowering plants and the switch from carnivorous to omnivorous diet (Wilson & Hölldobler 2005). Today, more than 14 000 ant species are described (Bolton et al. 2006) and there remains a tremendous number of still undiscovered ant species worldwide.

What makes ants so successful?

The key to their prosperity is the big number of individuals working together as a unit. One single tiny ant alone is inconspicuous and weak and always runs the risk of being captured. Millions of individuals working together in contrast get noted, shape their environment (e.g. "leafcutter ants" Kleidermann et al. 2001) and can overpower much bigger, stronger animals (e.g. „army ants" see Franks 1986).

Eusociality and conflicts in ant societies

In fact, ants are not only social, they are eusocial (that means "really social") - a term used for a social system where three traits are achieved (Wilson & Hölldobler1990):

first, members of the eusocial group cooperatively care about their brood; second, several generations live together; third, there is a division of labor at least between individuals that reproduce and those caring about the maintenance of the colony. But beneath all the advantages that ant collaboration brings - like in every other society, also in ant societies problems arise (Heinze et al. 1994):

Considering for example the question of who is allowed to reproduce: Hamilton's famous inclusive fitness theory works quite well for explaining worker sterility in monogynous, monandrous colonies (Hamilton 1964). Ants have a haplo-diploid sex determination system where diploid individuals turn into females and haploids turn into males. Consequently sisters are more related to their sisters (r=0.75) than to their daughters (r=0.5) which should let them favor raising sisters instead of producing its own offspring. But even in colonies where one single reproducing queen is inseminated by one male only, conflicts of interest over sex allocation arise. Workers are related to their brothers (r=0.25) three times less than to their sisters (r=0.75) and should approve a three times more female biased sex allocation whereas the queens optimum is an evenly distributed sex allocation as its relationship towards son and daughters is the same (r=0.5). In such monogynous, monandrous colonies the relatedness asymmetry (the relatedness of workers to female offspring divided through their relatedness to male offspring) is very high (Boomsma & Grafen 1991). A high relatedness asymmetry means that workers are more related to sisters, which should result in a higher interest in raising female offspring (Boomsma & Grafen 1991).

The relatedness asymmetry can be reduced by polygyny and polyandry, which results in declining relatedness towards sisters. It can further be reduced by worker reproduction as workers are more related to sons than to brothers. This consequently reduces the conflict between workers and queens over sex allocation. In several, mostly older investigations it is stated that workers prevail in the queen-worker conflict as they are able to manipulate the primary sex ratio (sex ratio of the eggs laid by the queen) by eliminating male larvae (Aron et al. 1994, 1995, Chapuisat et al. 1997). Other observations see the queen as mainly responsible for sex allocation (Menten et al. 2005): she can force workers to rear male brood by laying a huge amount or even exclusively haploid eggs (Passera et al. 2001). Nevertheless workers alter the primary sex ratio in many species so that the effective secondary sex ratio (sex ratio of eclosing pupae) lies just between the queen's and the worker's optimum (Mehdiabadi et al. 2003, Rosset & Chapuisat 2006).

There are also other conflicts in ant colonies that are put towards a certain equilibrium in different ant species (Ratnieks at al. 2006). In all conflicts arising in ant societies individuals try to maximize their inclusive fitness, which means for the reproducing individuals to maximize their reproductive success. This requires different prior conditions in males and queens.

Queens have to find and mate with a fit, appropriate male (see Chapter 3 & 5), find a good nesting place, escape predators during dispersal and succeed in colony founding. Furthermore, a queen can try to optimize sex ratio of its offspring to maximize inclusive fitness (Chapter 6), taking in account given conditions, like population sex ratio or local mate or resource competition.

Finally, a long living and high fertility augments the queen's reproductive success (Chapter 4).

For males, in contrast, access to fertile, healthy queens and the chance to mate with them is the basic condition for numerous offspring (Chapter 2).

The genus *Cardiocondyla*

The story of reproduction in ants is so fascinating with each ant species having its different methods and own idiosyncrasy. Although recent interest and research in ants increases, many things still remain unknown. In my PhD I examined different life history traits concerning reproduction in the ant genus *Cardiocondyla*.

This genus belongs to the Myrmicinae, tribe Formicoxinini, and comprises more than 100 species (Seifert 2003). The genus seems to be originally from Asia from where they spread to Australia Europe and Africa. Due to several tramp species *Cardiocondyla* species are nowadays distributed worldwide. Because of their very small body size, their relatively small colonies, and the individually foraging strategy these ants are often overlooked. Searching intensely for the genus will certainly reveal several new species.

In Chapter 1 I introduce the recently discovered species *Cardiocondyla pirata* from the Philippines.

The genus *Cardiocondyla* is perfect for the maintenance in the lab. The colony sizes are rather small (up to few hundreds of individuals), the lifespan of a queen is relatively short (around one year) and colonies are easy to keep in small plaster nests.

What makes this genus so interesting is the presence of a special wingless male form, the ergatoid male. Ergatoid males in ants are very rare and are also known only in the ant genera *Formicoxenus*, *Hypoponera* and *Technomyrmex* (Buschinger 1979, Hamilton 1979, Yamauchi et al.1996).

Cardiocondyla species with dimorphic male types, normal winged males and ergatoid males are supposed to be more ancestral (Heinze et al. 2005). Wing loss of males, e.g. the presence of only ergatoid males in some species seems to have evolved several times independently (Oettler et al. 2010). Whereas winged males usually mate at maximum with a few queens outside the nest and die shortly after mating, ergatoid males normally stay inside the nest their whole life. Due to a lifelong spermatogenesis they can mate with all virgin females that eclose inside the nest (Heinze & Hölldobler 1993). Ergatoid *Cardiocondyla* males are very long-lived and their lifespan can in some species exceed one year (Yamautchi et al. 2006).

Male mating tactics

Ergatoid males in *Cardiocondyla* display a huge variety of alternative species-specific reproductive tactics. In all known species with exception of a few monogynous, monandrous species where ergatoid males are tolerant against each other, (e.g. *Cardiocondyla batesii,* Schrempf et al. 2005b) ergatoid males are adapted to fight against other ergatoid males to monopolize mating with all virgin females. According to a phylogenetic analysis of Oettler et al. (2010) *Cardiocondyla* species can be grouped in two clades depending on male mandible form (Fig.1).

Males in clade A, equipped with sickle-shaped mandibles, grasp and hold their rivals and besmear them with a secretion from their hindgut. Workers attracted by the smell kill in such way marked males (Kinomura & Yamauchi 1987, Yamauchi & Kawase 1992). Fighting behavior of males in clade B, characterized by sheer-shaped mandibles, is more variable among species. Males use their strong mandibles to actively kill male pupae and also engage in lethal fights with competitors (Yamauchi & Kinomura 1993, Heinze et al. 1993, 1998). But also the monophyletic group of monogynous species with tolerant males belongs to that clade.

In Chapter 2 I introduce a species were the male tactic includes elements from both groups. *Cardiocondyla venustula* males besmear rivals but actively kill eclosing pupae as well and also show a novel tactic so far unknown in ants: males stick to and defend "territories" inside the nest and mate there with freshly eclosing queens.

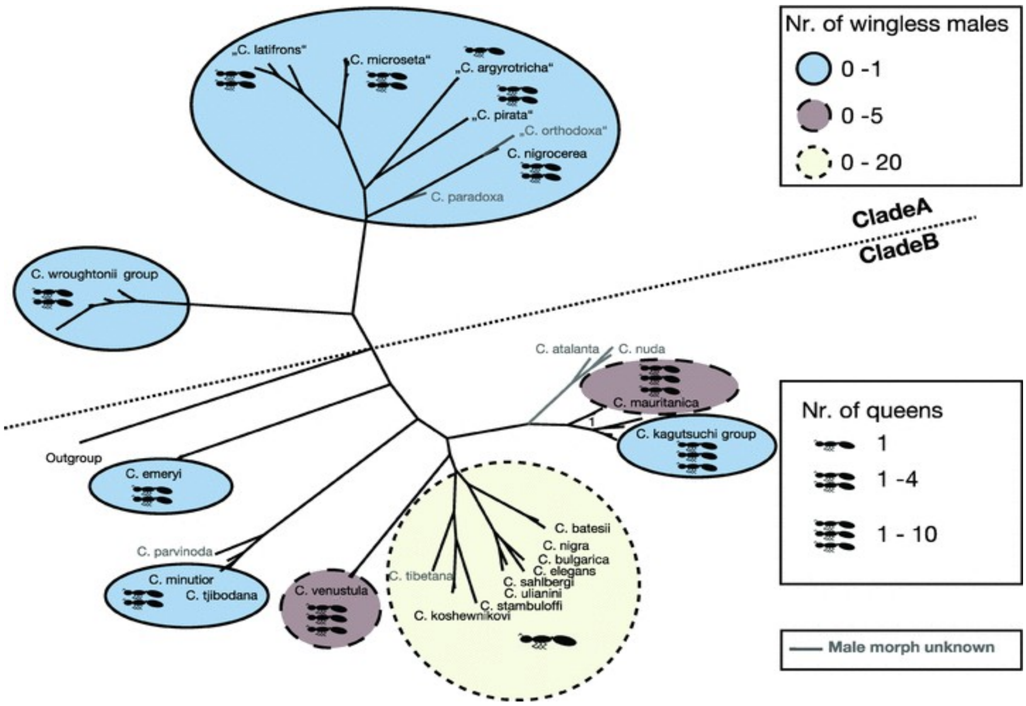


Fig.1: **Phylogenetic tree, showing the relationships among different *Cardiocondyla* species (after Oettler et al. 2010).**

Sex determination and inbreeding

Another specialty in *Cardiocondyla* is that mating takes place inside the nest, leading to a high amount of inbreeding (Schrempf et al. 2005b, 2006). Owing to their haplodiploidy, inbreeding in ants and other Hymenoptera was thought to be especially damaging because it can lead to a high production of diploid males assuming a single locus complementary sex determination (slcsd) system. This system is known for many Hymenoptera (Wilgenburg et al. 2006) and claims that there is only one polymorphic locus responsible for sex determination. Heterozygosity at that locus turns individuals into females whereas males are characterized by a hemizygous sex determination genotype. If - under inbreeding conditions - the queen and her mate are sharing the same allele on the sex locus, 50% of the diploid offspring will be homozygous at this locus and turn into diploid males that constitute a load to the colony because males do not contribute to colony maintenance. Although in the majority of ant species mating takes place in mating swarms where unrelated partners meet, there are several ant species where mating is between highly related individuals. If mating takes place in the maternal nest, inbreeding often cannot be avoided (Yamauchi et al. 1991). Nevertheless ant species have evolved different ways how to cope with that. In *Paratrechina longicornis,* for example, the genome of queens and males always remain separated. Queens are produced clonally and males never inherit maternal alleles (Pearcy at al. 2011).

Inbreeding and outbreeding in *Cardiocondyla*

In *Cardiocondyla,* freshly eclosing queens mate with the available ergatoid male after their cuticle hardened and, depending on species, disperse afterwards (Heinze et al. 2002) or stay inside the nest contributing to egg laying (Kinomura &Yamauchi 1987). Due to mating inside the nest, partners are closely related or even full siblings. Schrempf et al. (2006) could only find some evidence for inbreeding depression after several generations of mating between siblings, showing that sl-csd is absent in *Cardiocondyla obscurior* and probably also in other *Cardiocondyla* species. Considering the absence of sl-csd and the fact that in haplodiploids lethal alleles are eliminated through forced expression in haploid males, inbreeding depression in the genus *Cardiocondyla* seems to be rare.

Certain outbreeding events in this system where inbreeding prevails may be enabled by winged males that mate away from their mother colony or ergatoid males that escaped from their rivals from their natal nest and succeed in mating with virgin females in a neighboring nest. Very few studies about dispersal behavior of males and females in nature exist. Lenoir et al. (2007) could show that ergatoid males of *Cardiocondyla elegans* invade alien colonies and virgin queens were actively taken from neighboring colonies by workers. Furthermore, foreign queens in *Cardiocondyla nigra* were found in the same nest chamber as workers belonging to the colony (Schrempf 2014). In *Cardiocondyla batesii* (Schrempf et al. 2005b) queens fertilize their eggs with sperm from unrelated males which also let assume that males from alien colonies entered the nest. In my own observations with several *Cardiocondyla* species I never found a hostile reaction of any colony member (except of the inhabitant male) when adding an alien ergatoid male of the same species to a nest. Most outbreeding events (excluding winged male dispersal) usually must occur between colonies of close vicinity because dispersal radius of single ants spreading on foot is limited. Considering this and the preference towards sib mating in *Cardiocondyla*, a great genetic difference is expected (proofed for different populations of *C.obscurior* pers. comm. Schrempf) between separated populations which consequently let assume a high adaption towards local conditions.

In Chapter 3, I compared life history traits and colony parameters of inbreeding versus outbreeding colonies in the species *Cardiocondyla* cf. *kagutsuchi*. In my results I actually discovered more failed colony foundings in outbred colonies probably due to an inferior fit of genomes of the mating partners caused by genetic differences between the populations. Mating among siblings led to a slightly higher queen lifespan in inbred versus outbred colonies. In *Cardiocondyla obscurior* mating itself is suggested to increase longevity of the queens by the conception of seminal fluid proteins from the male (Schrempf et al. 2005a). A long living mate is of great interest for every male as a queen stores the sperm received from mating all life long for fertilization of her eggs. Better coevolution between mates can be assured through mating with close relatives, leading to better adapted seminal fluid proteins that can prolong the queen's lifespan even more.

In Chapter 4 I could proof that long living queens also produce more offspring. Comparing lifespan and reproductive success in inbred queens of *Cardiocondyla* cf. *kagutsuchi* shows that more fecund queens lived longer than less fecund queens. This supports the assumption that there is no trade-off between lifespan and reproductive success in social insects in contrast to solitary insects and other animals, even humans (Meeûs, 2000).

Risk of mating inside the nest

Despite the benefits of mating inside the nest, the already discussed coevolution of males and females, the security of a sheltered environment and the convenience of finding an appropriate mating partner can turn into handicap if there is no partner available.

Queens and workers can alter the colony sex ratio towards the need of the colony by trying to maximize their inclusive fitness. In *Cardiocondyla* species with fighting males the theory of local mate competition predicts that males are usually produced in very low numbers, just to ensure fertilization of all virgin females (Cremer at al. 2002). Especially in species where males are very long-lived and therefore only sporadically produced, virgin females pay the big price of not being inseminated if the colony male dies. Usually ant queens are supposed to have a narrow time window for successful mating (Wilson 1971, Thornhill & Alcock 1983). As queens usually are not accepted if they immigrate to alien nests they are doomed to stay uninseminated and die finally. Interestingly *Cardiocondyla "argyrotricha"* has evolved a so far unknown strategy in ants that I present in Chapter 5. Queens of this species are able to raise a new colony alone, only accompanied by some workers, by laying unfertilized eggs and mating with the first male that ecloses. After insemination queens are able to produce diploid eggs and manage to establish a flourishing colony.

Offspring sex ratio in *Cardiocondyla*

As mentioned above in *Cardiocondyla* colonies sex ratio is following local mate competition theory rather than theory about relatedness asymmetry (discussed in the beginning). The adaption of sex ratio towards the maximum inclusive fitness of the individual that is able to manipulate it is a powerful instrument inside an ant colony. As discussed before, queens and workers usually have a delayed individual optimum and try to shift sex ratio towards their own privilege through providing a fixed primary sex ratio (queen) or its manipulation, which results in a different secondary sex ratio. A shift in sex ratio cannot only be achieved by the alteration of diploid versus haploid eggs, but also by a shift in female caste determination - that means the percentage of diploid larvae that is growing into a queen or a worker. This was long thought to be fully controlled by environmental cues like temperature, overwintering status of the queen or diet (Crozier & Pamilo 1996). As diet is usually provided by workers, this is a toehold where workers may be able to bias caste determination towards their optimum. More recent studies reveal that there are environmental and genetic triggers that control caste determination, which seems to be very species specific (Schwander et al. 2010).

We took advantage of the already mentioned manageable size of *Cardiocondyla* colonies and the relatively short lifespan of the queen to get a closer look on the lifetime sex ratio in colonies of *Cardiocondyla* cf. *kagutsuchi*. In Chapter 6 I present the results of experiments about genetic versus environmental caste determination in *Cardiocondyla* cf. *kagutsuchi*. I established a series of experiments after observing that several young queens stemming from colonies with a highly queen biased sex ratio produced also mainly queens in their own colonies. A series of queens from queen-biased colonies mated with the same male than a series of queens from far less queen biased colonies. After mating each queen was placed in a new nest with workers from a third colony, unrelated to the colonies the queen and male were taken from. It turned out that the proportion of diploid to haploid offspring did not differ in the two treatments of our experimental colonies. However, the fate of the diploid individuals to develop into queens or workers differed between the two series. In colonies where the mother queen originated from a queen-biased colony a significantly higher proportion of diploid larvae turned into queens and vice versa- queen mother's from less queen biased colonies produced a lesser extent of daughter queens. This suggests that caste determination in *Cardiocondyla* cf. *kagutsuchi* has a strong genetic basis.

Summary: Aims of this thesis

Description of a new species:	Chapter 1
Reproductive strategies:	Chapter 2 &5
Life history traits of colonies:	Chapter 3, 4 & 6

The research interest in ants has been steadily growing the last years, and a lot of new insight has been gained. Crucial for many questions upon ants and their colony life is the possibility of maintenance of whole colonies in the laboratory. The genus *Cardiocondyla* is exceptionally appropriate for addressing different questions about colony and queen's life history traits as the rather small colonies with a short queen lifespan can be kept well under artificial conditions.

In this thesis, **Chapter 1** starts with an introduction of the newly described *Cardiocondyla pirata*, a species found on the Philippines during a collection trip.

Reproductive tactics of ergatoid males in the genus *Cardiocondyla* are very variable and complex and species can be separated in two clades according to the male's fighting tactics (Oettler et al. 2010). **Chapter 2** presents a so far unknown strategy displayed by ergatoid males of *Cardiocondyla venustula*: males mix the two fighting strategies that were observed in ergatoid males of other species and additionally defend small territories inside the nest. A similar territorial behaviour has never been observed in other ant males.

Chapter 5 deals with a special reproductive tactic of *Cardiocondyla "argyrotricha"* queens. Virgin queens that had no chance to mate can establish their own colony by laying haploid eggs. They mate with the first eclosing male and start to lay diploid eggs. In this manner they are able to raise a flourishing colony.

Chapter 3 investigates the difference in colony traits between in-and outbred colony strains in *Cardiocondyla* cf.*kagutsuchi*. **Chapter 4** and **Chapter 6** working with the same species dealing with the heritability of caste determination and the trade-off between lifespan and reproductive success.

Chapter 1:

Cardiocondyla pirata n.sp. – a new Philippine ant with enigmatic pigmentation pattern (Hymenoptera, Formicidae)

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Abstract

A new species of the ant genus *Cardiocondyla* Emery, 1869 – *Cardiocondyla pirata* n.sp. – is described from the Philippines. The species belongs to an Indo-Malayan group of six species that is characterized by workers having a strongly bilobate postpetiolar sternite and a thickset mesosoma with strongly convex dorsal profile as well as wingless, ergatoid males with sickle-shaped mandibles. The female castes show a pigmentation pattern not known from any ant worldwide. If having any adaptive value, a possible function of this structure is supposed to be visual dissolution of body shape in order to irritate predators .

Keywords *Cardiocondyla* – ergatoid males – Indo-Malayan region

Introduction

105 available names are listed in the ant genus *Cardiocondyla* Emery, 1869 and 68 of these are currently considered to designate bona species (Bolton 2012). While there is a rather good taxonomic knowledge of the species groups distributed in the Palaearctic (Seifert 2003), the situation in the Oriental, Indo-Malayan and Australasian faunal regions is poorly known. This is indicated by the fact that there is a minimum of 15 morphologically well-separated, but yet undescribed species from this region in the collection of the Senckenberg Museum of Natural History Goerlitz (unpublished protocols of the senior author).

During a field study of ants in the Hortarium of the Los Baños University / Philippines, one of the authors collected two nest samples of a *Cardiocondyla* species that shows a pigmentation pattern unknown in any ant worldwide. The new species belongs to a species group that is distributed from Thailand across the whole Indo-Malayan region and contains a minimum of six yet undescribed species. This species group is characterized by a strongly bilobate postpetiolar sternite, a thickset mesosoma with strongly convex dorsal profile and wingless, ergatoid males with sickle-shaped mandibles.

Methods

Recording of morphological data

Nineteen morphometric characters were investigated. In bilaterally recorded characters, arithmetic means of both body sides were calculated. All measurements were made on mounted and dried specimens using a pin-holding stage, permitting full rotations around X, Y, and Z axes. A Leica M165C high-performance stereomicroscope equipped with a 2.0 planapochromatic objective (resolution 1050 lines/mm) was used at magnifications of 120-384x. The mean relative measuring error over all magnifications was 0.3%. A Schott KL 1500 cold–light source equipped with two flexible, focally mounted light–cables, providing 30°–inclined light from variable directions, allowed sufficient illumination over the full magnification range and a clear visualization of silhouette lines. A Schott KL 2500 LCD cold–light source in combination with a Leica coaxial polarized–light illuminator provided optimum resolution of tiny structures and microsculpture at highest magnifications. Simultaneous or alternative use of the cold-light sources depending upon the required illumination regime was quickly provided by regulating voltage up and down. A Leica cross-scaled ocular micrometer with 120 graduation marks ranging over 52 % of the visual field was used. To avoid the parallax error, its measuring line was constantly kept vertical within the visual field. Measurements of body parts always refer to real cuticular surface and not to the diffuse pubescence surface.

Z-stack photographs were made with a Leica Z6 APO photomicroscope equipped with 2.0x planapochromatic objective and the automontage software Leica application suite version 3.

Definition of numeric characters

More detailed explanations of the character recording are given in Seifert (2003). Here we repeat the verbal definitions:

CL: maximum cephalic length in median line; the head must be carefully tilted to the position yielding the true maximum; excavations of hind vertex and/or clypeus reduce CL.

CW: maximum cephalic width; in *Cardiocondyla*, the maximum is found usually across and including the eyes, exceptionally posterior of the eyes.

CS: cephalic size; the arithmetic mean of CL and CW, used as a less variable indicator of body size.

dFOV: mean inner diameter of foveolae or mesh-like surface structures on vertex at about half way between the median line of head and the inner eye margin. These structures are either real foveolae or meshes of a reticulum and usually have the base of a decumbent pubescence hair in their centre. In species whose foveolae or mesh-like structures are reduced (e.g. in the *C. stambuloffii* group) the mean diameter of the small punctures or tubercles at hair bases is measured as dFOV. At least seven measurements are averaged.

EYE: eye-size: the arithmetic mean of the large (EL) and small diameter (EW).

FRS - distance of the frontal carinae immediately caudal of the posterior intersection points between frontal carinae and the lamellae dorsal of the torulus. If these dorsal lamellae do not laterally surpass the frontal carinae, the deepest point of scape corner pits may be taken as reference line. These pits take up the inner corner of scape base when the scape is fully switched caudad and produce a dark triangular shadow in the lateral frontal lobes immediately posterior of the dorsal lamellae of scape joint capsule.

MpGr: Depth of metanotal groove or depression, measured from the tangent connecting the dorsalmost points of promesonotum and propodeum.

ML - mesosoma length in the alates; measured in lateral view from the caudalmost portion of propodeum to the frontalmost point of the anterior pronotal slope (i.e., not to the frontalmost point of the whole pronotum that is usually concealed by the occiput!).

MW - maximum mesosoma width of alates frontad of the tegulae.

PEH: maximum petiole height. The straight section of ventral petiolar profile at node level is the reference line perpendicular to which the maximum height of petiole node is measured at node level.

PEL: diagonal maximum length of petiole in lateral view, measured from anterior corner of subpetiolar process to dorsocaudal corner of caudal cylinder.

PEW - maximum width of petiole.

PLG: mean length of pubescence hairs on dorsum of first gaster tergite as arithmetic mean of 6 measurements at least.

PPH: maximum postpetiole height; the lateral suture of dorsal and ventral sclerites is the reference line perpendicular to which the maximum height of postpetiole is measured.

PPW: maximum width of postpetiole.

PoOc: postocular distance. Use a cross-scaled ocular micrometer and adjust the head to the measuring position of CL. Caudal measuring point: median occipital margin; frontal measuring point: median head at level of posterior eye margin. Note that many heads are asymmetric; therefore average the left and right postocular distance.

SL: maximum straight line length of scape excluding the articular condyle given as the arithmetic mean of both scapes.

SPBA - the smallest distance of the lateral margins of the spines at their base. This should be measured in dorsofrontal view, since the wider parts of the ventral propodeum do not interfere the measurement in this position. If the lateral margins of spines diverge continuously from the tip to the base, a smallest distance at base is not defined. In this case SPBA is measured at the level of the bottom of the interspinal meniscus.

SP: maximum length of propodeal spines; measured in dorsofrontal view along the long axis of the spine, from spine tip to a line, orthogonal to the long axis, that touches the bottom of the interspinal meniscus. Left and right SP are averaged. This mode of measuring is less ambiguous than other methods but yields higher spine length values in species with reduced spines.

sqPDG: square root of pubescence distance on dorsum of first gaster tergite. The number of pubescence hairs n crossing a transverse measuring line of length L is counted; hairs just touching the line are counted as 0.5. The pubescence distance PDG is then given by L/n. In order to normalize the positively skewed distributions, the square root of PDG is calculated. Exact counts are promoted by clean surfaces and flat, reflection-reduced illumination directed slightly skew to the axis of the pubescence hairs. Counting is performed at a magnification of 320x. Tergite pubescence is easily torn-off in *Cardiocondyla*. An effort should be made to evaluate undamaged surface spots. In specimens with mostly removed pubescence, PDG can be calculated from the mean distance of hair base pits (BD) and PLG using the formula $PDG = BD^2 / PLG$.

Results

Cardiocondyla pirata n.sp.

Etymology

The species epithet refers to the black ribbon across the eye reminiscent of a pirate's blindfold.

Type material

Holotype worker labeled "PHI: 14.1643°N, 121.2375°E, Los Banos, University Park, 58 m, Hortarium, Frohschammer 2008.07.23 #39" and "Holotype *Cardiocondyla pirata* Seifert, 2013"; 4

workers, 3 dealate gynes and 1 ergatoid male labeled “PHI: 14.1643°N, 121.2375°E, Los Banos, University Park, 58 m, Hortarium, Frohschammer 2008.07.23 #39” and ”Paratype *Cardiocondyla pirata* Seifert, 2013”; 3 workers labeled “PHI: 14.1643°N, 121.2375°E, Los Banos, University Park, 58 m, in hole of a stone at riverside, Frohschammer 2008.07.23 #32” and ”Paratype *Cardiocondyla pirata* Seifert, 2013”; all material in Senckenberg Museum of Natural History Goerlitz.

Description and differential diagnosis

Measurements and indices in square brackets are arithmetic mean (see also Table 1).

Worker (Figs. 1-2, Tab.1):
Unmistakable pigmentation pattern for an ant worldwide. Lateral head at horizontal level of eye with an extended, longitudinal, dark brown ribbon that is as broad as the eye; this ribbon is flanked below and above by broad bands without any pigment (as result appearing whitish). Vertex, scape, postpetiole, gaster, procoxae, tibiae and femora except their proximal and distal portions light yellowish brown. Mesosoma light orange brown. Petiole, meso- and metacoxae, clypeus, spines, funiculus as well as proximal and distal portions of femora without pigmentation (appearing whitish). Very small size [CS 397 µm]. Head moderately elongated [CL/CW 1.132]. Postocular distance relatively small [PoOc/CL 0.408]. Eye rather small [EYE/CS 0.226]. With maximum CL and CW in visual plane, outlines of head roughly heart-shaped, with strongly concave posterior margin and an almost straight anterior clypeal margin (a distinct concavity appears after a tilt to frontodorsal viewing position when the three clypeal macrosetae become fully visible). Frontal carinae much more approached than in any related species [FRS/CS 0.242], subparallel and slightly diverging frontad of the FRS level. Mesosoma thickset, its dorsal profile evenly convex. Anterior pronotum in dorsal view rounded, without pronounced corners. Propodeal spines straight and much shorter than in any related species [SP/CS 0.208], in dorsal view slightly diverging, in lateral view straight and with their axis deviating by 40° from longitudinal axis of mesosoma. Petiole in lateral view rather massive, clearly higher than postpetiole, with a short peduncle, a slightly concave anterior profile and a convex dorsal node that steeply slopes down to the caudal cylinder; the node in dorsal view semiglobular and slightly wider than long. Postpetiole in dorsal view with a straight or slightly concave anterior margin, rounded sides and much wider than long; its sternite with pronounced anterolateral corners that are formed by bilateral lobes which strongly protrude

compared to anteromedian level. Whole surface of head, mesosoma and petiole with a very fine (mesh diameter on vertex only 8-9 µm) but deeply sculptured reticulum, thus appearing at lower magnifications perfectly matt. Postpetiole less deeply sculptured. Scapes, coxae, femora and tibiae with fine microreticlum and appearing matt at lower magnifications. First gaster tergite very finely microreticulate-shagreened, also appearing matt at lower magnifications. All cuticular surfaces including those of the appendages with decumbent, dilute pubescence. Pubescence on 1st gaster tergite much longer and denser than in any related species [PLG/CS 7.21%, sqPDG 3.92], on anterior surface directed caudad and on posterior one caudomediad. For morphometric data of 6 workers (three from each sample) see Tab. 1.

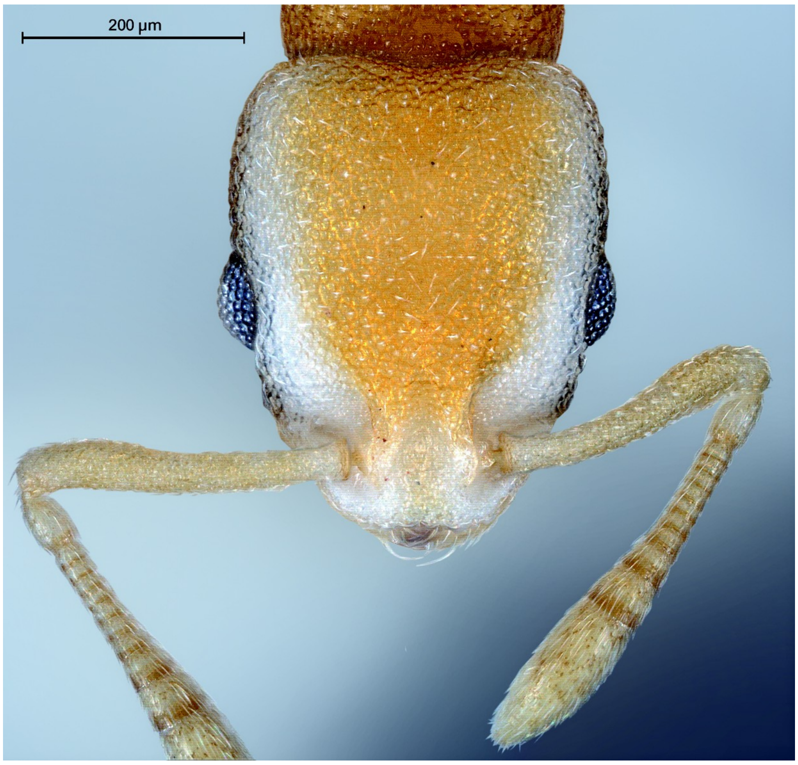

Fig. 1: Head of holotype worker in dorsal view.

Fig. 2: Lateral aspect of holotype worker.

Gyne (Figs. 3, 4, Tab. 1):

Unmistakable pigmentation pattern most similar to that described in the worker. Very small size [CS 437 µm]. Head shape comparable to worker but head more elongated, CL/CW 1.152. Postocular distance relatively small [PoOc/CL 0.401]. Frontal carinae much more approached than in any related species [FRS/CS 0.248], subparallel and slightly diverging frontad of the FRS level. Mesosoma shorter than in the next related species [ML/CS 1.301]. Propodeal spines straight and much shorter than in any related species [SP/CS 0.229], in dorsal view slightly diverging, in lateral view straight and with their axis deviating by 30° from longitudinal axis of mesosoma. Petiolar and postpetiolar shape comparable to worker but with the usual gyne-specific shape transformation: increased segment width and height relative to their length and postpetiole in dorsal view with a more concave anterior margin. All body surfaces appearing matt at lower magnification. Cuticular sculpture on all body surfaces similar to worker but several larger foveolae of 15-21 µm diameter, showing a central tubercle as basis of a pubescence hair, are interspersed within the fine reticulum of head and mesosoma. All cuticular surfaces, including those of the appendages, with decumbent, dilute pubescence. Pubescence on 1st gaster tergite much longer and denser than in any related species (PLG/CS 7.68%, sqPDG 2.63), on anterior surface directed caudad and on posterior one caudomediad. For morphometric data of 3 gynes see Tab. 1.

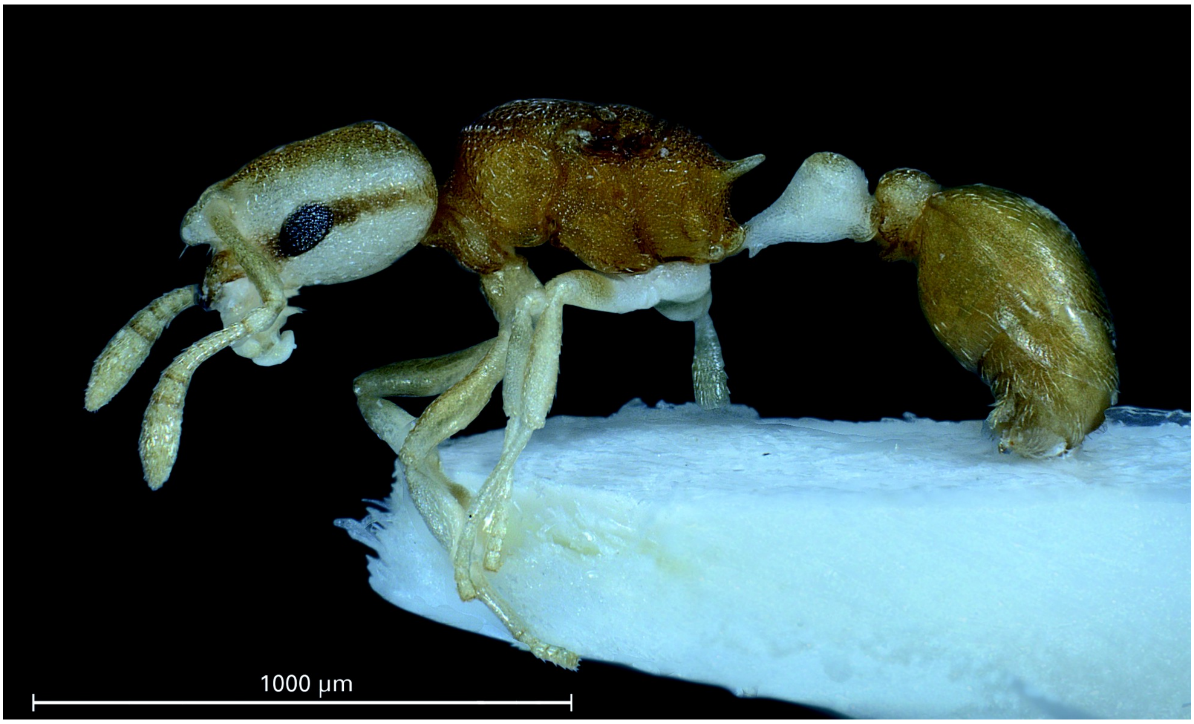

Fig.3: Lateral aspect of a paratype gyne. Postpetiole and gaster are distorted to a ventrolateral viewing position making visible both lobes of postpetiolar sternite.

Fig.4: Dorsolateral aspect of a paratype gyne. Only one lobe of postpetiolar sternite is visible.

Male (Figs. 5,6, Tab. 1):

Ergatoid. With exception of the blackish eyes, whole body concolorous pale yellowish. Nanitic size [CS 341 µm]. Antennae with 11 segments. Mandibles long and sickle-shaped, toothless. Head short [CL/CW 1.070]. Postocular distance relatively small [PoOc/CL 0.420]. Eye small [EYE/CS 0.210]. With maximum CL and CW in visual plane, outlines of head roughly trapezoid, with only weakly concave posterior margin and sides of head converging frontad. Anterior clypeal margin with a broad angular excision forming an angle of about 145°. Clypeus strongly extending caudad to about half length of frontal carinae. Frontal carinae much more distant than in female castes [FRS/CS 0.347] and almost parallel. Mesosoma very thickset and short, its dorsal profile evenly convex. With mesosoma in dorsal view, anterior pronotum rounded, without pronounced corners; pronotum and anterior mesonotum nearly twice as wide than the distance between the parallel sides of dorsal propodeum. Propodeal spines short, reduced to triangular dents. Petiole in lateral view more elongated, with a distinct peduncle, a concave anterior face and a high and short node that shows a rounded dorsum and falls almost perpendicularly down to the caudal cylinder. Petiolar node in dorsal view nearly 2.5fold wider than long, in anterior view strongly diverging dorsad and with an emarginate dorsal crest. Postpetiole in dorsal view 1.7 fold wider than its median length, with a slightly concave anterior margin and rounded sides; its sternite with pronounced anterolateral corners that are formed by bilateral lobes which strongly protrude compared to anteromedian level. Sculpture on all body surfaces similar to worker but sculpture and microsculpture on postpetiole and 1st gaster tergite less developed - as result these surfaces moderately shining. All cuticular surfaces including those of the appendages with decumbent, dilute pubescence. Pubescence on 1st gaster tergite much longer than in worker [PLG/CS 9.53%, sqPDG 3.66], on anterior surface directed caudad and on posterior one caudomediad. For morphometric data of the single investigated male see Tab. 1.

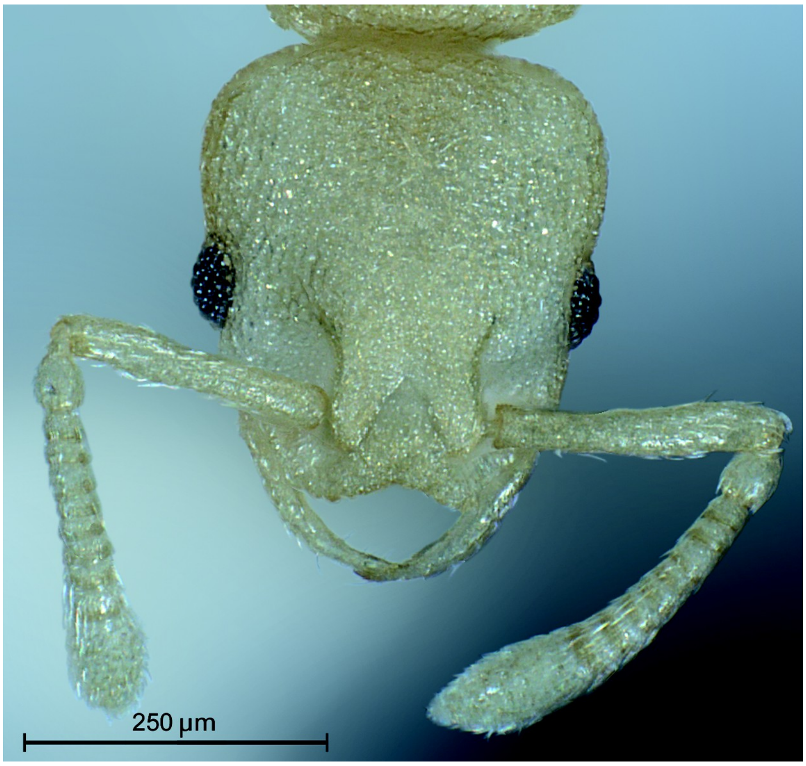

Fig.5: Head of the paratype male in dorsal aspect.

Fig.6: Lateral aspect of the paratype male. The head is distorted to a dorsolateral viewing position.

Discussion

Cardiocondyla pirata n.sp. cannot be confused with any ant worldwide because of its unique pigmentation pattern. This clear identification is supported by diagnostic structural and shape characters: there is no overlap with the five other undescribed species of this species group in the characters FRS/CS, SP/CS and PLG/CS and there is little overlap in PEW/CS and sqPDG (Tab.1). One complete colony consisting of three dealate queens, 15 workers and brood was collected in the field. From a second colony, only a sample was taken in EtOH. The first colony produced over 20 female sexuals and one ergatoid male in the lab, but thereafter died. Hence, there are no long-term observations on the life history of this interesting species. Considering the situation in related species of the Oriental and Indo-Malayan region (Heinze et al. 2010, Oettler et al. 2010), we may predict for *C. pirata* n.sp. the following biological traits: (a) there are only ergatoid males - winged males, which are an ancestral trait in *Cardiocondyla*, are no longer developed, (b) ergatoid males are long-lived, mate always inside the nest and try to kill rivals using their sickle-shaped mandibles in order to monopolize the matings and (c) nests should contain 1-4 queens.

The adaptive significance of the extraordinary pigmentation pattern of these tiny ants remains a puzzle. The poor resolution of the visual system (workers and males have only 50-65 ommatidiae per eye) and the dominance of chemical and tactile recognition cues in these ants as well as the fact that mating happens in darkness of the nest certainly exclude a function as an intraspecific recognition signal. A possible function could be visual dissolution of body shape by alternating dark and light pigment in order to escape the attention of a predator. The unpigmented petiole in particular, being in living condition rather translucent, permits the visual impression that anterior and posterior body are separate objects. Remains the question: Which predator with a high-performance visual system could consume these tiny ants?

Acknowledgements

We wish to thank Roland Schultz / SMNH Goerlitz for providing z-stack photos and Ekkehart Mättig /SMNH Goerlitz for help in processing of these photos. S. F. was supported by DFG (He 1623/22). We thank David General and the University of the Philippines Los Baños for the permission and their support of field work.

Tab. 1: Morphometric data of workers, gynes and a male of *Cardiocondyla pirata* n.sp. Worker data of five undescribed, most closely related species are given - the strings in capitals are code designations of these species in the files of B. Seifert. Arrangement of data: arithmetic mean ± standard deviation [lower extreme, upper extreme]. Measurements of *C. pirata* workers radically differing from those of related species are given in bold.

	sp.: ARGE, ARGY, ARPI, LATI, MISE	***Cardiocondyla pirata* n.sp.**	
	worker (n=174)	worker (n=6)	gyne (n=3)
CS [µm]	423 ± 45 [358,556]	397 ± 4 [392,402]	437 ± 3 [433,440]
CL/CW	1.113 ± 0.027 [1.040,1.182]	1.132 ± 0.010 [1.120,1.147]	1.152 ± 0.005 [1.146,1.155]
SL/CS	0.829 ± 0.018 [0.785,0.892]	0.807 ± 0.005 [0.800,0.816]	0.792 ± 0.011 [0.782,0.804]
PoOc/CL	0.418 ± 0.011 [0.389,0.455]	0.408 ± 0.005 [0.404,0.416]	0.401 ± 0.002 [0.398,0.402]
EYE/CS	0.228 ± 0.009 [0.204,0.247]	0.226 ± 0.002 [0.223,0.229]	n.r.
dFov [µm]	16.4 ± 1.8 [10.9,20.0]	8.6 ± 0.3 [8.2,9.0]	13.7 ± 2.8 [10.9,16.5]
FRS/CS	0.325 ± 0.020 [0.263,0.357]	**0.242 ± 0.007** **[0.235,0.254]**	0.248 ± 0.006 [0.242,0.254]
MW/CS	n.r.	n.r.	0.797 ± 0.016 [0.784,0.815]
ML/CS	n.r.	n.r.	1.301 ± 0.005 [1.297,1.306]
MGr/CS	0.38 ± 0.36 [0.0,1.48]	0.0 ± 0.0 [0,0]	n.r.
SPBA/CS	0.358 ± 0.020 [0.303,0.414]	0.373 ± 0.005 [0.364,0.378]	0.438 ± 0.015 [0.422,0.452]
SP/CS	0.366 ± 0.062 [0.232,0.476]	**0.208 ± 0.004** **[0.204,0.214]**	0.229 ± 0.005 [0.223,0.232]
PEW/CS	0.310 ± 0.020 [0.267,0.362]	0.359 ± 0.004 [0.355,0.367]	0.392 ± 0.002 [0.391,0.394]
PPW/CS	0.459 ± 0.028 [0.397,0.531]	0.468 ± 0.004 [0.462,0.475]	0.532 ± 0.007 [0.524,0.537]
PEH/CS	0.355 ± 0.018 [0.315,0.422]	0.343 ± 0.006 [0.335,0.354]	0.391 ± 0.006 [0.386,0.398]
PPH/CS	0.299 ± 0.031 [0.231,0.359]	0.320 ± 0.008 [0.308,0.328]	0.361 ± 0.005 [0.358,0.366]
sqPDG	5.34 ± 1.04 [3.42,8.25]	3.92 ± 0.13 [3.74,4.12]	2.63 ± 0.07 [2.55,2.68]
PLG/CS [%]	4.37 ± 1.21 [2.14,6.75]	**7.21 ± 0.26** **[6.87,7.54]**	7.68 ± 0.24 [7.49,7.95]

Chapter 2:

Male fighting and "territoriality" in the ant *Cardiocondyla venustula*

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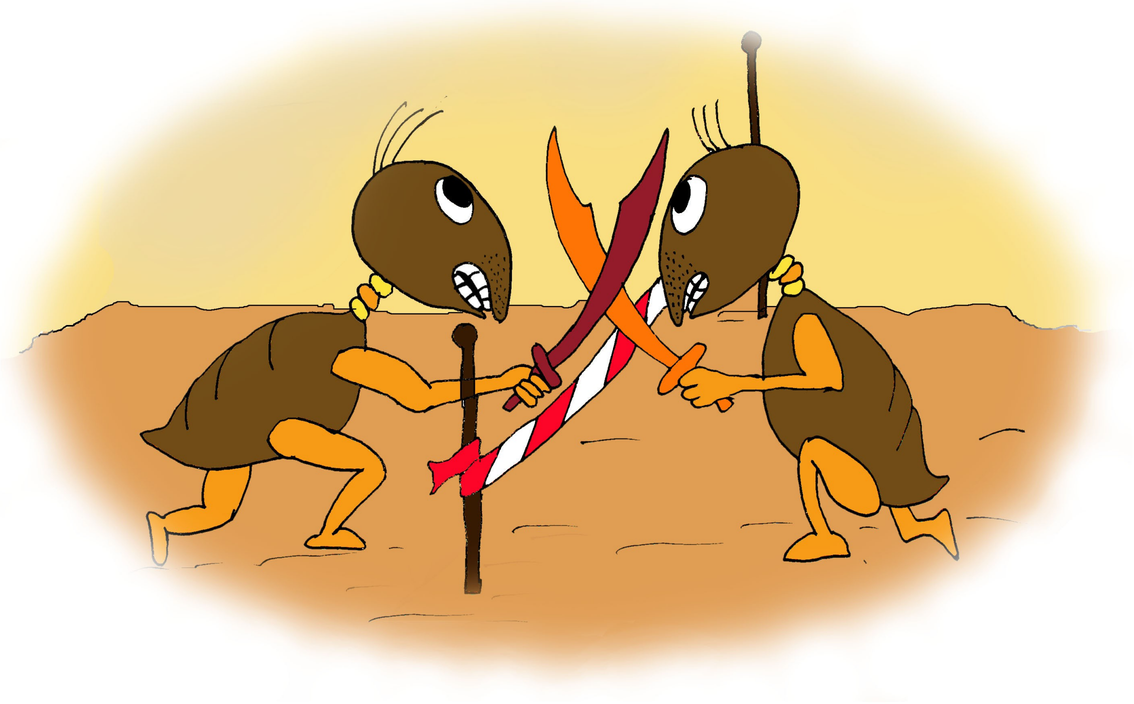

Published in Naturwissenschaften,(2009), 96(1), 159-163.

Abstract

The ant genus *Cardiocondyla* is characterized by a bizarre male polymorphism with wingless fighter males and winged disperser males.Winged males have been lost convergently in several clades, and in at least one of them, wingless males have evolved mutual tolerance. To better understand the evolutionary pathways of reproductive tactics, we investigated *Cardiocondyla venustula,* a species, which in a phylogenetic analysis clusters with species with fighting and species with mutually tolerant, wingless males. Wingless males of *C.venustula* use their strong mandibles to kill freshly eclosed rival males and also engage in short fights with other adult males, but in addition show a novel behavior hitherto not reported from social insect males: they spread out in the natal nest and defend "territories" against other males. Ant males thererfore show a much larger variety of reproductive tactics than previously assumed.

Keywords Territoriality Male reproductive tactics Sexual selection Formicidae

Introduction

The sex life of male ants is usually short and unexciting compared to that of most other male animals. Ant males do not fight for access to females, have a limited sperm supply sufficient for only one or a few copulations, and die after mating with one or a few young queens, if at all, leaving the latter with enough sperm to fertilize their eggs without ever re-mating (Hölldobler & Bartz 1985, Boomsma et al. 2005). The wingless (ergatoid) males of the ant genus *Cardiocondyla* are a striking exception to this rule. While winged *Cardiocondyla* males resemble ordinary ant males in morphology, physiology and behavior, wingless males have an ongoing spermatogenesis (Heinze & Hölldobler 1993) and may engage in fatal fighting to monopolize mating with a large number of female sexuals (Kinomura & Yamauchi 1987, Stuart et al. 1987, Heinze et al. 1993). Which factors shaped the evolution of this peculiar reproductive biology is unknown, but variation in male tactics among species of *Cardiocondyla* allows investigating under which ecological conditions fighting is beneficial and under which it is not.

Fighting appears to be an ancestral trait (Heinze et al. 2005), which persists in several paleotropic and African species, including a number of invasive species that today occur worldwide in the tropics and subtropics (Heinze et al. 2006). In *Cardiocondyla obscurior*, *C. wroughtonii* and *C.* sp., wingless males use their saber-shaped mandibles to pierce the soft body of freshly emerged males and to hold adult males to besmear them with hindgut secretions, which again elicit aggression by workers (Yamauchi and Kawase 1982, Kinomura & Yamauchi 1987, Stuart et al. 1987, Yamauchi et al. 2006). In *C. mauritanica*, *C. minutior*, *C. emeryi*, *C.* cf. *kagutsuchi*, and C. *tjibodana* (Yamauchi & Kinomura 1993, Heinze et al. 1993, 1998; unpublished observations), wingless males have shear-shaped mandibles, which are predominantly used to crush the cuticle and cut off the legs and antennae of freshly emerged males. In a more derived monophylum comprising several taxa from xeric desert- and steppe-habitats throughout Eurasia, aggressive male competition has been lost and males co-exist peacefully in the nest (Forel 1892, Arnol'di 1926, Marikovsky & Yakushkin 1974, Schrempf et al. 2005b, Lenoir et al. 2007).

In a phylogeny based on mitochondrial and nuclear gene sequences, the African *C. venustula* clusters with the palearctic clade of "peaceful" *Cardiocondyla* and several taxa, such as *C. mauritanica* and *C.* cf. *kagutsuchi,* in which male fighting does occur (J. Oettler, in prep.). Determining how *C. venustula* males interact is therefore of considerable importance for elucidating the evolutionary pathways of male reproductive strategies. Here, we investigate wether and how males of *C.venustula* compete for access to female sexuals.

Material and Methods

Study organism

Cardiocondyla venustula (Wheeler, 1908) was originally described from Puerto Rico, but is presumably of African origin and has been introduced to the Caribbean islands, Florida, and other areas around the world probably more than 100 years ago (Seifert 2003, own observations). Though it was reported to be widespread in Hawai'i (Nishida 2002), almost all investigated material belongs to a paleotropic tramp species of the *C.* cf. *kagutsuchi*-group (e.g., Krushelnycky et al. 2005, Yamauchi et al. 2005). Only specimens collected in 2001 and 2006 at an elevation of appr. 1100m on Nualolo Trail, Koke'e State Park, Kaua'i, including winged and wingless female sexuals sitting on vegetation or running on the ground, were identified as *C. venustula* (Krushelnycky et al. 2005, B. Seifert, pers. comm.). Their mtDNA sequences were similar or identical to those of *C. venustula* from Ethiopia (Debre Markos, Senbete, Weliso) and South Africa (Kwazulu-Natal: Hlalanathi, Scottburgh, St. Lucia; Mpumalanga: Machadodorp) (M. Suefuji, A. Trindl and J. Heinze, unpubl.).

Methods

Male behavior was observed in experimental colonies originating from a colony collected at Nualolo Trail in September 2006, which consisted of a single dealate queen, numerous female sexuals, workers, brood, and two relatively large, wingless males with strong mandibles and angular shoulders (Fig. 1). In the laboratory, the ants were kept in a 9.6 cm x 9.6 cm x 2.8 cm plastic box with a cavity in the plaster floor covered by red plastic serving as nest with artificial temperature cycles of 15°C night / 25°C day. The colony was provided with pieces of cockroaches and droplets of honey twice per week and grew very quickly so that the colony had to be split into numerous smaller subunits.

Casual observations suggested that males separate in different parts of the nest. To make the quantification of male location easier and because natural colonies of *Cardiocondyla* also consist of several chambers (e.g. Marikovsky & Yakushkin 1974), we established a total of 13 "stock colonies," each consisting of workers, brood, and dealate and winged queens from the original colony and housed them in separate boxes with a plaster floor and a multi-chambered nest cavity. Males eclosing from the brood were marked with rings of 25µm copper wire at the joints between alitrunk, petiole, postpetiole, and gaster. Their location in the nest was monitored regularly and interactions among males were studied by opportunistic and scan sampling. Marking appeared to neither affect the behavior of males nor that of their nest mates.

In addition, we established small experimental nests consisting of workers, brood, and one or a few (up to eight) queens from one of the stock colonies, housed them in Petri dishes with plaster floor and a barbell-shaped nest cavity, and added two 1 or 2 day old males (n = 10) or two male pupae of similar developmental stage (n = 13) to determine how young males interact. In 20 of these nests, males were sufficiently different in coloration, size, or other characters for distinction by eye, in the other colonies the males were marked with wire loops as above. We noted the position and behavior of each male to twelve times per day directly after the beginning of the experiment and later at least once per day until one male had died or been expelled from the nest for more than three days. Emerging male pupae were removed daily.

Results

During the observation period, stock colonies produced large numbers of female sexuals (up to 100) and frequently contained two or more, up to ten wingless males (2, 4, 5, 8, 9, 10) and up to 18 wingless male pupae at the same time. Winged males were never produced. Similarly, two colonies of *C. venustula* collected 2008 in Hlalanathi and St. Lucia, South Africa also did not contain any winged male but one or two wingless males, respectively.

The behavior of males showed enormous variation. In one “stock colony”, in which interactions among males were examined in greater detail, and in 12 of 23 experimental nests, males separated into different parts of the nests, which they inhabited consistently for several days (e.g., males green, blue, yellow in Fig. 1). Inside this “territory”, males walked around and antennated the brood and mated with virgin females, but apart from it they moved more quickly and with extended antennae, antennated nestmates more rapidly, and retreated quickly when encountering another male. In four observations, a confrontation between males at the border of their “territories” led to a brief fight similar to those described below, but lasting only for a few seconds. In six additional observations, males only opened their mandibles and then retreated back into their own “territory”. Males, which inside their “territory” met another male, approached it rapidly with open mandibles, tried to bite and grip it and followed the intruder until it had moved away. Several other males appeared to have no such "territory" and were almost constantly on the move, apparently avoiding contact with other males and occasionally hiding in the brood pile. Most of such "floaters" finally left the nest after fighting with other males (e.g., individual olive in Fig. 1). Unlike males, workers and queens moved freely through the nest and also relocated brood between different nest parts.

The pattern of emergence apparently influenced how males behaved. Males, which had been one or two days old when the experiment started, almost permanently separated in different parts of the nest in nine of ten colonies (separate during 71-100% of the scans over 2 to 39 days). In contrast, only four of 13 pairs of males, which had emerged on the same day in the experimental nests, spaced out (separate during 78 – 100% of scans over 2 – 22 days; Yates corrected $\chi 2$= 3.69, p= 0.055). Instead, young males frequently engaged in fights, and in eight of 13 experimental nests one male was found decapitated or dismembered on the day following emergence. Two males co-existed significantly longer in nests originally containing two 1 or 2 day old males (median, quartiles: 12, 8, 20 days) than in nests with two male pupae (median, quartiles: 2, 1, 8 days; permutation test, p= 0.0207).

Fighting among adult males resembled that known from other *Cardiocondyla species*,in particular *C.obscurior* (e.g. Kinomura & Yamauchi 1987): males grabbed the opponent with their mandibles and attempted to besmear it with secretions from the tip of the abdomen. In 19 of 21 observed fights, aggression stopped immediately after besmearing, the males parted and started to groom themselves (e.g., individual red in Fig. 1 on day 5). In two cases, males continued to hold each other for several minutes more. Secretions apparently elicited interest and occasionally also aggression from workers. In 14 cases, workers were seen first hesitantly and then intensively grooming besmeared males. One male was seen to decapitate a freshly ecloing male with its strong mandibles, and at least three severed and killed young males were found in some of the "stock colonies". Nevertheless, in at least one case, we observed one male eclosing safely on the border of two territories.

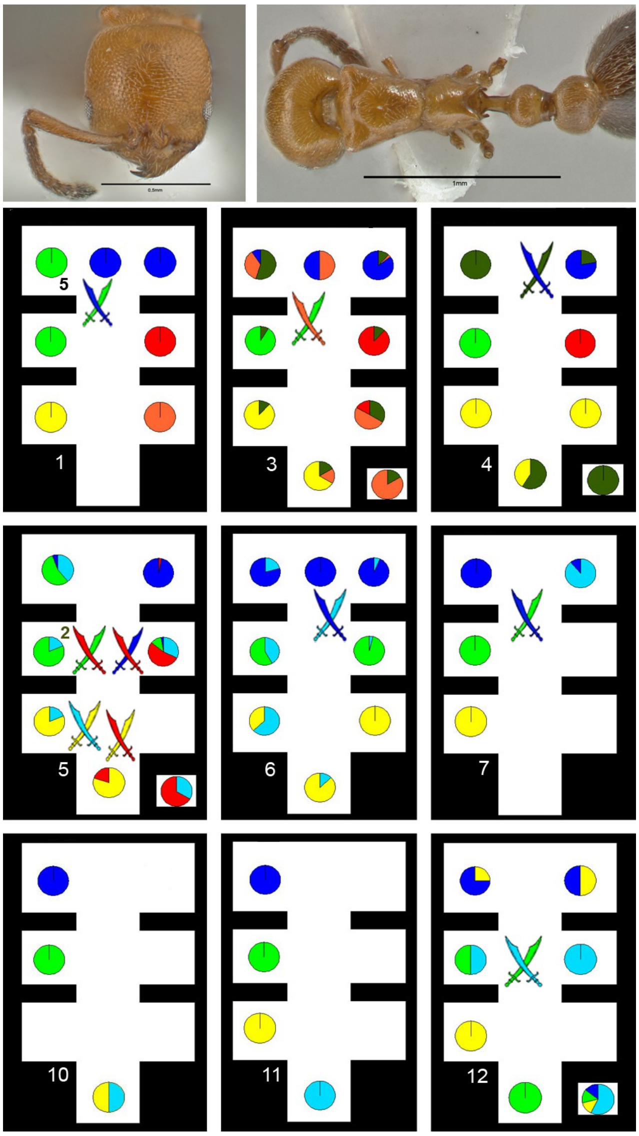

Fig. 1 **Frontal view of the head and dorsal view of the thorax of a wingless male of the ant *Cardiocondyla venustula,* and schematic pattern of the distribution of individual males (depicted in different colors) in a multimale colony over a period of 12 days. *Pie diagrams* indicate the percentage of scans during which males were seen in a certain nest chamber. Whereas some males spread out and consistently stayed in certain parts of the nest, others moved throughout the nest and engaged in fights (as indicated by crossed swords). *Pie diagrams in the lower right corner* indicate individuals staying outside of the nest.**

Discussion

Male competition in *C. venustula* combines elements of male behavior in species with regular lethal fighting and / or the elimination of freshly emerged males and species with male tolerance. While males readily eliminated young rivals and in particular young males engaged in lethal fighting, older males appeared to space out and to defend areas, which we here provisionally refer to as "territories". Interactions in or at the border of such home ranges were usually much less violent than fighting among young males and in several cases mandible opening seemed to be enough to deter intruders.

To our knowledge, the defense of "territories" or "home ranges" within the natal nest is novel for social insect males. Males of many bees and wasps guard territories outside the maternal nest, in which they try to monopolize mating with all visiting females (e.g. ‚Kasuya 1981, Sugiura 1991, Alcock 1994, Strohm & Lechner 1999). Similarly, dominant ant queens may chase subordinate queens away from the brood pile into the periphery of the nest (e.g., Hölldobler 1962, Hölldobler & Carlin 1985, Heinze & Smith 1990, Yamauchi et al. 2007). Hamilton's casual remark (1979) that males of the ant *Hypoponera punctatissima* fight for occupancy of the chambers in which female sexuals eclose is probably the closest match to the situation in *C. venustula*.

Males of hitherto studied species of *Cardiocondyla* freely move around in the nest and either ignore each other or incessantly patrol the brood pile, kill young rivals and, at least in *C. obscurior* and a few other species, engage in lethal fighting with other adult males wherever they encounter them (e.g., Heinze et al. 1993, Heinze 1997). Why is *C. venustula* different? Complete male tolerance in palearctic species appears to be associated with the evolution of single-queen colonies (Schrempf & Heinze 2007), but according to preliminary data African colonies of *C. venustula* may contain several queens (J. Heinze, unpublished), like in other species with fighting males. In contrast to tropical species of *Cardiocondyla*, in which a few male and female sexuals are produced year-round, laboratory colonies of *C. venustula* produced sexuals seasonally and in large numbers. This might make it impossible for individual males to inseminate all female sexuals and at the same time eliminate all rivals (see also Heinze et al. 1993). In addition, the shear-shaped mandibles of *C. venustula* males appear to be less efficient in interactions among adults than the saber-shaped mandibles of *C. obscurior* males. While males of *C. obscurior* bite and hold adult rivals for minutes or even hours and besmear them with secretions that elicit deadly attacks by workers (Yamauchi & Kawase 1992), males of *C. venustula* male usually grabbed opponents only for a few seconds and besmearing rarely resulted in the death of the opponent. As in other species with fighting males with shear-shaped mandibles (Yamauchi & Kinomura 1993 , Heinze et al. 1993, 1998), attacks appeared to be directed mostly against young males, whose soft cuticula can easily be crushed with shear-shaped mandibles. Because of the inefficiency of weapons and the synchronous eclosion of large numbers of sexuals, it might be more advantageous for adult males to control a small part of the nest. While *Cardiocondyla* males are typically much smaller than conspecific queens (e.g., Heinze et al. 1993, Mercier et al. 2007), the body size of *C. venustula* males, though enormously variable, often approaches or even exceeds that of queens (unpublished). Whether large size is important during home range establishment and defense and whether males compete for good places in the nest and form dominance hierarchies still needs to be determined.

Acknowledgements

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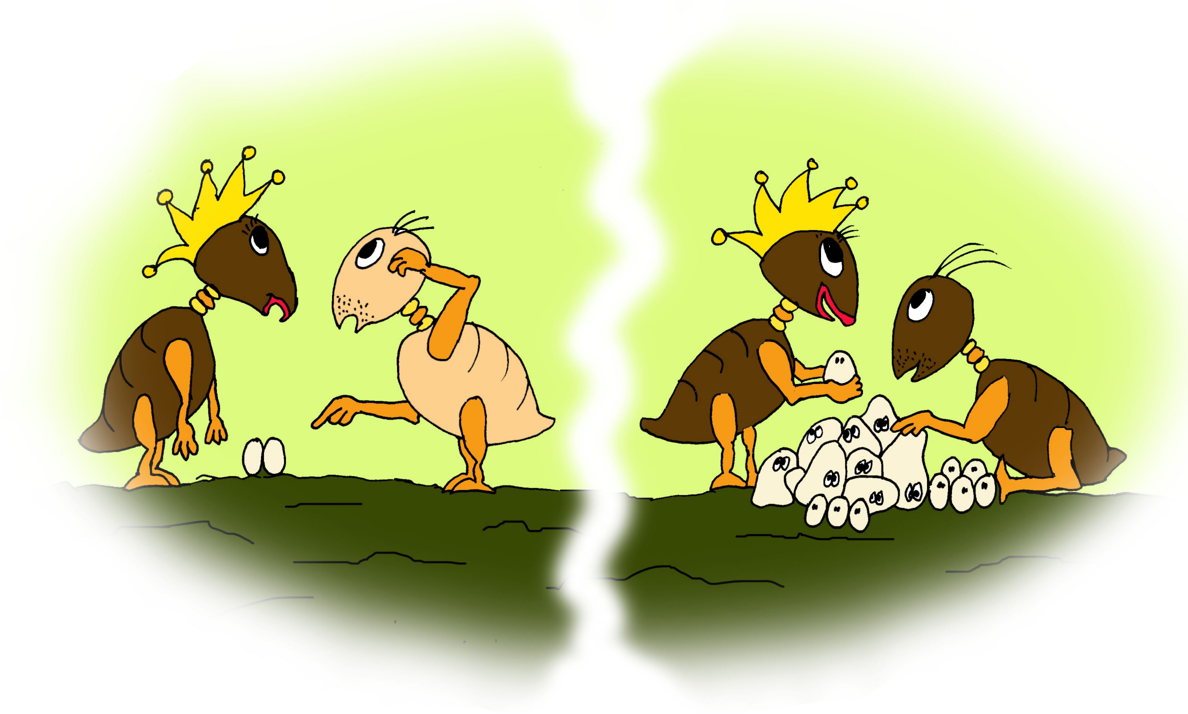
Chapter 3:

In- and Outbreeding in *Cardiocondyla* cf. *kagutsuchi* *

Sabine Frohschammer, Alexandra Schrempf, Abel Bernadou & Jürgen Heinze



* Manuscript

Introduction

Outbreeding as well as inbreeding can have both- positive and negative effects on the fitness of individuals and populations.

Outbreeding- mating with unrelated partners- increases the heterozygosity in the progeny. Outbred individuals often show higher fitness than their parents. This is referred to as “hybrid vigor.” Elevated fitness of hybrids is thought to be due to three causes. Beneficial epistatic interactions mean the favorable interaction of alleles from different loci. Overdominance is the advantageous combination of two different alleles at one loci, where both have a positive influence on the individual’s phenotype. Dominance is the masking of recessive deleterious- through dominant alleles (Hedgecock at al. 1995, Birchler et al. 2006).

In populations, genetic variability can help to adapt to changing environmental conditions (Carvalho 1993, Lande & Shannon 1996). The importance of outbreeding is emphasized especially in conservation biology, as small, isolated populations often face a loss of genetic variability (Edmanns 2007, Rotheray et al. 2012). However, beside these beneficial traits, inter-population crosses can also be harmful. Fitness decline in the offspring of outbreeding individuals has been discovered in several studies with plants and animals (e.g., Waser & Price 1994, Fischer & Matthies 1997, Gharrett et al. 1999, Montalvo & Ellstrand 2001). This fitness decline, referred to as outbreeding depression, can be due to the breakup of locally coadapted gene complexes (Edmands 1999, Jong & Nielsen 2002). It also may be a consequence of alleles selected for a a certain phenotype that is best adapted towards the inhabited environment. Mating between individuals from different environments may lead to a hybrid offspring, that often is adapted to neither parental environment, which means that intermediate genotypes are disfavored (Fenster & Dudash 1994). Long term separation of populations without gene flow can lead to reproductive isolation barriers ending in speciation (Coyne & Orr 2004). Hence, for each species, it is expected that there is an optimal outbreeding distance (Price &Waser 1979, Bateson 1983), which maximizes population fitness.

Inbreeding - mating between close relatives- leads to increased homozygosity and a decrease of allelic richness. Inbreeding bears the risk of great fitness loss for individuals through expression of deleterious recessive alleles (Charlesworth & Charlesworth 1999). To avoid this inbreeding depression, plants and animals have evolved a variety of methods that prevent them from mating with close kin (Holmes & Sherman 1983, Pusey and Wolf 1996). Examples reach from recognizing and rejecting kin as mates (Simmons 1989, Keller & Passera 1993) to the spatial and temporal separation of different sexes (Cockburn et al. 1985, Perrin & Mazalov 1999, 2000). In the haplodiploid order Hymenoptera, the amount of deleterious alleles is thought to be reduced because of "purifying selection" through the forced expression of alleles in haploid males. Inbreeding yet yields another risk for haplodiploids with single locus complementary sex determination (sl-CSD), which is assumed for several Hymenoptera (van Wilgenburg et al. 2006). In this system males are haploid and consequently have only one single allele at the sex locus. Diploid larvae that are heterozygous at the sex locus develop into females whereas homozygosity at the sex locus leads to inviable or sterile diploid males. In social Hymenoptera, males do not contribute to colony maintenance (Tarpy & Page 2002), and the production of sterile males instead of females constitutes a great load towards the colony.

Similar to outbreeding, inbreeding can also have positive aspects for individuals and populations. Mating within the population can fix positive adaptations within a habitat (Verhoeven 2011, Lenormand 2012) and allows to avoid risky dispersal (Bengtsson 1978). Mating with kin augments inclusive fitness of an individual through an indirect fitness gain by increasing the mating success of a relative (Hamilton 1964, Lehmann & Perrin 2003, Kokko & Ots 2006). In social insects, mating with close kin compensates for reduction of relatedness with polygyny, or polyandry (Trontti et al. 2005, Thurin & Aron 2009, Thurin et al. 2011). Indeed, there is evidence for an indifference towards or even preference for inbreeding in various ant species. For example, in the Argentine an*t Linepithema humile* queens fail to use genetic cues to avoid sib-mating and queens mate randomly with both- related and unrelated males (Keller & Fournier 2002). Due to mating in the nest and low dispersal of males, colonies of the polygynous ant *Plagiolepis pygmaea* also show high values of inbreeding (Trontti et al. 2005). In *Hypoponera opacio*r the second highest level of inbreeding in ants could be detected (Foitzik et al. 2011) but the population did not notably suffer from inbreeding depression over the 12 year that colonies were observed (Foitzik et al. 2010, 2011, Kureck et al. 2012). In its natural environment*,* highly inbred nest of *Hypoponera opacior* shift offspring allocation towards winged sexuals that will outbreed (Kureck et al. 2012).

The highest level of inbreeding in ants has been found in *Cardiocondyla batesii* (Schrempf et al. 2005b). It has been demonstrated that a related species, *C. obscurior* has evolved an alternative mechanism of sex determination other than sl-CSD. In the latter, evidence for inbreeding depression, such as reduced queen lifespan, higher brood mortality and lower fertility of males, was observed only after several generations of sib-mating in the lab (Schrempf et al. 2006).

In this experiment we studied the effects of inbreeding versus outbreeding on traits of the individual and colony phenotype in the ant *Cardiocondyla* cf. *kagutsuchi,* an ant with regular sib-mating and potentially restricted male dispersal in this clade, as winged males never occurred in the laboratory over at least three years. We compared different colony parameters, such as queen productivity, queen lifespan, and the success of colony establishment between inbred and outbred colonies.

Material and Methods

The species *Cardiocondyla* cf. *kagutsuchi* belongs to the palaeotropic *C. nuda*-group (*C. atalanta, C. mauritanica*, and *C.* cf. *kagutsuchi*) and is part of a complex of sibling species widely distributed throughout Southern and Eastern Asia (Seifert 2003). In some taxa of this complex, normal winged males co-occur with wingless (ergatoid) or brachypterous, short winged males (Heinze et al. 2005, Yamauchi et al. 2005) exist, but our experimental colonies belong to a clade of *C.* cf. *kagutsuchi* with only ergatoid males. Colonies were collected from three different sites: two from the Hawaiian Islands Hawai´i and Kaua´i, USA, and one from Okinawa, Japan.

Colonies from Kauai were collected in 2006 near Pu'u o Kila lookout and the trail heads of Awa'awapuhi and Nualolo trail at an elevation of approximately 1100 m. Colonies from Hawai´i were found at the Kilauea Iki Overlook. All colonies were excavated from their nests in the upper layers of the soil.

Colonies from Okinawa Island were collected in 2006 by M. Suefuji.

C. cf. *kagutsuchi* has been introduced to these places by human activities and the lack of genetic variation among them indicates that they go back to small founder populations on each of these islands. Colonies from within the various sites where thus at least distantly related.

Colonies of *Cardiocondyla* cf. *kagutsuchi* are facultative polygynous and usually contain only one ergatoid male, which mates with all virgin females (Yamauchi et al. 2005).

Obtained stock colonies were housed in three chamber plaster nests and fed with cockroaches and honey twice a week. All ant nests were reared in the laboratory at 15°C-25°C and day-night cycle (12h/12h).

Experiments started in January 2007 and lasted until December 2007. Sexuals were taken from 6 different stock colonies from Kaua´i, from 3 colonies from Hawai´i, and 6 colonies from Okinawa.

We established a series of 52 outbred colonies, in which founding sexuals originated from different populations and another series of inbred colonies (N= 30) in which sexuals were taken from the same nest and were therefore closely related. Experimental colonies were supplied with one queen and one male pupa each, 10 workers and a few brood items from the stock colony of the queen (and the male in case of inbreeding). After eclosing, sexuals mated inside the nest (Fig.1), the queen shed its wings and started to lay eggs. Dead workers were replaced by new workers from the stock colony until the experimental colony had begun to produce its own workers. Colonies were monitored twice per week and the presence of the queen and the male, and the number of workers and brood items were noted. Colonies were allowed to grow without restriction, but sexual pupae were removed, to avoid confusing sexual offspring with the founding pair.

Fig 1: Mating couple of *Cardiocondyla* cf. *kagutsuchi.*

In several colonies, the queen died during the first days or weeks of the experiment, and / or failed to lay eggs. In 64 colonies at least eggs were produced. In 3 colonies, these only developed into males and the queens were probably not inseminated (2 inbred, 1 outbred colony). For the statistical analysis only nests were taken into account that produced at least one worker or queen pupae.
The following parameters were compared between inbred and outbred colonies:
lifespan of the queen, age of the queen when shedding its wings, start of queen egg laying, average number of eggs per scan, number of larvae, pupae, and workers produced (calculated from 20 days after appearance of first brood item until the death of the queen), date of appearance of first worker, male and queen pupa relative to queen eclosion, sum of all queen and male pupae produced and the numerical sex ratio (proportion of female sexuals in the offspring).

Normally distributed data were analyzed with t-tests, and non-normally distributed data and lifespans were compared with Mann-Whitney U-tests. Correlations among various traits were compared by Spearman rank correlation tests, as it is less sensitive to outliers than other non-parametric correlations.

Due to the complexity of the dataset and the close correlations among various life history traits, we also used generalized linear mixed models (GLMM) to analyze the lifespan of queens and sex ratio. As the lifespan of queens was count data and sex ratio was proportional data, we used Poisson and binomial error distributions, respectively. Due to overdispersion, the standard errors were corrected using a quasi-GLM model (Zuur et al. 2009). For the analysis of queen lifespan, we started with a full model including population of queen origin, appearance of first male pupae, number of total sexuals, and worker number at queens' death as fixed effect factors. The model for sex ratio included breeding treatment (two levels: inbreeding / outbreeding) and populations as fixed effect factors. Several experimental colonies were set up from the same stock colonies. To account for the resulting pseudo-replication, the factor colony was entered as a random effect factor in both models. For each dependent variable, we obtained a minimal model by a stepwise backward elimination procedure, i.e., by successively removing all non-significant terms ($p > 0.05$). All analyses were performed with the statistical software R 2.11.0 (R Development Core Team, 2010) and the glmmPQL function from the MASS library of R.

Results

Egg number and wing loss

Colonies that produced eggs but no larvae contained significantly fewer eggs (average eggs per scan) than colonies that were able to raise larvae and pupae in both treatments (t-test, inbreeding: $n_{pupae}=14$, $n_{no\_pupae}=6$, T=4.088, p=0.003; outbreeding: $n_{pupae}=12$, $n_{no\_pupae}=18$, T=3.183, p=0.004). Inbred and outbred colonies did not differ significantly concerning the timing of wing loss ($n_i=20$ M=16 days, SD=5.2; $n_o=15$, M=14 days, SD=60; t-test: T=-1.136, p=0.264) average ($n_i=20$, M=30 days, SD=11.0; $n_o=15$, M=25 days, SD=12.4; t-test: T=-1.273, p=0.212) and maximum egg number ($n_i=20$ M=53 days, SD=17.7; $n_o=15$, M=45 days, SD=21.8; t-test: T=-1.316, p=0.197). Egg productivity varied greatly among colonies (average eggs present per scan: min=3.5, max=47.8), which probably explains why we could not find any significant differences between in- and outbred colonies concerning the number of eggs 24, 36, and 48 days before the death of queens [bqd]).

Averaging across colonies of one breeding line at distinct dates evened differences in egg production and we received one average value for every time point in both treatments. This was done by taking values of every colony on a certain time (e.g. 3, 6, 9, 12 etc. days after first egg layed) and building the average value over all inbred and all outbred colonies. These average values were compared among in and outbred colonies. During the first 52 days of egg production, in- and outbred colonies did not differ among each other. In both setups the average number of eggs present per scan increased with time (Spearman`s rank correlation test, n=14, inbreeding: r_s=0.92, p<0.001; outbreeding: r_s =0.94, p<0.001). During the last 64 days of a queen’s life, inbred colonies contained on average 6.2 eggs (SD=2.7) more per scan (n= 17 matched pairs t-test: T= -5.3, p<0.001) than outbred colonies. Considering egg production from day 64 to day 8 before the queen’s death, in outbred colonies egg production was still significantly correlated with time, while there was no significant association in inbred colonies (Spearman`s rank correlation test, n=15, outbreeding: r_s =0.632, p=0.011, inbreeding: r_s=-0.435, p=0.105).

In both breeding lines the number of eggs counted during the next to last and the last scan declined significantly compared to the scan 8 days bqd (matched pairs t-test: n_o=15, 8 bqd against 4 days bqd: T=3.5, p=0.004, 4 days bqd against 0 days bqd: T= 3.9, p=0.002; n_i=19, 8 days bqd against 4 days bqd: T=2.9, p=0.009, 4 days bqd against 0 days bqd: T= 2.5, p=0.021). The highest egg number per scan present in a colony was found at 77% of the queen’s lifespan (quartiles 63, 90.5%) in outbred colonies and 72% in inbred colonies (quartiles 59, 80%) (n_o=15, n_i=20, Mann-Whitney U-test, U=116, p=0.268).

Number of larvae, pupae, worker

Inbred colonies were significantly more successful in raising a colony (Fig.2). Out of all 61 queens that produced eggs (and were not surely unfertilized- queens that only produced male pupae were excluded), only 37 managed to raise larvae and pupae. In the remaining 24 colonies eggs never hatched. In 27 inbred colonies, 21 colonies produced pupae (77.8%) compared to only 16 of 34 outbred colonies (47.1%) (chi^2=5.48, p=0.019).

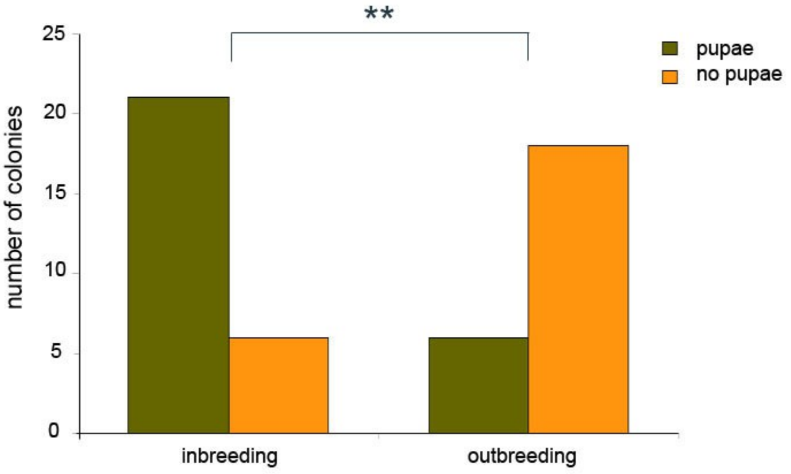


Fig.2 : founding success (successful development of pupae) in both breeding lines.

Successful inbred and outbred colonies- where eggs hatched - did not differ in average number of larvae, worker pupae and workers. They also did not differ in the time of eclosion of first worker and first male pupa, but outbred colonies produced first female pupa significantly earlier than inbred colonies (median, quartiles: 97, 62, 108 days, vs. 115, 101, 164 days; n_o=15, n_i=15, Mann-Whitney U-test, U=46.0, p=0.005). Higher egg production of inbred colonies towards the end of the queen's life resulted in higher worker number at queen's death at marginal significance (n_i =19, M=53, SD=39.7; n_o=15, M=31, SD=26.1; t-test: T=-1.834, p=0.076). Taking only colonies into account that produced all types of pupae yielded a significant difference (n_i=14, n_o=12, median, quartiles: 75, 38.5, 72.5 days, vs. 31, 11.5, 44.5 days; Mann-Whitney U-test, U=35.0, p=0.005).

Sex ratio, maximum worker number

There was a significant difference in sex ratio between in- and outbreeding colonies. Outbred colonies showed a significantly more female-biased sex ratio, both before and after death of the queen (before queen death: n_i=16, n_o=10 median, quartiles: 0.23, 0.01, 0.61 % female sexuals, vs. 0.65, 0.37, 0.90 % female sexuals, Mann-Whitney U-test, U=33.0, p=0.012; after queen death: n_i=16, n_o=15 median, quartiles: 0.35, 0.07, 0.78 % female sexuals, vs. 0.89, 0.81, 1.00 % female sexuals, Mann-Whitney U-test, U=57.5, p=0.012). In 6 colonies (4 inbred, 2 outbred), queens showed signs of sperm depletion, producing only male pupae at the end of their life (inbreeding: n_{male_end}: 9, 10, 21, 75; outbreeding: n_{male_end}: 48, 50). After excluding these colonies from the analysis, differences in sex ratio before the queen`s death persisted and even increased after death of the queen (until queen death: n_i=12, n_o=8 median, quartiles: 0.41, 0.11, 0.70 % female sexuals, vs. 0.83, 0.50, 0.95 % female sexuals, Mann-Whitney U-test, U=16.0, p=0.012; after queen death: n_i=12, n_o=13 median, quartiles: 0.61, 0.18, 0.82 % female sexuals, vs. 0.91, 0.84, 1.00 % female sexuals, Mann-Whitney U-test, U=28.5, p=0.005; Fig.3). In the following analysis, regarding sex ratios, colonies with sperm depletion were excluded.

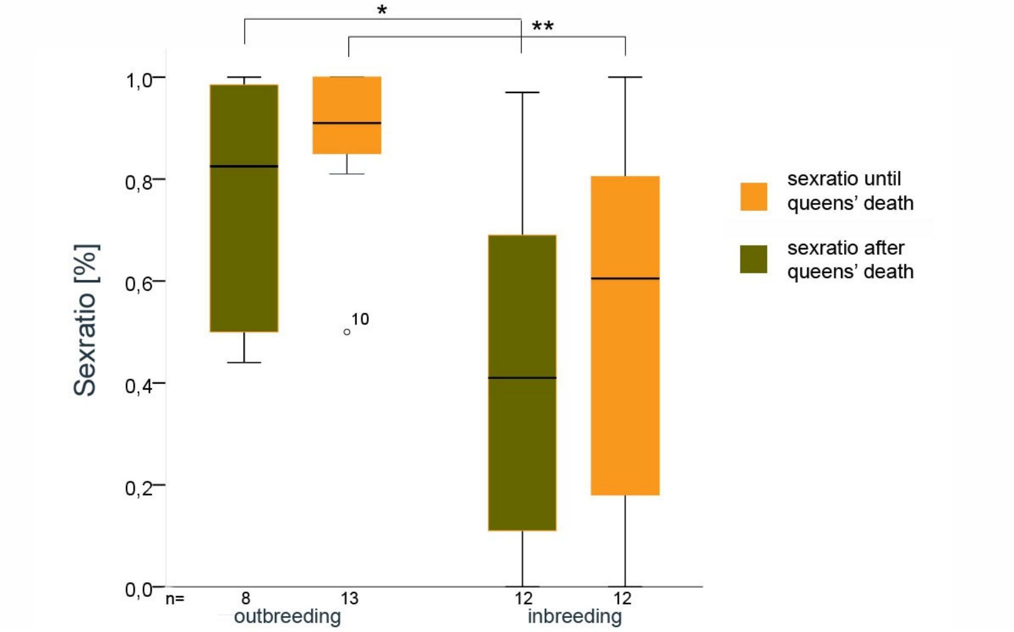


Fig.3: Sex ratio of in-and outbreeding colonies before and after the queens death (only colonies without sperm depletion).

Sex ratio differences were due to a significantly higher production of males in inbred colonies (n_i=13, n_o=13; t-test: T=-2.768, p=0.016). The average number of males produced by inbred colonies was 25 (SD=26.8) but only 4 in outbred colonies (SD=5.9). The production of female sexuals was not significantly different between both treatments (n_i=13 M=45, SD=90.5; n_o=13 M=26, SD=29.1; t-test: T=-0.712, p=0.480). Maximum worker number of colonies that produced at least one sexual was significantly higher in inbred colonies (n_i=13 M=82, SD=40.5; n_o=13 M=48, SD=36.9; T=-2.236, p=0.035; Fig.4).

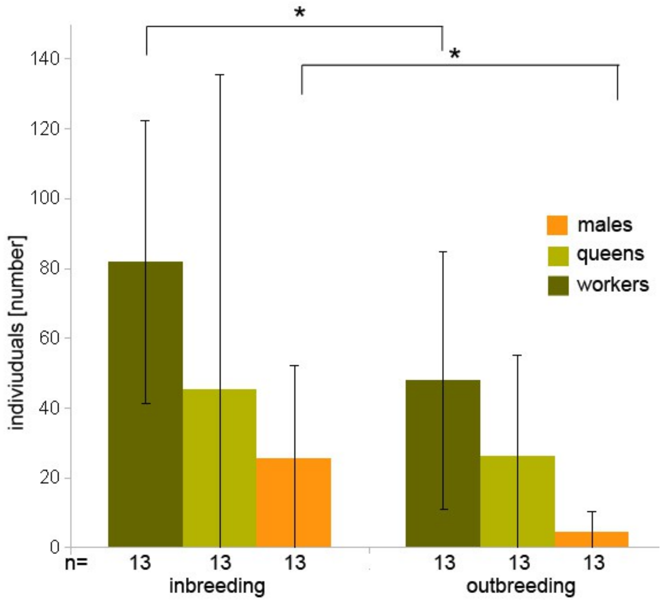


Fig.4 : number of sexuals and maximum worker number in both breeding lines.

In a generalized linear mixed model including breeding treatment and population, only breeding treatment remained as significant factor for sex ratio (breeding treatment, inbreeding *vs*. outbreeding: *t*= -2.20, *p*= 0.04), but because of the relatively large p- values these results need to be considered with caution (Zuur et al. 2009). Figure 5 shows that we found a higher sex ratio in outbreeding colonies, regardless from which population the queen came from.

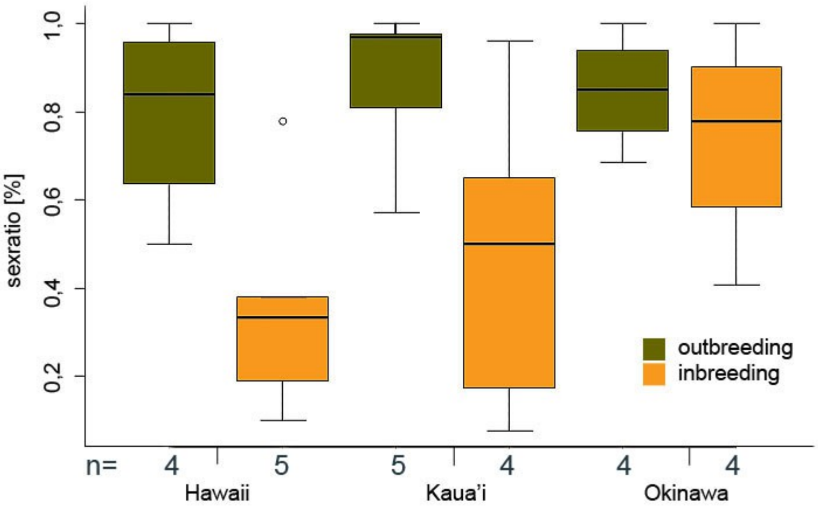


Fig.5: Sex ratio expanded by population of origin of the queen and treatment.

Sex ratio of inbred colonies did not change significantly after the queen' s death (before queens' death [bqd], n_i=12, median, quartiles: 0.41, 0.11, 0.70 % female sexuals, vs. after the queen's death [aqd], n_i=12, median, quartiles: 0.61, 0.18, 0.82 % female sexuals, Wilcoxon matched pairs test: Z=-0.889, p=0.374), whereas outbred colonies showed a significantly higher sex ratio after the death of the queen (bqd, n_o=8, median, quartiles: 0.83, 0.50, 0.99 % female sexuals vs. aqd, n_o=13, median, quartiles: 0.91, 0.84, 1.00 % female sexuals, Wilcoxon matched pairs test: Z=-2.028, p=0.043). From day -33 until day 27 (taking day 0 as day of queens death) sex ratio increased with time in both breeding lines (Spearman`s rank correlation test, n=21, inbreeding: r_s=0,799, p<0,001; outbreeding: r_s =0.595, p=0.004). Sex ratio differences at different dates between in- and outbred colonies were highly significant (n=21, median, quartiles: 0.42, 0.39, 0.46 % female sexuals, vs.0.78, 0.72, 0.83 % female sexuals, Wilcoxon matched pairs test: Z=-4.015, p<0.001).

Inbred ant queens produced sexuals over a longer time period during their lives than outbred colonies (n_i=17, M=66days, SD=50.8, n_o=15, M=34days, SD=32.8; t-test: t= -2.042; p=0.05). Considering this period as percentage of queen's lifespan differences between the breeding lines vanished (median, quartiles: inbreeding; 0.40, 0.28, 0.49 %queen lifespan, outbreeding; 0.27, 0, 0.42 %queen lifespan, Mann-Whitney U-test: U=84.5, p=0.105).

Sex ratio after queen's death and sex ratio over whole colony life in inbred colonies correlates negatively with the average number of workers (Spearman`s rank correlation test, total sex ratio: n=13, p=0.019, r=-0.637, sex ratio after queen's death: n=12, p=0.001, r=-0.823). In inbred colonies, sex ratio after the queen's death was also negatively correlated with the lifespan of the queen (Spearman`s rank correlation test, n=12, p=0.024, r=-0.644).

In outbred colonies, total sex ratio and sex ratio after queen's death were correlated with the appearance of first male and worker pupae (Spearman`s rank correlation test, collective sex ratio: first worker n=13, p=0.029, r=0.605, first male, n=10, p=0.049, r=-0.0634; sex ratio after queens' death: first worker n=13, p=0.030, r=0.600, first male, n=10, p=0.039, r=-0.0656). Sex ratio until queen's death was negatively correlated with average number of workers (Spearman`s rank correlation test, n=8, p=0.040, r=-0.731).

Lifespan of queens and males

Queens that mated with a sibling (inbred colonies, n_i=20) showed a slight but insignificant longer median lifespan (median, quartiles 118, 143, 170.3 days) than queen's that mated with a male from a different population (outbred colonies, n_o=15, median, quartiles 95, 123, 150 days; Mann-Whitney U-test, U=107, p=0.158). When only colonies were taken into account that produced all types of pupae, inbred queens lived significantly longer that outbred queens (n_i=14, median, quartiles 155, 121, 180.3 days; n_o=12, median, quartiles 120.5, 91.3, 147.3; Mann-Whitney U-test, U=41.5, p=0.027; Fig.6).

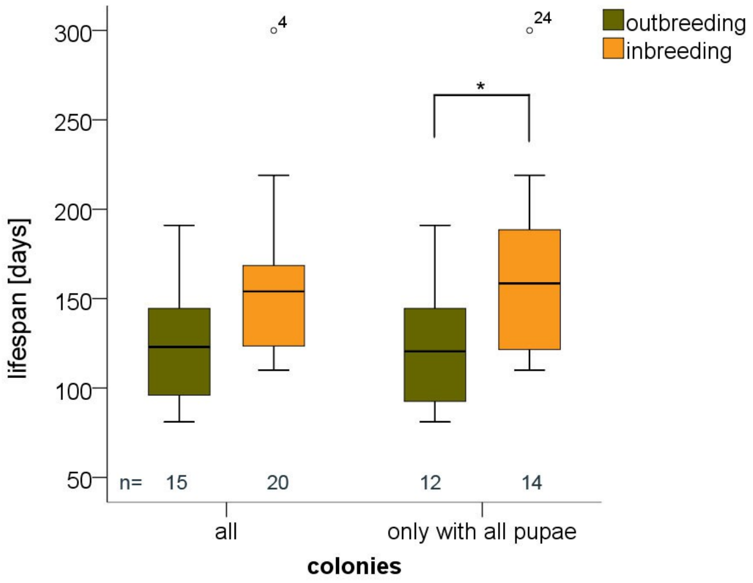


Fig.6: Lifespan of queens of all colonies and lifespan of queens exclusively of colonies that produced all types of pupae.

Queens in both breeding lines that could establish a colony, i.e. were able to produce pupae, did live significantly longer that queens that failed to produce pupae (colonies without pupae n_i=6, median, quartiles 81, 52.8, 117.3; n_o=18, median, quartiles 75.5, 60, 94.8, Mann-Whitney U-test, inbreeding: U=14, p=0.003, outbreeding: U=6, p=0.001).

Queen longevity in inbred colonies correlated with the timing of first male pupae (n=16, r_s=0.654, p=0.006), the average number of workers (n=19, r_s=0.694, p=0.001), worker number at queen death (n=19, r_s=0.523, p=0.021) and the number of all sexuals (n=17, r_s=0.555, p=0.021). The average number of workers and the number of workers at queen death were correlated (n=19, r_s=0.876, p<0.001). In a generalized linear mixed model, total sexual production (t=7.8, p< 0.001) and the number of workers at the queen's death (t=3.2, p=0.025) remained as significant factors.

Queen lifespan in outbred colonies was only correlated with the maximum number of eggs, albeit at marginal significance (n=15, r=0.462, p=0.083).

Discussion

We could show that queens of the ant *C.* cf. *kagutsuchi* were able to establish flourishing colonies, regardless of whether they mated with a brother or a male from a different population.

We could not detect any evidence for inbreeding depression. This is not surprising as inbreeding appears to be common in *C.* cf. *kagutsuchi* and other *Cardiocondyla* species. In *C. obscurior*, inbreeding depression could be shown only after several generations of sib-mating (Schrempf et al. 2006). In contrast, our experiments revealed outbreeding depression at a very early stage of the hybrid generation. Although both inbred and outbred colonies resembled each other in many colony parameters, we could find three striking differences:

first, the lifespan of queens of outbred colonies was slightly shorter than that of queens from inbred colonies; second, outbred queens were less successful in colony founding, and third, sex ratio was more female biased in outbred colonies as only few males were produced.

A longer lifespan of inbred queens could possibly be explained by the co-evolution of males and females of closely related reproductive units (population or even colony). Seminal fluid proteins transferred through mating are a known to enable males to manipulate the behavior and physiology of females to increase their own reproductive success (Poiani 2006). In fruit flies, seminal fluid proteins transferred during mating can increase the egg laying rates of females (Herndon & Wolfner 1995), while shortening their lifespan (Fowler & Partridge 1989). The male benefits from this manipulation as the female invests all its resources in the production of eggs fertilized with the sperm obtained during the present copulation. In ants, males benefit from long living queens as queens store sperm after mating in their spermathecae throughout their lives. Mated queens of *C. obscurior* live longer than unmated queens, and it was suggested that males transmit substances that the queen's lifespan extend (Schrempf et al. 2005a). In crickets, where females receive a nutrition-rich spermatophore as nuptial gift, females actively choose males whose ejaculates more strongly increase their lifespan and reproductive success (Wagner and Harper 2003). In our experiment, females of the outbred line were only presented one single male from a distant population. An unrelated male might further be equipped with seminal fluid proteins to which the queen is not adapted which therefore might not benefit the queen as strong as those of males from their own population. Reproductive proteins evolve very rapidly due to high selection pressure and it is assumed that they might also play an important role for speciation (Swanson & Vacquier 2002, Clark et al. 2006a).

The most conspicuous and probably the most important result was the large number of colony foundations in outbred colonies that failed because eggs did not hatch. In both breeding lines, queens, whose eggs did not turn into larvae and therefore failed to establish a colony, laid significantly fewer eggs and lived significantly shorter than did successful queens. This might reflect variation in queen quality (Shukla et al. 2013). However, it is suggestive that queens that mated with an unrelated male had a 60% higher colony founding failure rate. A similar result was found in the highly inbred ambrosia beetle *Xylosandrus germanus*, in which outbreeding depression manifests in reduced hatching rates (Peer & Taborsky 2005). Failed egg development in the outbreeding line might reflect incipient speciation with post-copulatory reproductive isolation. The genomes of the mating partners may no longer match properly due to genetic differences between the populations. Many phenotypic traits and biochemical pathways are coordinated by more than one gene which is called "within genotype epistasis". Inheritance of alleles from one parents that does not fit to an allele inherited from the other parent can lead to inviable offspring conveyed in egg mortality in certain queen/male combinations. There may also be parent-offspring deleterious interactions in the zygote resulting in among-genotype epistasis (Wolf 2000) that also can have lethal effects. Another post mating isolation barrier is cytoplasmic incompatibility caused by symbiotic bacteria, which are very widespread in ants (Zientz et al. 2005). Bacteria with their rapid generation cycles diverge much more quickly into different strains than their symbiotic partners and restricted gene flow between populations with symbiotic bacteria often leads to considerable bacterial differentiation. The consequence is cytoplasmic incompatibility in crosses between mating partners with different bacterial strains (Gotoh et al. 2007, Perlman et al. 2008, Brucker & Bordenstein 2012). If nuclear genes in the zygote are not adapted to certain strains of endosymbionts, this incompatibility may result in nonviability of the F1 generation through frozen egg development (Bordenstein 2003). In their recent investigation, Brucker and Bordenstein (2013) could show a strong co-adaptation between the host genome in genus *Nasonia* and the microbiome of their gut. Hybridization destroys this co-adaptation and results in hybrid lethality and possibly in speciation. *C .kagutsuchi* contains endosymbiontic *Wolbachia* (Yoshizawa et al. 2009) and probably also other endosymbiontic genera, which was shown in other *Cardiocondyla* species (Oettler unpubl.).

Given that *C.* cf. *kagutsuchi* is a species complex it would be interesting to search for endosymbionts and their possible role in speciation of these ants.

The third notable difference between inbred and outbred colonies concerned the production of sexuals. This result however has to be considered with care. There is a heritable compound for sex ratio in *C.* cf. *kagutsuchi* (Frohschammer and Heinze 2009). Low number of stock colonies and unbalanced design (different stock colonies used in the two breeding lines) might predominantly explain differences in sex ratio in this experiment. Probably there are also some other reasons that shall be discussed below.

The significantly more female-biased sex ratio of outbred colonies was due to the production of more males in inbred colonies. The optimal progeny sex ratio - optimal in the sense that it maximizes the queen's fitness - is affected by local mate competition between related males and by relatedness asymmetries as they occur for example under inbreeding (Hamilton 1967, 1979). In many Hymenoptera, haploid males develop from unfertilized eggs and receive half of the chromosome set of their mother. Thus, queens are related to their sons by r=0.5. Diploid daughters inherit half the genome of each of their parents. Consequently, a queen is also related to its daughter by r=0,5. However, if a queen is related to the male she mates with, the relationship between mother and daughter increases as the daughter may inherit also genes from its father, which are identical by descent between mother and father. Hence, the optimal sex ratio in inbreeding haplodiploids is more female-biased (Herre 1985). Workers are then also more related to their sisters and should prefer to raise new queens instead of males.

The results of our experiments with inbred colonies producing a less female-biased brood than outbred colonies stand in contrast to this prediction. In *C. obscurior,* inbred colonies were more female- biased, but several colony strains began to produce predominantly males after several generations of inbreeding (Schrempf et al. 2006). Denver and Taylor (1995) showed in their model that the male proportion under sib mating increases if there is inbreeding depression. If the genetic load due to inbreeding is too high, females can profit from the exclusive production of males (Greeff 1996). In *C.* cf. *kagutsuchi,* female sexuals typically mate with their brothers inside the nest before either starting to breed in their natal nest or dispersing to found a new colony. Males usually kill dark male pupae and freshly eclosing brothers. If many male pupae are produced at the same time, the dominant male may not be able to execute all rivals and some safely eclosed males may escape from the nest (Yamauchi et al.2005, own observation). Alien *C.* cf. *kagutsuchi* males are generally accepted by queens and workers of other nests. An exchange of sexuals is also known from other *Cardiocondyla* species (Lenoir et al. 2007). As *C.* cf. *kagutsuchi* colonies are usually not far from each other, at least a few males may find their way to other colonies to mate. If there is a given load due to inbreeding queens inclusive fitness would profit from higher male production as males can disperse and outbreed.

In *Hypoponera opacior*, another ant with regular inbreeding, the effects of inbreeding appear to be regulated by an active increase of investment into dispersing sexuals, particularly in the dispersive generation (Kureck et al. 2012). Furthermore, higher production of sons will be reasonable if there is a certain local resource competition among dispersing queens.

We did not determine the ploidy of males and therefore cannot exclude the production of diploid males in inbred colonies. The absence of a single locus complementary sex determination mechanism was shown for *C. obscurior* (Schrempf et al. 2006), but though this is presumably also the case for other species of this genus, controlled inbreeding experiments have not yet been conducted. However, in the case of diploid males, worker number should be reduced as 50% of all diploid individuals would develop into diploid males. In our studies, worker number was higher in inbred than in outbred colonies.

The other factor for sex ratio equation- the number of female sexuals- did not differ between in and outbreeding colonies. Higher worker number in inbred colonies points to a higher queen- bias in diploid offspring. The commitment if a diploid egg will turn into a queen or worker is elicited by environmental and heritable triggers, to different species-specific degrees. In the ant *Pogonomyrmex* different genetically determined lineages are thought to exist (Anderson et al. 2006) where sexuals are produced only through intra-lineages mating whereas from inter-lineages mating only workers result. Different lineages could not be proved, but Schwander and Keller (2008) agree that phenotypic plasticity in *Pgognomyrmex* rugosus is affected by the combination of the parental genomes.

More and more studies prove that ant queens in several species have more impact on caste determination as it was assumed some decades ago (Schwander et al. 2010). Via its genetic predisposition and that of its chosen mate but also via maternal effects that may be the queens age of the overwintering status (Schwander at al.2008)

In *C.*cf. *kagutsuchi* a maternal heritable component of caste determination could be verified (Frohschammer & Heinze 2009). Queens stemming from colonies where caste determination was more queen-biases also produced a higher amount of queens in their diploid offspring.

Nevertheless the percentage of produced female sexuals can partly also be due to differential feeding of female larvae by the workers (Hammond et al. 2002). *C.* cf. *kagutsuchi* females can mate and remain inside their natal nest contributing to egg laying. For the persistence and growth of a colony investment into female sexuals is therefore most important. An increased number of female sexuals translates into faster colony growth. If workers could recognize the reproductive potential of their queen and realize e.g. that the queen will not be very long lived, workers would profit from an early start and high amount of female sexual production. We could indeed observe a significantly earlier start of the production of female sexuals in outbred colonies. The equal production of female sexuals in both breeding lines but the significant lower production of workers in outbred colonies compared to the inbreeding line may indicate the decision of workers to manipulate allocation in favor of female sexuals.

In conclusion, *C.* cf. *kagutsuchi* appears to be well adapted to regular sib-mating. We could not find any negative effects of inbreeding but rather a slight outbreeding depression at a very early stage of F1 generation. Ineffective colony founding and a shorter lifespan of the queens of outbred colonies may be due to genetic differences between the founding queen and its mate. In successful outbred colonies, workers might be able to balance female allocation to even out the costs caused by the queens mating with distant related males.

Further studies, in particular selected outbreeding observations over more generations, might shed light on the long- term success or failure of outbreeding colonies.

Chapter 4:

Queen lifespan and total reproductive success are positively associated in the ant *Cardiocondyla* cf. *kagutsuchi*

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Abstract

While reproduction and longevity are negatively correlated in a large number of animals, this fundamental trade-off appears to be absent from social insect queens. Here we examine the distribution of the lifespans of queens and their mates and the age trajectory of reproduction in experimental colonies of the Pacific tramp ant *Cardiocondyla* cf. *kagutsuchi.* Queens of *Cardiocondyla* are generally short-lived (0.5 - 2 years), which allows determining their lifetime reproductive success and establishing mortality tables. We show that the queen's total number of sexual offspring is positively associated with its lifespan. Regular counts of eggs, larvae, and pupae suggest that more fecund queens live longer than less fecund queens and that an early onset of sexual production does not negatively affect queen lifespan. This corroborates the view that the costs of reproduction are not born by the queen itself. The number of eggs present in colonies increased with queen age until shortly before death, indicating negligible reproductive senescence. Sex ratios were strongly affected by the origin of queens but neither by colony size nor total productivity. Several queens produced only males late in their lives, suggesting the occurrence of sperm depletion. Male lifespan was not correlated with any other studied trait. Our study shows that the ant genus *Cardiocondyla* provides a suitable model system to investigate the evolution of lifespan in social insect queens and males.

Introduction

Reproductive lifespan and age-specific fecundity are among the most fundamental life history traits of organisms. The correlations and trade-offs among them and how they evolve are not completely understood. Recent theoretical and empirical work suggests that social insects might provide ideal model systems for investigating the proximate and ultimate causes of variation in longevity (e.g., Keller & Genoud 1997, Bourke 1999, 2007, Jemielity et al. 2005, Münch et al. 2008, Rueppell 2009, Parker 2010). For example, the enormous differences between the average longevities of reproductives and non-reproductives of ants, honey bees, and termites allows testing evolutionary theories of aging and determining the physiological and epigenetic correlates of lifespan polyphenism (e.g, Keller & Genoud 1997, Heinze & Schrempf 2008, Parker 2010). Furthermore, social insects might be particularly suitable to explore models of kin or group selected adaptive senescence and programmed individual death (e.g., Travis 2004, Dytham & Travis 2006, Mitteldorf 2006, Ronce & Promislow 2010, Markov 2012).

With exception of a few well-studied examples, such as honey bees (e.g., Page & Peng 2001, Remolina & Hughes 2008, Rueppell 2009), information on mortality rates, queen age and colony size at maturity, or the age-trajectory of reproduction is fragmentary at best, if available at all (e.g., Tschinkel 1991, Keller 1998). Such data are often not easily accessible, as many social insect species have extremely long-lived, populous, and often subterraneous colonies (but see Cole 2009). Annual species, such as bumble bees (Müller & Schmid-Hempel 1992, Lopez-Vaamonde et al. 2009), and perennial species with small colony size and short generation time, such as *Cardiocondyla* ants, might therefore be useful alternatives to learn more about the complex interrelations among colony growth rate, queen longevity, and reproductive success in social insects.

The ant genus *Cardiocondyla* comprises about 100 species with a strikingly large variation of life histories (Seifert 2003, Oettler et al. 2010). Colonies of xerothermic species from Southern Europe and Western Asia usually contain only a single, multiply-mated queen (monogyny, polyandry), which may survive for more than two years. In contrast, colonies of Southeast Asian *C. obscurior* usually have multiple, singly-mated queens with an average lifespan of only 182 days (Schrempf et al. 2005a, 2011). The lifespans of the wingless, locally mating *Cardiocondyla* males are similarly variable: while males of some species survive only for a few weeks (Schrempf et al. 2007), those of others have been observed to live for more than one year (Yamauchi et al. 2006), making them by far the most long-lived males among the social Hymenoptera.

We have recently used the cosmotropical tramp *C. obscurior* to determine how egg laying rate and lifespan are associated and how the social environment influences queen longevity and life time reproductive success (Schrempf et al. 2011, Heinze & Schrempf 2012). Here, we extend our studies to its distantly related congener *C.* cf. *kagutsuchi.* Both species are facultatively polygynous tramp ants but differ in several important life history traits, such as the morphology and behavior of their wingless males (sickle-shaped vs. shear-shaped mandibles and lethal fighting among adult males vs. attacks only towards freshly eclosed rivals, Oettler et al. 2010). Most importantly, *C. obscurior* nests in extremely ephemeral sites in plants, such as rolled lemon tree leaves or the cavities under the bracts of aborted coconuts, while *C.* cf. *kagutsuchi* inhabits more stable nests in the soil (Seifert 2003). This likely affects the external mortality risk and thus the life expectancy of queens (e.g. Keller & Genoud 1997). Furthermore, while we standardized colony size in *C. obscurior* by removing worker pupae we allowed colonies of *C.* cf. *kagutsuchi* to grow without restriction.

We recorded the lifespan of queens and their mates, colony growth patterns, the timing of sexual production, lifetime reproductive success, and sex ratio in experimental colonies of *C.* cf. *kagutsuchi* from Hawaii, Kauai, and Okinawa. From models on colony growth and sex allocation we expected colonies to switch to the production of sexuals only after reaching a certain worker number and particularly large colonies to rear a more female-biased sex ratio than small colonies. In accordance with our previous results – and in contrast to many solitary animals – we expected that no trade-offs exist between early and late reproduction and between longevity and total reproductive success. Finally, we examined whether the fecundity of *C.* cf. *kagutsuchi* queens declines towards the end of their life, as expected from models of reproductive senescence.

Material and Methods

In 2006, complete colonies of *C.* cf. *kagutsuchi* Terayama, 1999 were excavated from their nests in the upper level of soil in various sites on the two Hawaiian Islands Hawaii and Kauai and on Okinawa, Japan. This particular taxon of *C.* cf. *kagutsuchi* is a tramp species that has been introduced to various Pacific islands through human activities (Seifert 2003) and has only wingless males, in contrast to other taxa of this clade from Southeast Asia (Yamauchi et al. 2005).

Populations exhibit very little genetic diversity (Yamauchi et al. 2005), suggesting founder effects in their recent history.

In the laboratory, all colonies were kept in three-chambered boxes in the same incubator under near-natural conditions (e.g., 12h light 15°C / 12h dark 25°C, unregulated humidity). More than 100 experimental colonies were set up from four stock colonies each from Okinawa and Kauai and three stock colonies from Hawaii. Young queens and males of tropical *Cardiocondyla* are produced throughout the year (see, e.g., Heinze & Delabie 2005 for *C. obscurior*) and because of the limited number of sexuals that emerged at the same time, individual experimental colonies were started between January 2007 and January 2008. As expected from the aseasonal production of sexuals, neither queen lifespan (Kruskal-Wallis test, $H_{10,55}$=5.949, p=0.820) nor total sexual production ($H_{10,55}$=9.702, p=0.467) were affected by the month in which the particular experimental colony was established, and the start of the experiment was therefore not further included in the analysis. An unmated, female sexual pupa was placed into a new nest box containing ten workers, several larvae and a male pupa from the same colony. Workers that died during the first few days of the experiment were replaced by new workers from the stock colony to maintain the initial number of ten workers. Replacement stopped when the colony had begun to produce own workers. Unlike many other social insects, male and female sexuals of *Cardiocondyla* readily mate in the nest both in the field and under laboratory conditions. Prolonged inbreeding without the production of diploid males is the rule in all previously studied species (e.g., Schrempf et al. 2005b, 2006, Lenoir et al. 2007, Schrempf, unpubl.). Sib-mating as in our experiment therefore presents a natural condition. Queens mated within the first few days of their adult lives and shortly thereafter shed their wings. The timing of this event was taken as the starting point of the queen's reproductive life. To avoid confusion with the parental pair, we took care to remove all freshly eclosing males and female sexuals.

We monitored the experimental colonies every two to three days for the presence of brood and workers and the survival of the queen. Colonies that failed during the first few days after being set up were removed from the analysis, leaving a total of 69 colonies for the analysis of the following life history traits: lifespan of queen and its mate and date of eclosion of the first male or female sexual pupa (relative to the date of wing loss), number of eggs per scan (starting 20 days after the appearance of the first eggs), maximum number of eggs, number of workers per scan (starting 20 days after the appearance of the first pupae), number of workers at the time of the queen's death, total number of male and female sexuals produced, sex ratio (female sexuals / all sexuals), and number of workers at the eclosion of first sexual pupae. As *Cardiocondyla* eggs develop into larvae within approximately one week and workers do not have ovaries (see also Heinze & Schrempf 2012), egg counts allow estimating the actual fecundity of queens. Not all traits could be recorded for all colonies, e.g., some colonies did not produce sexuals or male longevity could not be measured because a male pupa was overlooked and the two adult males could not be distinguished. Dead queens were quickly dismembered by workers and some corpses disappeared completely. Only ten queens were sufficiently intact to measure body size (Weber's thorax length, Weber 1938). Because several queens had the same body size, we determined by gamma correlation whether body size effects longevity.

Prior to multivariate statistic analysis, we investigated interrelations among traits by Spearman rank correlation tests. Several pairs of measures were highly correlated because they provided different estimates of the same underlying biological trait (average and maximum number of eggs, $n=56$, $r_S=0.917$, $p<0.0001$; average number of workers and number of workers at the time of the queen's death, $n=55$, $r_S=0.791$, $p<0.0001$; total sexual production and number of female sexuals, $n=49$, $r_S=0.797$, $p<0.0001$). We therefore excluded one factor from each of these pairs and also other recorded traits (e.g., average number of larvae and pupae) from the subsequent analysis to avoid redundancy.

Because of missing data, unbalanced design and the complex interrelations among the remaining life history traits (lifespan, worker number, sexual production, sex ratio etc.) we analyzed our data in two different ways. First, we conducted several univariate non-parametric analyses. Because of the absence of censored data, life expectancies could be compared by median and Mann-Whitney U-tests. Using median tests we initially investigated whether population and stock colony affect any of the dependent variables (e.g., longevity of queens and males, total sexual production, sex ratio, maximal number of eggs present per scan, number of workers at death, etc.). Thereafter we investigated possible associations among traits by separate Spearman rank correlation tests, using Statistica 6.0. p-values were corrected for the 15 pairwise tests using Holm's sequential Bonferroni method (Holm 1979), yielding p*-values.

Second, we used generalized linear mixed models (GLMM) to analyze the longevity of queens and males, total sexuals and sex ratio. As the lifespan of queens and males as well as the total number of sexuals were count data and sex ratio was proportional data, we used Poisson and binomial error distributions, respectively. Because of overdipersion, the standard errors were corrected using a quasi-GLM model (Zuur et al. 2009). For longevity of queens and males, we started with a full model that included populations, sex ratio, total sexuals, number of workers at the time of the queen's death, and maximum number of eggs as fixed effect factors. The model used for total sexuals included populations, number of workers at the time of the queen's death, and maximum number of eggs. The model for sex ratio was similar to that used for the total sexuals, except that it also included total sexuals as an additional fixed effect factor. Because experimental colonies were set up from stock colonies of different populations and to account for pseudo-replication, the factor colony was entered as a random effect factor in all models.

For each dependent variable, we obtained a minimal model by a stepwise backward elimination procedure, i.e. by successively removing all non-significant terms ($p>0.05$). All analyses were performed with the statistical software R 2.11.0 (R Development Core Team, 2010) and the glmmPQL function from the MASS library of R.

Results

Effects of population and colony

Of the more than 100 queens originally used in the experiment, only 69 laid eggs, 55 produced worker offspring, and 49 produced male or female sexuals or both. Population (Hawaii, Kauai, Okinawa) appeared to have an effect on the maximal number of eggs ($\chi^2=6.456$, $p=0.039$) and queen longevity ($\chi^2=8.775$, $p=0.012$). Colony had a strong effect on sex ratio (median test, df=10, $\chi^2=26.991$, $p=0.003$, see also Frohschammer & Heinze 2009). None of the other traits were significantly affected by colony or population.

Lifespan of queens and males

Queens from Okinawa lived slightly longer than queens from Hawaii and Kauai (median, quartiles: 139, 123, 158 days vs. 123, 81, 154 days; Mann-Whitney U-test, $n_1=19$, $n_2=50$, $U=325.5$, $p=0.045$; Fig. 1). Hazard rate (the probability that after surviving until the beginning of a certain time interval a queen will die during this time interval) increased with time (due to sample size only studied in the combined sample from Hawaii and Kauai, Spearman rank test, n=11 intervals, $r_S=0.769$, p=0.002).

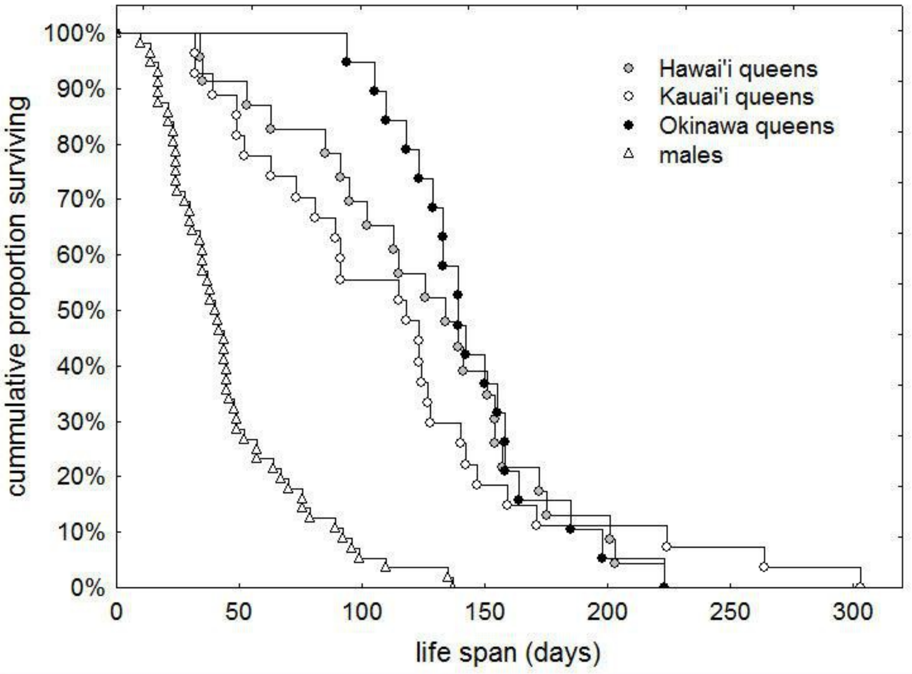

Fig. 1: Survival rates of queens and males of the ant *Cardiocondyla* cf. *kagutsuchi* from three populations.

In univariate tests, queen longevity was strongly correlated with the maximal number of eggs (n=69 r_s=0.658, p<0.0001, p*<0.0001), the number of workers present at death (n=69, r_S=0.548, p<0.0001, p*<0.0001) and the total number of sexuals (n=49, r_S=0.552, p<0.0001, p*<0.0001, Fig. 2). The latter three traits were all closely correlated among themselves (workers and eggs, n=69, r_S=0.531, p<0.0001, p*<0.0001; workers and sexuals, n=49, r_S=0.411, p=0.003, p*=0.036; sexuals and eggs, n=49, r_S=0.398, p=0.005, p*=0.047). In a generalized linear mixed model including population, maximal eggs, sex ratio, total number of sexuals and worker number at death, the latter two traits remained as significant factors (total number of sexuals, t=5.541, p<0.0001; worker number at death t=3.417, p=0.002).

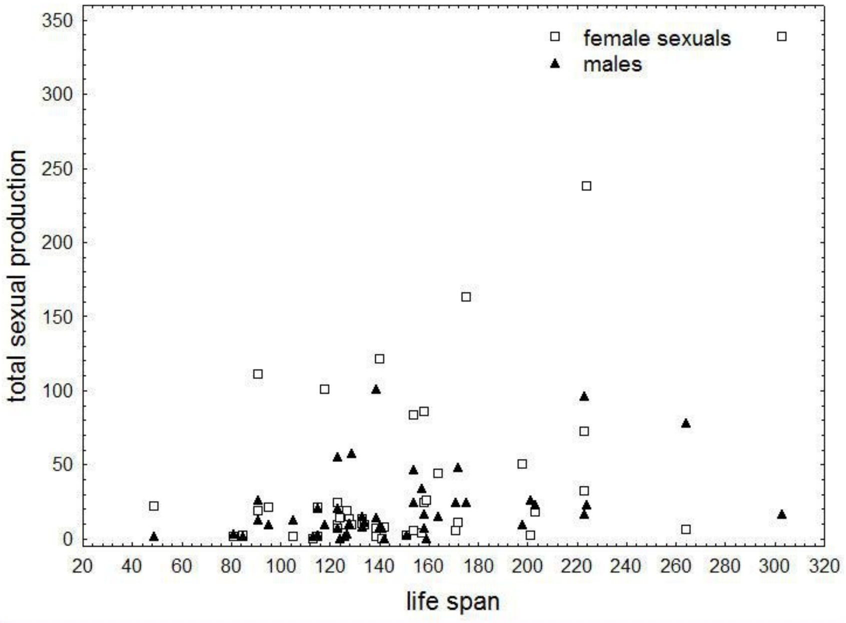

Fig. 2: Total number of female sexuals and males produced by 46 queens of the ant *Cardiocondyla* cf. *kagutsuchi* throughout their lives.

Median lifespan of males was 40.5 days (quartiles 24, 57 days, n=56), i.e., significantly shorter than that of their queens (Wilcoxon matched pairs test, T=21, $p<0.0001$, Fig. 1). In univariate tests, male longevity appeared to be completely independent from any other measured trait. A generalized linear mixed model revealed sex ratio (t=2.188, p=0.037) and total number of sexuals (t=-2.328, p=0.027) as significant factors, but because of the relatively large p-values these results need to be considered with caution (Zuur et al. 2009).

Though only ten queens could be measured, queen longevity appeared to be correlated with their thorax length ($\Gamma=0.512$, p=0.049). In this small sample, total sexual production was not correlated with body size ($\Gamma=0.166$, p=0.646). In a linear regression, body size explained only 13% of the variation in queen lifespan and the association between longevity and body size was no longer significant when the largest queen was removed from the data set ($\Gamma=0.434$, p=0.243).

Pattern of reproduction

The number of eggs present in the colony increased with the day of life of the queen (Spearman's rank correlation test, significant at the 0.05 level after Holm's sequential Bonferroni correction for multiple testing in 35 of 47 colonies, median $r_S=0.746$). It reached a maximum after 74.5% of the lifespan of queens (median, quartiles 62.0, 93.1%). Okinawa colonies (n=16) contained on average more eggs per scan than colonies from the Hawaiian Islands (Hawaii and Kauai, n=40, median, quartiles 35.2, 27.5, 40 vs. 25.5, 14.9, 30.1; U=150, p=0.002). This, however, did not translate into an increased worker number at death of the queen (χ^2=2.829, p=0.243). Colonies contained fewer eggs on the day of the queen's death than one week before (Wilcoxon matched pairs, n=41, T=83, p<0.0001, Fig. 3).

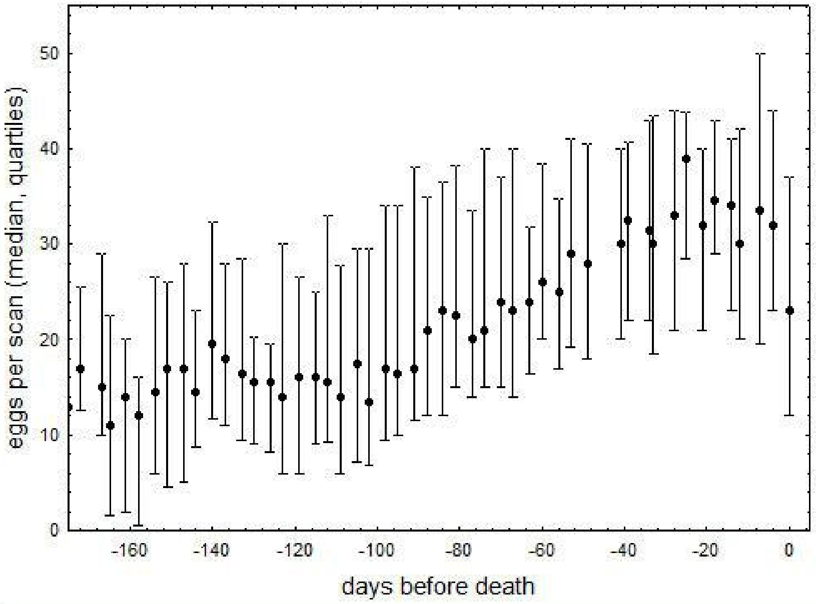

Fig. 3: Eggs (median, quartiles) present in experimental single-queen colonies of the ant *Cardiocondyla* cf. *kagutsuchi* relative to the time of the queen's death.

The number of workers present per colony was significantly correlated with the day of life of the queen in 38 of 47 colonies (Spearman rank correlation test with Holm's correction for multiple testing, p<0.05, median r_S=0.928) and on average reached its maximum at the day of the queen's death (median, quartiles 58, 29.25, 80). Queen productivity (maximal number of eggs per scan) and the number of workers at death were positively correlated (n=69, r_S=0.531, p<0.0001).

Queens produced new workers (n=55, median, quartiles, 49, 42, 54 days) significantly earlier than males (n=46, 92.5, 87, 102 days; Wilcoxon matched pairs test, n=44, T=0, p<0.0001) and female sexuals (n=43, 101, 67, 115 days; T=2.5, p<0.0001). There was no difference between the timing of the production of the first male and female sexual pupae (n=39, T=234.5, p=0.281, Fig. 4). Males were produced at a median worker number of 26.5 (n=44, quartiles 19, 33) and female sexuals at a median worker number of 35 (n=43, quartiles 10, 49). In a generalized linear mixed model, the total number of sexuals was significantly affected by the maximal number of eggs (t=4.262, p=0.0001).

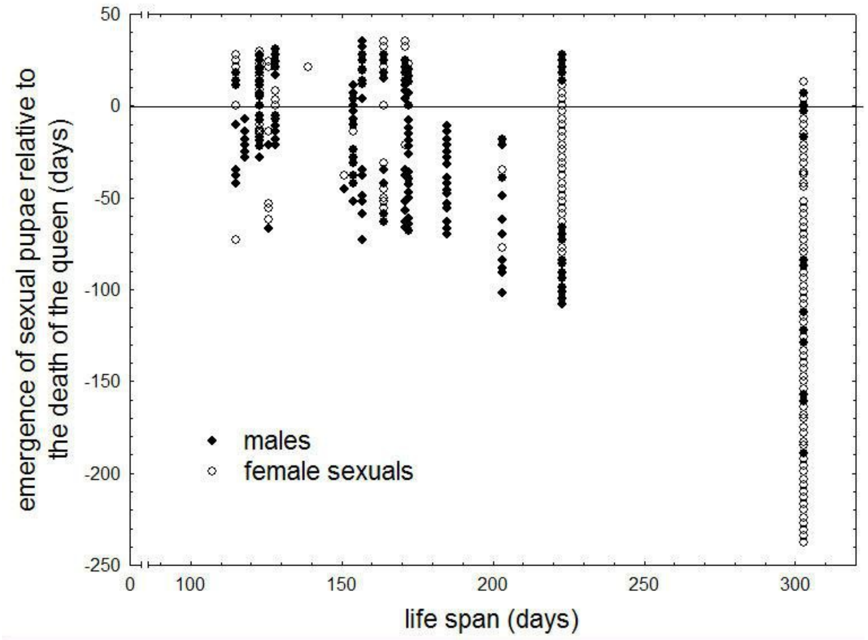

Fig. 4: Date of emergence of female sexual and male pupae produced by queens of the ant *Cardiocondyla* cf. *kagutsuchi* with different lifespan, relative to the time of the queen's death. Each vertical line depicts the age-trajectory of sexual production of an individual queen (n = 16).

Sex ratios varied widely and ranged from 0 to 1 (median, quartiles 0.5, 0.14, 0.85). They were affected by colony (see above) and were positively associated with the total number of sexuals (n=49, r_S=0.283, p=0.048), but this was no longer significant when only colonies were taken into account that had produced five or more sexuals (n=41, r_S=0.220, p=0.166). In a generalized linear mixed model with colonies producing five or more sexuals, the sex ratio was not affected by the total number of sexuals (t=1.651, p=0.109).

Queens that started to produce sexuals early in their life did not trade-off the early onset of reproduction against longevity (eclosion of first male pupa vs. lifespan, n=49, r_s=0.0611, p=0.677; eclosion of first female sexual pupa vs. lifespan: n=38, r_s=- 0.094, p=0.576). In contrast, those queens that produced their first sexuals proportionally early in life produced more sexuals (percentage of lifespan vs. total sexuals, first male, n=45, r_s=-0.532, p<0.0002; first female sexual, n=43, r_S=-0.398, p=0.008).

A considerable fraction of the offspring particularly of short-lived queens eclosed after the queen's death (Fig. 4). Overall, sex ratios produced before and after a queen's death were highly correlated (n=26, r_S=0.867, p<0.0001). Before queen death, usually only one or two male pupae were present in a colony, but shortly before and after queen death eight colonies simultaneously contained many more (up to 12) male pupae, resulting in highly male-biased posthumous sex ratios (between 0 and 0.25).

Discussion

Our study shows that total reproductive success and lifespan of queens are positively associated in the ant *Cardiocondyla* cf. *kagutsuchi*. This is not merely a consequence of longer-lived queens being able of producing offspring over a longer time span. In univariate tests, lifespan was also positively correlated with the number of eggs present during observation scans, meaning that more fecund queens lived longer than less fecund queens. Furthermore, an early onset of the production of sexuals did not negatively affect total reproductive success or longevity. These data not only corroborate the well known phenomenon that reproductives in social insects outlive non-reproductives (e.g., Tsuji et al. 1996, Keller & Genoud 1997, Hartmann et al. 2003, Heinze & Schrempf 2008, Parker 2010) but in addition indicate that lifespan positively correlates with the degree of queen fecundity. This stands in striking contrast to the commonly observed trade-off between reproduction and survival in solitary insects and other animals (e.g., Carey 2001). In addition, our study failed to reveal a trade-off between the production of sexuals and workers – instead, both were positively correlated (for a similar phenomenon in a halictid bee see Strohm & Bordon-Hauser 2003).

In principle, a positive association between longevity and fecundity might reflect variation in the genetic quality of individuals and varying resource availability. For example, queens with particularly good genes or living in a very rich environment might be able to invest more in both longevity and fecundity than individuals with lower quality and restricted resources (e.g., Reznick et al. 2000). We can exclude this explanation for the apparent lack of a cost of reproduction in our study. First, all stock and experimental colonies were kept under standardized conditions and equally fed diluted honey and pieces of insects ad libitum. Second, a large genetic influence on longevity and fecundity is unlikely at least in the queens from Hawaii and Kauai, as they came from several highly inbred stock colonies and the factor colony did not have a significant effect on either trait. Nevertheless, queens might have been treated differently during larval development. Indeed, longevity appeared to be correlated with queen body size in the few individuals in which thorax length could be measured. In several species of insects, female body size and fecundity are positively correlated (e.g., Bosch & Vicens 2006), but the magnitude of the association between body size and lifespan varies tremendously across taxa (e.g., Khazaeli et al. 2005). More data are needed to determine whether the variation in queen longevity and fecundity in *Cardiocondyla* is indeed a mere consequence of size and, if so, which factors create size variation among close relatives and under standardized conditions.

The linkage of lifespan and fecundity might also reflect the involvement of common molecular mechanisms in the regulation of both traits (e.g., Corona et al. 2007). Furthermore, it supports the view that, in eusocial insects, the costs of reproduction are at least partly born by the workers (Heinze & Schrempf 2008). The fecundity of queens of *C.* cf. *kagutsuchi* might therefore have increased with the number of workers present in the colony. Indeed, worker number and queen egg laying rate were positively correlated. However, in a previous study with *C. obscurior* it was shown that the number of eggs increased with queen age even when worker numbers were kept constant (Heinze & Schrempf 2012). The association between queen age and egg laying rate did not differ strikingly between this and our study, even though we allowed worker numbers to increase. Though intraspecific comparisons of the queens' reproductive trajectories under different experimental designs still have to be made, the similarities between *C. obscurior* and *C.* cf. *kagutsuchi* might indicate that the reproductive output of single-queened colonies is more limited by the egg laying capacity of the queen than by the availability of foragers and nurses. Indeed, colonies of *C.* cf. *kagutsuchi* from Okinawa had a median of 34 workers and usually contained several queens (Oettler et al. 2010).

The increase of egg number with queen age also stands in contrast to the common decline of the reproductive efforts of female insects (e.g., Dixon & Agarwala 2002, Akman Gündüz & Gülel 2005, Novoseltsev et al. 2005) but matches previous observations in solitary Hymenoptera with parental care (Bosch & Vicens 2006) and both annual and perennial social Hymenoptera (Lopez-Vaamonde et al. 2009, Heinze & Schrempf 2012). In contrast to *C. obscurior* (Heinze & Schrempf 2012), the number of eggs present in colonies of *C.* cf. *kagutsuchi* reached a maximum after about ¾ of the queen's lifespan and thereafter remained constant. The slight decrease immediately before the queen's death might be due to our sampling of egg counts only every two to three days, i.e., since the death of the queen a few eggs may have developed into larvae and presumably does not reflect reproductive senescence.

After a short "ergonomic" phase of about 50 days, during which queens produced exclusively worker offspring, sexuals were reared throughout the whole lifespan of queens. Apparently no minimum worker number was necessary for brood to be reared to sexuals. Sex ratios over the whole lifespan of queens varied tremendously between colonies and overall were much less female-biased than expected for a species with local mate competition (e.g., Cremer & Heinze 2002, Schrempf et al. 2005b, Lenoir et al. 2007). Several unproductive colonies produced only one or two males in addition to workers, but when these colonies were excluded, sex ratios were no longer positively associated with total sexual production. As shown previously (Frohschammer & Heinze 2009), sex ratios were strongly influenced by colony: queens stemming from the same stock colony produced similar sex ratios. This appears to be due to heritable variation in the propensity of diploid brood to develop either into queens or workers (Frohschammer & Heinze 2009). The surprisingly balanced sex ratio might in part be explained by our regular removal of all sexual pupae, but in a few colonies it might result from sperm limitation. In some colonies, sex ratios became highly male-biased shortly before and after the death of the queen. It was suggested (Finch 1998) that social insect queens may die when they have run out of sperm. This might indeed have been the case for a minority of queens in our study, but others continuously laid fertilized eggs until their death. Egg hatchability did not decrease with queen age, as in a subset of colonies larva-egg ratios steadily increased with day of life (unpublished data).

The mortality curve of males resembled that of queens but males lived significantly shorter. The oldest male in our study survived for 137 days, which is about one third of the maximum lifespan of queens. Compared to other males of social Hymenoptera (e.g., Rueppell et al. 2005, Shik et al. 2012), *Cardiocondyla* males are relatively long-lived. Male lifespan did not correlate with any other measured trait, reflecting the fact that ant males do not actively take part in the social life of the colony.

In conclusion, queens of *C.* cf. *kagutsuchi* do not individually trade-off longevity and fecundity. Our egg counts suggest negligible reproductive senescence, and the production of diploid offspring from eggs laid shortly before the queen's death proves that sperm limitation is not a major factor causing queen death. It is therefore puzzling why queens do not live longer, given that those of related species may reach lifespans of up to two years (Schrempf & Heinze 2007). The similarly short lifespan of *C. obscurior* queens matches predictions from evolutionary theories of aging (Keller & Genoud 1997), as the species nests in ephemeral sites, such as folded lemon leaves or tree galls, and queens might be exposed to high external mortality risks during the resulting frequent moves to new nesting sites. Soil nests as in *C.* cf. *kagutsuchi* are presumably much more stable, but other as yet unknown differences in life history details between the two species might compensate for an eventually lower risk of mortality from external hazards.

Evolutionary models of senescence suggest that death may be adaptive in species with resource inheritance and limited dispersal, such as facultatively polygynous *C.* cf. *kagutsuchi*. Indeed, experimental manipulations in *C. obscurior* show that the lifespan of queens is reduced in multi-queen societies (Schrempf et al. 2011). However, the models require that the fecundity of reproductives declines with age (e.g., Dytham & Travis 2006, Bourke 2007, Markov 2012). Functional genomic analyses and detailed comparisons of life histories and the age trajectories of queen fecundity across different species *of* Cardiocondyla might help to identify factors that shape the evolution of queen life expectancy in this genus.

Acknowledgements

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Chapter 5:

Virgin ant queens mate with their own sons to avoid failure at colony foundation

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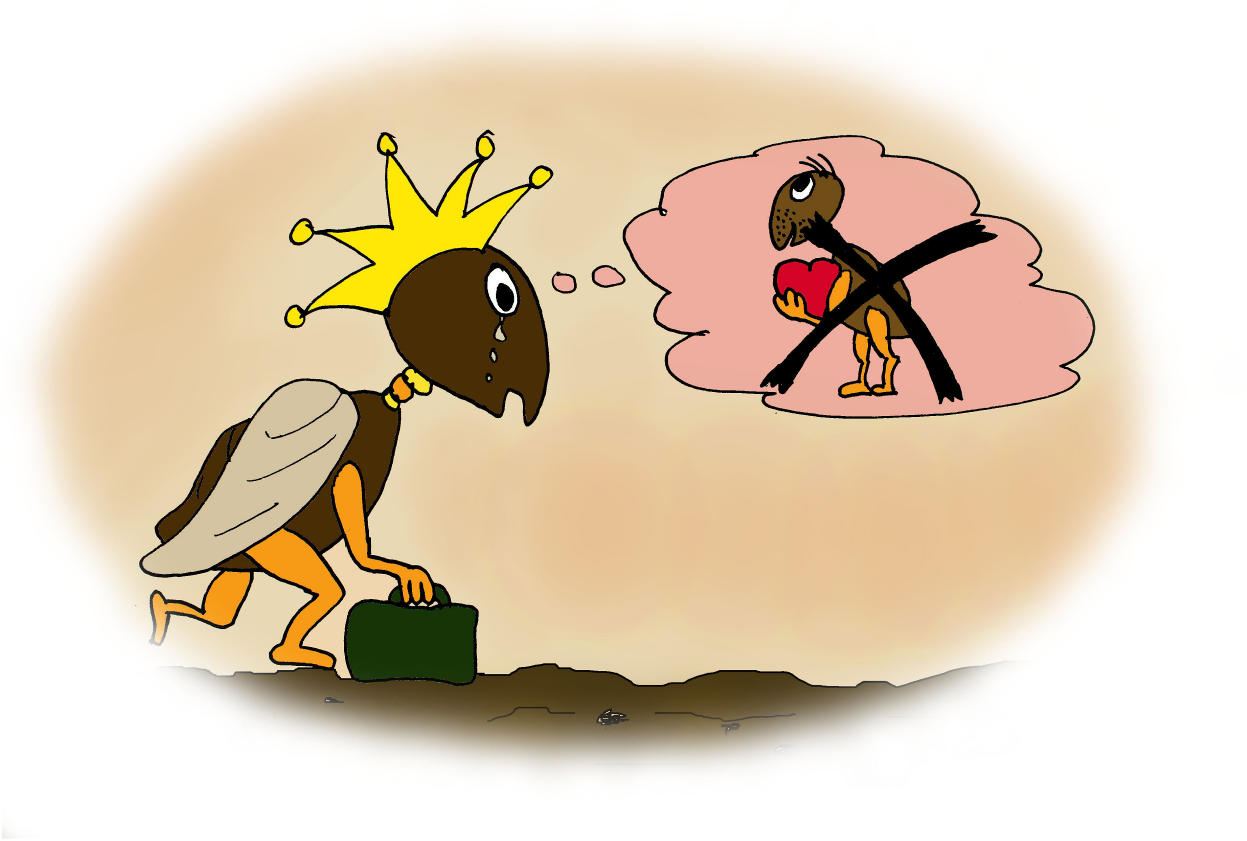

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Abstract

Mother-son mating (oedipal mating) is practically non-existent in social Hymenoptera, as queens typically avoid inbreeding, mate only early in life and do not mate again after having begun to lay eggs. In the ant genus *Cardiocondyla* mating occurs among sib in the natal nests. Sex ratios are extremely female-biased and young queens face the risk of remaining without mating partners. Here, we show that virgin queens of *C. "argyrotricha"* produce sons from their own unfertilized eggs and later mate with them to produce female offspring from fertilized eggs. Oedipal mating may allow *C. "argyrotricha"* queens to found new colonies when no mating partners are available and thus maintains their unusual life history combining monogyny, mating in the nest, and low male production. Our result indicates that a trait that sporadically occurs in solitary haplodiploid animals may evolve also in social Hymenoptera under appropriate ecological and social conditions.

Keywords *Cardiocondyla* · ants · mother-son mating · inbreeding · colony foundation

Introduction

Queens of most social Hymenoptera (ants, bees, and wasps) mate usually only once at the beginning of their life away from their natal nests. Thereafter, they produce workers and later also new queens from fertilized eggs and haploid males from unfertilized eggs without ever mating again (Wilson 1971). Because of the standard mechanism of single-locus complementary sex determination (sl-CSD), inbreeding is particularly detrimental in social Hymenoptera, and mating with close relatives is usually avoided (van Wilgenburg et al. 2006). In contrast, mating in the ant genus *Cardiocondyla* occurs in the natal nest and regularly involves close sibs. Wingless *Cardiocondyla* males engage in fatal fighting with their rivals and the only survivor monopolizes mating with all virgin queens reared in the nest (Yamauchi et al. 2006). Especially in single-queened (monogynous) species, mother queens are selected to adjust their offspring sex ratio to an extreme female bias, as brothers locally compete for mating chances ("local mate competition", Hamilton 1967). Indeed, queens of monogynous *Cardiocondyla* lay only relatively few male-destined, haploid eggs (Schrempf et al. 2005b), and data from the field suggest that colonies may lack male offspring despite containing several young virgin queens. Virgin queens thus face the risk of failing to acquire a mating partner, in particular when they eclose after the death of their mother. Here, we show that virgin queens of *C. "argyrotricha*" (provisional name of a recognized morphospecies currently described by B. Seifert) emerging in nests without a male may establish a new colony by raising males from their unfertilized eggs and later mating with their own sons.

Materials and Methods

All workers and brood used in the experiment came from nests that originally were set up by splitting a single stock colony of *Cardiocondyla "argyrotricha"* from the botanical garden of UPLB, Los Baños, Philippines. Colonies of *C. "argyrotricha*" consist of 20 to 60 completely sterile workers (*Cardiocondyla* workers generally lack ovaries, Heinze et al. 2006) and a single reproductive queen. Though males of this and related species are extremely long-lived compared to other ant males (up to one year, Yamauchi et al. 2006), about one third of colonies that contained winged virgin queens when collected in the field in various parts of Southeast Asia were without a male (median sex ratio 0.92, n = 10 colonies). In colonies with a male, matings typically occur between brothers and sisters.

Fig1: Dealate *Cardiocondyla "argyrotricha"* queen; picture provided with kind pemrmission of Lukas Schrader.

To examine the behaviour of virgin queens in the absence of males, we set up a total of 31 experimentally queenless colonies. Each colony consisted of ten workers, one or two queen pupae (of which one was removed after the first queen had emerged), and several second and third instar larvae (in order to be able to differentiate between initially provided larvae and larvae later produced by the emerged virgin queen). Experimental colonies were kept in climate chambers under near-natural conditions with 28 °C / 23 °C temperature and 12 h / 12 h day-night cycles and fed *ad libitum* with honey and pieces of cockroaches twice a week.

We checked experimental colonies for the presence of adults and brood three times a week. When necessary, new workers were added to keep initial worker numbers constant. The dates of emergence of the queen, the appearance of first eggs, the hatching of the first larvae and male pupae, the emergence of the first male and first worker pupae were noted. In addition, we counted the number of eggs, larvae and pupae produced by the queen until the first worker pupae appeared. Scans were continued for about four weeks after the emergence of the first worker pupae to control for male offspring (i.e., to check whether a large number of diploid males were produced). Queens mated with their own sons and thereafter produced diploid worker offspring in 13 of the 31 experimental colonies. Three of these colonies were not included in the statistical analysis because the numbers of brood items were censused only at more irregular intervals. In the other 18 colonies, queens died either before rearing males or before mating with their sons. Statistical tests were conducted with the software Statistica 6.0 and the slopes of regression lines were compared using the online tool Comp2Regs_Pgm at www.statstodo.com.

In a supplemental experiment we set up nine additional colonies as above. In four of these colonies we regularly removed all male pupae to determine whether virgin queens might ever produce female offspring. In the other five colonies we dissected the ovaries of queens 7 to 28 days after the eclosion of the first male pupae and presumed mating to determine whether their spermathecae contained sperm (Buschinger & Alloway 1978). In order to directly observe mother-son mating, we in one colony separated the male pupae shortly before eclosion from the queen and put them together only after the male had hatched. We videotaped the 55 day old queen mating with her 1 day old son 10 minutes after the latter had been returned into the nest.

Results

After eclosion, the virgin queens readily shed their wings and began to lay eggs within one or two weeks after emergence (median 11 days, quartiles 9.25-12, n=10 queens). The number of eggs increased with time only slightly if at all (Fig. 1, slope of regression line before eclosion of first offspring β_1=0.058, SE=0.026, sign. different from zero: t=2.192, p=0.043), and only 1 to 5 eggs (median 2.5, quartiles 1.75-4) hatched into male pupae several weeks later (first male pupa after queen emergence, median 46 days, quartiles 51-56; total production 44 males, median 4, quartiles 3-5.75). All other eggs were fed to the larvae, and not a single egg developed into a female pupa. The first adult male eclosed on average 63 days after queen emergence (median 56 days, quartiles 46.25-62.75), and mated thereafter with its mother. Dissection verified the presence of sperm in the spermathecae of the five additional queens, which in a supplemental experiment with the same design had mated with their own sons. In one case, we could directly observe and videotape a 55 day old queen mating with its 1 day old son.

After mating, egg numbers increased significantly more rapidly than before (Fig. 2, β_2=0.677, SE=0.027, sign. different from zero: t= 23.024, p< 0.00001; difference in slopes β_1 - β_2 =0.618, SE=0.057, t=10.871, p<0.0001). First adult workers eclosed on average 40 days after male emergence (median 38 days, quartiles 33-48.5, range 26-57). During the four weeks following the emergence of the first worker pupae, 9 of 10 colonies produced exclusively workers, suggesting that most eggs laid after the emergence of males have been fertilized. One colony produced two males in addition to workers, but throughout our experiment no new virgin queens were reared. Four additional queens that were prevented to mate with their sons by regular removal of all male pupae produced only male brood throughout the whole experiment (median 101.5 days after queen emergence, range 93.25-106.25) and their colonies failed, documenting that the production of female offspring requires the fertilization of eggs and is not due to parthenogenesis.

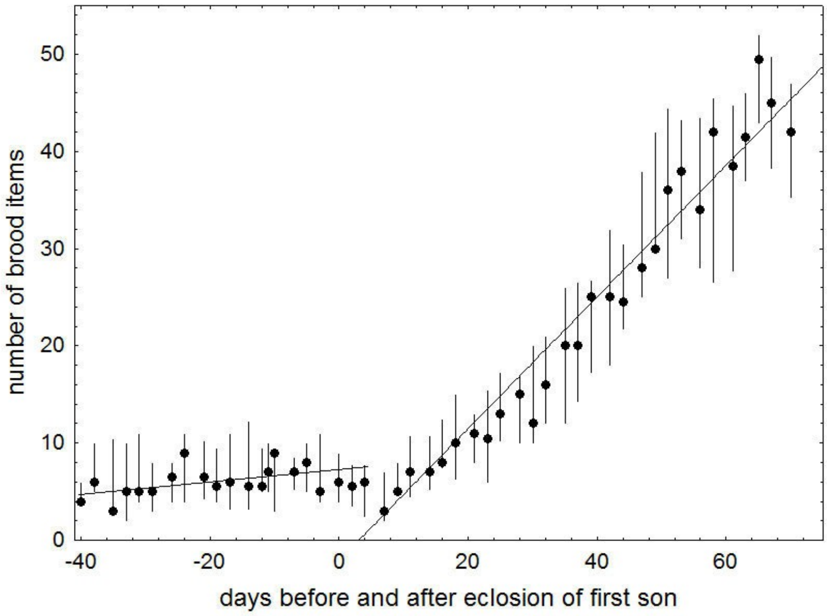

Fig. 2: Number of brood items (eggs, larvae, pupae) produced by initially unmated queens of the ant *Cardiocondyla "argyrotricha"* before and after the eclosion of their first sons at day 0 (*n* = 10 queens). Given are medians, upper (75%) and lower (25%) quartiles, and separate linear regression lines for the relationship between the number of brood items and time in the two periods before and after day 0

Discussion

Mother-son mating (oedipal mating) has previously been reported from a number of haplodiploid species, such as mites, termites, beetles, and parasitoid wasps (see, e.g., Adamson and Ludwig 1993, Kobayashi et al. 2013). It was suggested to be beneficial in habitats in which dispersing females face a particularly high risk of remaining without mating partners (Adamson and Ludwig 1993).

In social Hymenoptera, however, mother-son mating is a rare phenomenon. A thorough literature search revealed only three reports of oedipal mating in social Hymenoptera, one of them turning out to be an erroneous citation, and the other two not clearly showing that sperm was actually transferred during mating. Garófalo (1973) reports that one laboratory-mated queen of the bumble bee *Bombus atratus* laid eggs, which, when the experimenter provided them with pollen from a honeybee colony, developed into male and female offspring. Two males were later observed to mate with their mother, but as the queen had mated before it is not completely clear whether sperm was transferred during these copulations or whether most probably males just tried to copulate with available individuals. Liebig et al. (1998) provides anecdotal evidence suggesting mother-son mating in the ant *Harpegnathos saltator*, which regularly has mated workers in addition to queens. Workers were observed to first produce males but later switch to the production of female offspring. Finally, Wilson (1971) cites Peacock et al. (1954) for mother-son mating in the pharaoh ant, *Monomorium pharaonis*, but a careful reading of the original paper does not support this claim. Rather, Peacock et al. (1954) describe male production by virgin queens.

Oedipal mating appears to be very rare in social Hymenoptera because of two fundamental traits of their life history. First, queens are generally receptive only during a short time window following adult emergence and before laying eggs (Wilson 1971, Thornhill & Alcock 1983, Kronauer & Boomsma 2007). Second, as mentioned above, the typical sex determination mechanism of social Hymenoptera, sl-CSD, makes sib-mating especially harmful because under inbreeding half of all fertilized eggs develop into usually sterile or inviable diploid males instead of workers (van Wilgenburg et al. 2006). The initial production of only workers by *C.* "*argyrotricha*" queens that mated with their sons shows that, in contrast to the majority of social Hymenoptera, in *C.* "*argyrotricha*", sex is not determined by sl-CSD but another, as yet unknown mechanism. The absence of sl-CSD has also been revealed in other species of *Cardiocondyla,* where sister-brother mating is the rule (Schrempf et al. 2006). This pre-disposes *Cardiocondyla* ants to evolve the capability of mother-son mating under appropriate ecological and social conditions. Hymenopteran species more prone to diploid male load might have responded to the risks of female sexuals remaining without mating partners by evolving thelytokous parthenogenesis (Rabeling & Kronauer 2013).

Oedipal mating might underlie the evolutionary stability of the life history of *C.* "*argyrotricha*" combining monogyny, fatal fighting among males, and local mating. It still remains to be seen how widely distributed mother-son mating is within this genus and in other ants with sib-mating in the nest, but its occurrence in *C.* "*argyrotricha*" again highlights the striking plasticity of social insect life histories.

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Chapter 6:

A heritable component in sex ratio and caste determination in a *Cardiocondyla* ant

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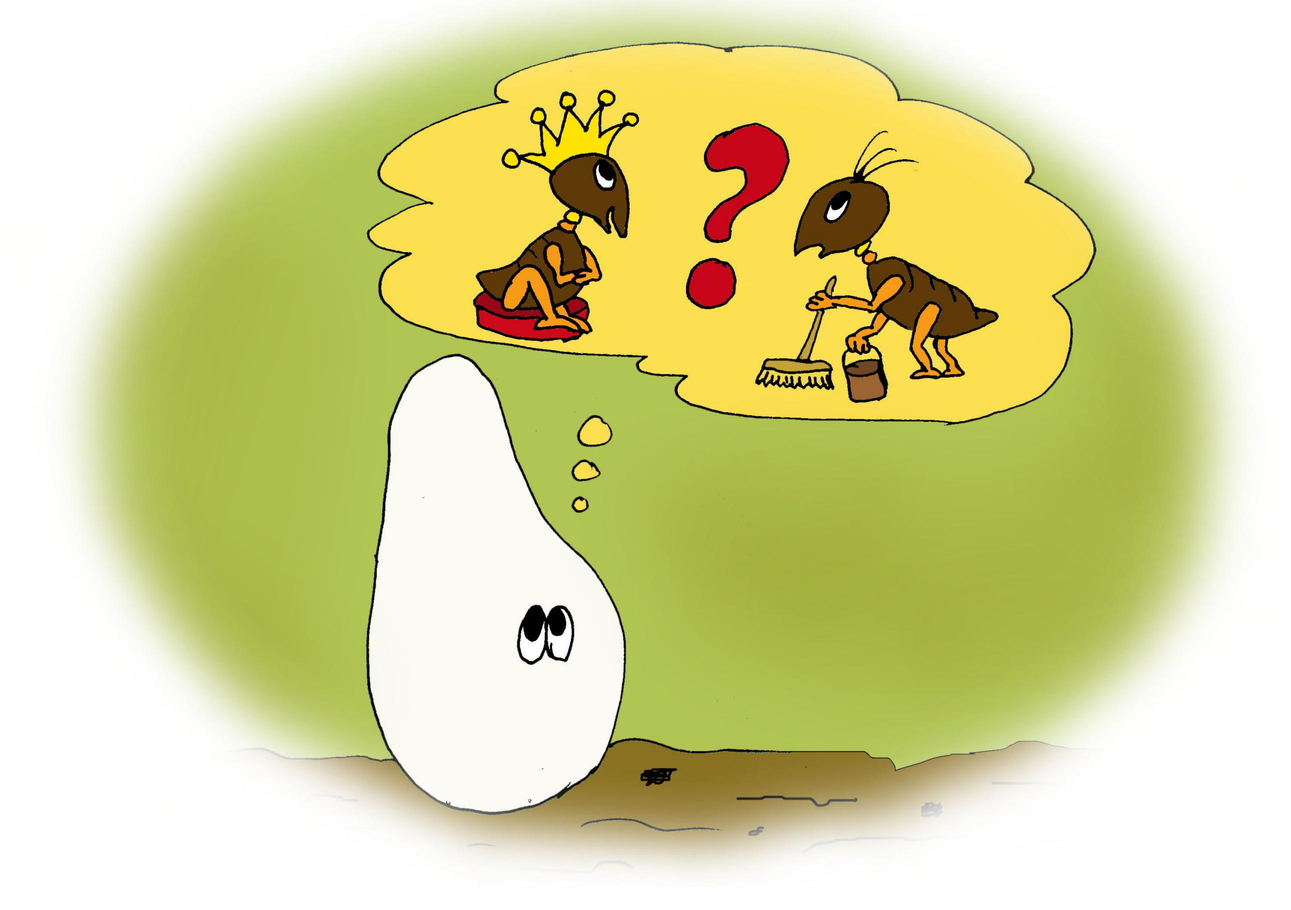

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Abstract

Studies on sex ratios in social insects provide among the most compelling evidence for the importance of kin selection in social evolution. The elegant synthesis of Fisher's sex ratio principle and Hamilton's inclusive fitness theory predicts that colony-level sex ratios vary with the colonies' social and genetic structures. Numerous empirical studies in ants, bees, and wasps have corroborated these predictions. However, the evolutionary optimization of sex ratios requires genetic variation, but one fundamental determinant of sex ratios - the propensity of female larvae to develop into young queens or workers ("queen bias") - is thought to be largely controlled by the environment. Evidence for a genetic influence on sex ratio and queen bias is as yet restricted to a few taxa, in particular hybrids.

Because of the very short lifetime of their queens, ants of the genus *Cardiocondyla* are ideal model systems for the study of complete lifetime reproductive success, queen bias, and sex ratios. We found that lifetime sex ratios of the ant *Cardiocondyla* cf.*kagutsuchi* have a heritable component. In experimental single-queen colonies, 22 queens from a genetic lineage with a highly female-biased sex ratio produced significantly more female-biased offspring sex ratios than 16 queens from a lineage with a more male-biased sex ratio (median 91.5% vs. 58.5% female sexuals). Sex ratio variation resulted from different likelihood of female larvae developing into sexuals (median 50% vs. 22.6% female sexuals) even when uniformly nursed by workers from another colony.

Consistent differences in lifetime sex ratios and queen bias among queens of *C.* cf. *kagutsuchi* suggest that heritable, genetic or maternal effects strongly affect caste determination. Such variation might provide the basis for adaptive evolution of queen and worker strategies, though it momentarily constrains the power of workers and queens to optimize caste ratios.

Introduction

Studies of intra- and interspecific variation of sex ratios in the social Hymenoptera (bees, wasps, and ants) provide among the most convincing support for the importance of inclusive fitness in evolution. Haplodiploid sex determination in the Hymenoptera (males arise from unfertilized eggs and females from fertilized eggs) results in a closer genetic relatedness of workers to their sexual sisters than to their brothers, while queens are equally related to their male and female offspring (Trivers & Hare 1976, Bourke & Franks 1995, Crozier & Pamilo 1996) . Consequently, workers in a colony with a single, singly-mated queen can optimize their inclusive fitness by allocating three times more resources into female than male sexuals, while the queens gain most from an equal sex allocation ratio. When queen numbers and queen mating frequencies vary among colonies relative to the population mean, evolution may lead to split sex ratios: under worker control, individual colonies specialize either for the production of male or female sexuals (Boomsma & Grafen 1990, Ratnieks & Boomsma 1997).

A large number of empirical studies strongly support the predictions from sex ratio theory (e.g., Meunier at al. 2008). In numerous species, workers appear to be capable of biasing sex allocation, i.e., via the selective culling of male larvae or bias- ing the development of sexuals from female larvae, while in other species a compromise between the interests of queens and workers is achieved (e.g., Sundström 1994, Sundström et al 1996, Hammond et al. 2002, Mehdiabadi et al. 2003).

Despite this compelling evidence, one fundamental assumption of sex ratio theory has remained largely untested: the optimization of sex ratios in evolution requires heritable genetic variation in this trait. While there is some evidence for genetic influences on sex ratio in solitary Hymenoptera (West & Herre 1998, Pannebakker et al. 2008), little is known on the heritability of sex ratios in social insects. On the contrary, one of the major determinants of sex ratios - the propensity of female larvae to develop into female sexuals rather than workers ("queen bias") - is usually fully controlled by the environment, e.g., colony size, temperature, pheromones, or food quality (Hölldobler & Wilson 1990). A lack of variation in the genetic threshold for caste determining influences might severely constrain the adaptive evolution of sex ratios. The importance of genetic variation on queen bias has rarely been documented, with the best evidence coming from hybrid lineages of harvester ants, where caste is exclusively determined by genotype (e.g., Volny & Gordon 2002, Julian et al. 2002, Helms Cahan & Keller 2003, Anderson at al. 2008), and crossfostering experiments in the acorn ant *Temnothorax curvispinosus* (Linksvayer 2006).

Due to local mate competition (Hamilton 1967) among wingless males, sex ratios in single-queen colonies of the ant genus *Cardiocondyla* are on average highly female biased but vary strongly between colonies (Cremer & Heinze 2002, Schrempf et al. 2005a). By comparing sex ratios among females belonging to different genetic lineages of *C.* cf. *kagutsuchi* we studied whether this natural variation might have a heritable basis. Sex ratio studies usually provide only short snapshots of sexual production during one or a few breeding seasons and do not take queen age into account, which might severely affect the likelihood of female larvae to develop into sexuals (Schwander et al. 2008). In contrast, the short lifespan of *Cardiocondyla* ant queens (<1 year; Schrempf et al. 2005a) and the fact that they continuously produce sexuals year-round allowed determining, for the first time, the complete lifetime reproduction of ant queens. *C.* cf. *kagutsuchi* queens from two different genetic lineages differed significantly in their lifetime sexual production and also in the likelihood of fertilized eggs developing into female sexuals rather than workers. This suggests heritable genetic or maternal effects on sex ratio and queen bias.

Material and Methods

C. cf. *kagutsuchi* is a complex of morphologically similar species, which are widely distributed throughout Southeast and East Asia (Yamauchi et al. 2005). One taxon, which has only wingless males, is a "tramp species" and has been introduced accidentally into many Polynesian islands. On Hawai'i it is one of the very few ants occurring in high densities in primary rain forests with O'hia trees (Metrosideros sp.) and Hapu'u tree ferns (Cibotium) and also at higher elevations on Mauna Kea volcano (Wetterer et al. 1998, Krushelnycky et al.2005).

Colonies used for this experiment were collected in 2006 near Pu'u o Kila lookout (A, D) and the trailheads of Awa'awapuhi and Nualolo trail (B, C) in Kaua'i, Hawai'i, USA, at an elevation of appr. 1100 m. Colonies were at least 250 m apart and because of mating in the nest and the low dispersal of mated queens can be considered to be, at most, distantly related. We monitored the brood production of the colonies under standard laboratory conditions (e.g., Cremer & Heinze 2002, 17°C/28°C cycles) for approximately one year, during which female sexuals mated with males from their own nests and replaced older queens. We chose two colonies (stocks A and B) with particularly different sex ratios and set up the following experiment: individual female sexuals from stocks A and B were each allowed to mate with a single male from a third colony C and thereafter placed each into a new plastic nest box with brood and 10 workers from a fourth colony D. All foreign brood was removed after the queen had started to lay eggs. The initial addition of workers and brood served to increase colony founding success and to accelerate colony growth. The first brood produced by stock A and stock B queens was thus uniformly reared by workers all from the same stock colony D. This removes the possibility for indirect effects, such as variation in brood care, contributing to the observed differences between lineages. Workers of *Cardiocondyla* do not possess ovaries and therefore did not contribute to the male offspring produced in these colonies.

We originally aimed at pairing each male from colony C both with a queen from stock A and a queen from stock B and to compare brood production between these dependent samples. As several matings failed and a few queens died without producing sufficient numbers of sexuals, only 11 samples were available for a matched pairs test. Numerical sex ratios in single-queen colonies of *Cardiocondyla* have previously been shown to be female-biased because of local mate competition among the wingless fighter males (Cremer & Heinze 2002, De Menten et al.2005).

Colonies were kept in incubators as described previously (e.g., Cremer & Heinze 2002) and provisioned with diluted honey and pieces of cockroaches twice per week. The presence of eggs, larvae, and sexual pupae and the number of workers was noted once per week. All sexual pupae were removed. We estimated "worker production" by subtracting the initial workforce, 10, from the maximum number of workers observed in the colony. Queens, which did not produce any female offspring (female sexual or worker) or less than five sexuals were excluded from the analysis.

Data were not normally distributed (Shapiro-Wilks' test, $p<0.05$) but variances were homogeneous (Brown-Forsyth test, $p>0.05$). We therefore used Mann-Whitney U-tests and Wilcoxon matched pairs tests to compare sex ratios and production data.

Results

Lifetime sex ratios (female sexuals/all sexuals) in 12 monogynous laboratory colonies set up from female sexuals from seven stock colonies collected on Hawai'i and Kauai ranged from 0.098 to 0.960, with a median of 0.459. To determine the basis of this variation we chose two large stock colonies, A and B, which differed strongly in sexual production and allowed young queens to mate with males from a third stock colony, C, and to found a new single-queen colony assisted by workers from a fourth stock colony, D. Throughout their lives, 39 queens from stock A produced in total 127 males and 1193 virgin queens, while 37 queens from stock B produced 253 males and 314 virgin queens. Numerical sex ratios were significantly different between queens from the two stocks even after all queens that had produced less than five sexuals each during their life were excluded (A: n=22, virgin queens/all sexuals, median, quartiles: 0.915, 0.778, 0.986; B: n=16, 0.585, 0.402, 0.735; Mann-Whitney U- test, U=98.0, p=0.021).

Queens from the two stocks differed neither in total lifetime offspring number (workers, males, and female sexuals, n1=22, n2=16; U=137, p=0.249) nor in total number of diploid offspring (workers and female sexuals, U=144, p=0.344), but in total production of workers (U=65, p=0.001, Fig. 1): queen bias of the female brood was considerably higher in stock A than stock B colonies, both in the total sample (% female sexuals, median, quartiles 50.0, 35.0, 75 vs. 22.6, 10.0, 31.6; U=72, p=0.002) and, at marginal significance, in a comparison between pairs of queens from stock A and stock B that had mated each with exactly the same male (n=11, Wilcoxon matched pairs test, T=12, p=0.062).

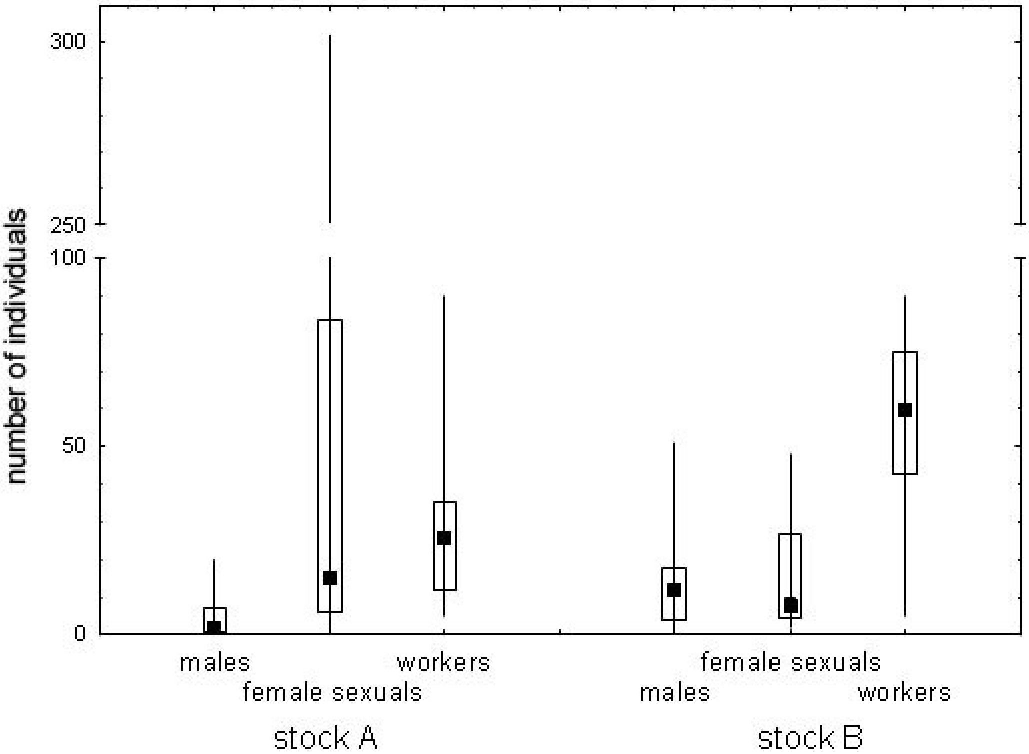

Fig. 1: Number of males, female sexuals, and workers (median, quartiles, range) queens of the ant *Cardiocondyla* cf. *kagutsuchi* from two different genetic lineages (stock A: 22 queens, stock B: 16 queens) produced during their lives. Stock A queens produced a significantly more female-biased sex ratio and female larvae with a significantly higher queen bias (female sexuals / all females) than stock B queens.

Queens from stock A produced female sexuals significantly earlier (median, quartiles: 42, 35, 50 days vs. 87.5, 59.5, 105.5 days; U=75.5, $p<0.003$) and at much lower worker numbers than colonies of stock B (median, quartiles: 2.5, 0, 5 workers vs. 30, 9.5, 45 workers; U=75.0, $p<0.003$). Furthermore, the time difference between the first appearance of first worker pupae and the first appearance of female sexual pupae was significantly shorter in stock A (median, quartiles: 6.5, 0, 14 days vs. 60, 17.5, 77.5 days; U=70.0, $p<0.002$). First female sexual pupae appeared at the same time as or even before the first worker pupae in 10 of 22 stock A colonies but only 2 of 16 stock B colonies of (Yates corrected $\chi2=4.05$, $p=0.044$). This all means that, though the first brood was uniformly reared by workers from a different colony D, which originally accompanied each queen, stock A colonies produced relatively more female sexuals than stock B colonies. Indirect sib effects through workers from the different stocks engaging differently in brood care could thus be eliminated.

Discussion

Our study documents consistent differences in lifetime sex ratios and queen bias between two different lineages of the ant *Cardiocondyla* cf. *kagutsuchi*. Queens of the two lineages differed significantly in their patterns of reproduction even though they all had mated with males from the same other colony, their first brood was uniformly reared by workers from the same other colony, and their colonies were kept under exactly the same environmental conditions in the laboratory. Sex ratios in *C.* cf. *kagutsuchi* were different but not because queens limited the number of fertilized eggs and thus forced the workers to rear a male- biased sex ratio, as previously observed in fire ants (Passera at al. 2001). Instead, queens of the different lineages produced similar total numbers of diploid offspring, but female larvae differed in the likelihood of developing into queens.

In previous studies, sex ratios and queen bias have usually been estimated only for one or a few subsequent breeding seasons, e.g., for relatively short fractions of the queens' lives. The extremely short lifespan of *C.* cf. *kagutsuchi* queens allowed us to determine, for the first time in ants, the exact lifetime reproductive success of each queen. We can therefore exclude a strong influence of queen age on sex ratio and queen bias (Schwander at al. 2008). Our data instead suggest that caste differentiation and indirectly also sex ratio have a "heritable" component.

Proximately, this can be due to maternal effects, e.g., queens of stock A better provisioning their fertilized eggs (e.g., Schwander et al. 2008) or somehow manipulating workers into more provisioning the larvae. Alternatively, stock A larvae might be more sensitive to queen-determining environmental or social influences. A third, indirect factor - workers of the two stocks engage differently in brood care (e.g., Linksvayer 2007) - may also have contributed to the total difference at later stages of brood production. However, queen bias was already different between the two lineages in the very first larvae, which were uniformly reared by workers from another colony. We therefore conclude that eggs or larvae themselves differ in their likelihood of developing into sexuals, either because of direct heritable, genetic or maternal, i.e., queen-derived effects on caste determination.

Genetic caste determination has recently been described from genetically heterogeneous populations of *Pogonomyrmex* harvester ants and *Solenopsis* fire ants, in which fertilized eggs with the genomes of different genetic lineages invariably develop into workers, while genetically homogeneous fertilized eggs invariably develop into queens (e.g., Volny & Gordon 2002, Julian et al. 2002, Helms Cahan & Keller 2003, Helms Cahan & Vinson 2003, Anderson at al. 2008). Furthermore, genotyping suggested that in colonies of honey bees and leafcutter ants headed by multiply mated queens, female larvae belonging to different patrilines differed in their tendency of developing into female sexuals (Moritz et al. 2005, Hughes & Boomsma 2008). Our research on *C.* cf. *kagutsuchi* differs from these studies in several aspects. In contrast to *Pogonomyrmex* and *Solenopsis*, all queens produced both workers and female sexuals from fertilized eggs, although in different proportions. In contrast to honeybees and leaf-cutter ants, the difference in the queen bias of female larvae was expressed not within, but between colonies with a single, singly-mated queen each, which excludes nepotistic brood rearing by workers of a particular genotype (e.g., Hannonen & Sundström 2003) as a potential proximate mechanism.

What maintains variation in queen bias in our study population of *C.* cf. *kagutsuchi*? For the evolutionary stability of such a polymorphism, queens, which produce brood with a high queen bias, are expected to have on average the same fitness as queens, which produce brood with a low queen bias. This might be the case if female sexuals from the two lineages differed in their probability of establishing colonies and producing sexuals themselves, e.g., when they followed alternative dispersal and founding strategies. For example, *Leptothorax* sp., an ant with a genetic queen polymorphism, winged queens engage in highly risky dispersal and solitary nest founding. In contrast, wingless queens mate near the maternal nest and found new colonies in a rather safe process called "budding," i.e., assisted by workers from the maternal nest (Heinze 1993). The efflux of workers from the maternal nest represents a sort of supplementary investment into the female sex and budding in ants appears to be generally associated with a less female-biased numerical sex ratio (e.g., Bulmer 1983). Wingless queens of *Leptothorax* sp. A indeed produce relatively more workers and fewer female sexuals than winged queens, presumably due to genetic variation in the threshold of queen development (Heinze 1993). At present little is known about colony founding by *C.* cf. *kagutsuchi* queens, but alternative dispersal strategies like in *Leptothorax* sp. A might be a reasonable explanation for the maintenance of heritable variation in sex ratios and queen bias.

Our study documents heritable variation in queen bias in female larvae of the ant *C.* cf. *kagutsuchi* and thus in the lifetime production of female sexuals by queens. One might argue that our results reflect idiosyncrasies of particular colonies, as young queens used in this study were taken from two original colonies, which in our study population differed most in their patterns of sexual production. However, exactly such natural variation provides the basis for adaptive evolution of sex and caste ratios required by sex ratio theory. Recent studies have documented genetic variation in several traits of the social phenotype of insect societies that were formerly believed to be mainly due to age or environmental influences, such as division of labour (Smith at al. 2008), queen size (Rüppell et al. 2001), and worker caste polymorphism (Fraser at al. 2000, Hughes at al. 2003). Our study shows that queen bias and numerical sex ratios may also be influenced by heritable variation. While such genetic variation provides the basis for adaptive evolution, it may momentarily limit the power of workers and queens to optimize caste ratios and might have to be considered as a constraint in studies on sex and reproductive allocation.

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General Discussion

Species richness and new discovery *Cardiocondyla pirata*

(Chapter1)

The ant genus *Cardiocondyla* is an excellent example for the enormous species richness of ants. Although ants of this genus are easily overlooked, the number of described species rose from 49 to 70 species in the last 12 years (Seifert 2003, Bolton 2015). Locating of ants of the genus *Cardiocondyla* in nature is quite difficult as individuals forage alone, their size seldomly exceeds 2 mm and the colour of their cuticle let them merge with their surroundings. While all so far known *Cardiocondyla* species are colored plainly the newly discovered *Cardiocondyla pirata* introduced in Chapter 1 breaks ranks. This species shows a unique coloration pattern - a sandy color with translucent body parts and a dark eye stripe - so far unknown in ants. In its natural surroundings in the primary rainforest of the Philippines the *C.pirata* nest was found in a stone crack of a sandy stone in a very shady place surrounded by a small river. Individuals of that species are even tinier (worker smaller than 1.5 mm) than other *Cardiocondyla* species and because of their translucent petiolus, legs and parts of the head, it appears as if gaster and mesothorax were different objects. The reason for this pattern is still speculative: in other animals translucency is often associated with camouflage (Rotheray 1986, Giguere & Northcote 1987). This also applies to stripes in mammals (Ortolani 1999). If camouflage is indeed the reason it still remains puzzling which predator preys on this tiny ant species.

Male fighting strategies in *Cardiocondyla venustula*

(Chapter 2)

In *Cardiocondyla* species that contain both male forms, winged and ergatoid males, environmental conditions ultimately determine into which type of male a haploid larvae will develop. Stressful conditions such as drop of temperature or diminishment of colony size lead to the production of winged disperser males (Cremer & Heinze 2003). The crucial stage of larvae determination is the second of three instars (Schrempf & Heinze 2006).

But what lead to the phenotypic fixation and the loss of the winged male form in the evolution of the genus *Cardiocondyla*? Among the Hymenoptera several species of fig wasps represent another taxa with only ergatoid males (Weiblen 2002). As females mate inside the figs males gain a high fitness if they can monopolize a lot of females for mating. Consequently males evolved a new strategy: ergatoid males stay on the fig waiting for females to eclose and for maximum mating success males gained the ability of killing rivals (Bean and Cook 2001). In *Cardiocondyla* females mate inside the nest. Hence, similarly to fig wasps, the best policy for males is staying inside the nest, mating with all eclosing females and eliminating all rivals. In species with apterous males and nest mating females, gene flow is further reduced leading to easier speciation. In fact, the most species rich branches of *Cardiocondyla's* phylogenetic tree are those where species with only wingless males are located (Oettler et al. 2010).

With the evolution of new species with monomorphic males, each species developed its idiosyncratic male reproduction tactic as an adaptation to environmental conditions and in accordance with the colony specific life history traits. Reproduction tactics of ergatoid males range from mutually tolerant males that share virgin queens towards species were one dominant male kills all rivals (see Introduction Fig.1). The unique strategy of ergatoid males in *Cardiocondyla venustula* that defend territories (Chapter 2) is especially interesting as this species is phylogenetically located between species with killing males and species with tolerant males (Oettler et al. 2010).

What could have driven *Cardiocondyla venustula* to develop such a complex male mating tactic? First, colonies of *Cardiocondyla venustula* are characterized by a seasonal production of sexuals like their phylogenetically neighboring species *C.mauritanica* and the group of male-tolerant species. In tropical species there is no annual cycle and queens are produced year round so that one single male is able to inseminate all of them. In contrast seasonal species get flooded by young queens during the sexual production phase. Therefore, during this time, a high male production is needed. In fact we could observe up to 10 adult males and 18 male pupae inside a nest of *Cardiocondyla venustula*. But unlike in species with mutually tolerant males, males of *C.venustula* do fight against each other. This may be due to the expectedly less close relationship of males. Whereas all tolerant species are monogynous and males are closely related, in *C.venustula* several queens reproduce which leads to greater genetic divergence among male offspring.

The policy of fighting and eliminating rivals seems to be another important point in the development of male territories. *Cardiocondyla venustula* males possess sheer-shaped mandibles that are adapted to kill pupae or freshly eclosing males. Adult males whose cuticle is already hardened cannot be killed with that kind of weapon. Tropical Asian *Cardiocondyla* species have sheer-shaped elongated mandibles which allows them to grab and hold even adult rivals and besmear them with hindgut secretion (see Introduction). Marked males are killed by workers thereafter. The tactic of catching and marking rivals is thought to be an ancestral trait (Oettler at al. 2010). *C.venustula* males nevertheless also use this tactic of besmearing rivals. However, the rather improper mandible form allows them to hold their rivals for a few seconds only. In comparison, a *C.obscurior* male (which has sickle-shaped mandibles) has been observed to hold on to its rival for 23 hours (Yamauchi and Kawase 1992). If a *Cardiocondyla venustula* rival can free itself from its attacker after a few moments, it can clean itself and in such manner wash away the odor that elicits worker aggression. This inability of *C.venustula* males to kill adult rivals makes the establishment and defense of a small territory inside the colony even more reasonable. The tactic of defending territories will provide each territory-holder with virgin females that eclose inside its domain and rival males only have to be attacked and chased away in a small defined area.

Male's territory defending tactic may be the best adapted policy in a polygynous, seasonal species where numbers of sexuals are very high. One single male will be unable to inseminate all virgin females and at the same time kill all male pupae. Furthermore, overlooked young males whose cuticle has once hardened can not be eliminated anymore such as the male mandible form is useless for that task. Interestingly the territorial strategy evolved in *C.venustula* but not in its neighbouring species *C.mauritanica* which is also characterized by sexual seasonality, polygyny and males' sheer-shaped mandibles.

Remarkably *C.venustula* males show big differences in body and head size (unpublished measurements). In fact males can even exceed queens in their body size - which is something extraordinary within the genus *Cardiocondyla.* Male polymorphism, however, is associated with alternative reproductive tactics (Gross 1996). In *C.venustula* only big males establish territories which applies also to other Hymenoptera (Alcock 1979). Small *C.venustula* males were only seen running from one territory to another. We referred to them as "floaters" that probably could sneak matings- something that is known for example from males in wool-carder bees (Severinghaus et al. 1981). Therefore the variability in male size may have ultimately lead to a system of male territories in *Cardiocondyla venustula* where only the biggest specimen can monopolize and defend a part of the nest. Male size differences are absent in *C.mauritanica,* but strikingly Seifert described (2003) in his taxonomic revision a second morphological slightly different species of *C.mauritanica* which he refers to as „an epigenetic modification rather than a different species". The described individuals were only workers, but remarkably this presumably "epigenetic modification" can also be found in the male caste of the phylogenetically neighboring species complex *Cardiocondyla* cf. *kagutsuchi* (Oettler et al. 2010). Based on the fact that in this group species with monomorphic, polymorphic and also "intermorphic" males exist, Oettler et al. (2010) contemplate that "male-determining mechanisms are not very stable in this taxon". Perhaps these instable determining mechanisms were an ancestral trait that could also be found in the branch containing *C.venustula* and lead there to a great variation in male body size (for phylogenetic location of species see Introduction, Fig.1).

Coevolution and speciation in *Cardiocondyla* cf. *kagutsuchi*

(Chapter 3)

Seifert describes the clustered distribution of *Cardiocondyla* nests as "isolated occurrence on small habitat patches within large desert systems" (Seifert 2003). I can confirm this distribution for all *Cardiocondyla* nests collected for this study. .An explanation for such a pattern could be the fact that queens, after having mated with the colony male, distribute on foot which limits dispersal distances (Heinze et al. 2002). This matches with the observation of many nests in close vicinity and a high relationship between colonies of one population. Inside a colony genetic variability is low due to frequent matings between siblings (Schrempf et al.2005b).

Close relationship of mates may lead to a tight coevolution between males and females. Mate's coevolution can result in a perfect matching of the male's seminal fluid proteins for the body of the female, which is supposed to prolong the life of the queen (Schrempf et al.2005a).

In the inbreeding line of my crossing experiments with the species *Cardiocondyla* cf. *kagutsuchi* (see Chapter 3), mating took place among siblings. I found a slightly higher lifespan of the founding queen than in the outbreeding line where unrelated individuals mated, which may hint at well adapted life prolonging seminal fluid proteins that the queen received from her genetically similar mate.

Whereas dispersal on foot and mating inside the nest cause close genetic relationships within populations, gene flow between populations seems to be restricted. This was shown for at least two *Cardiocondyla* species (Lenoir et al. 2007, Schrempf et al. 2005a). It might explain the high species richness of *Cardiocondyla*: genetical separation of isolated populations can, over generations, result in post mating isolation barriers, which finally precludes mating between individuals of the respective populations. Differences in the particular genpool may lead to lethal "within genotype epistasis" (between parental alleles) or "among genotype epistasis" (between mother and offspring alleles), bacterial caused cytoplasmic incompatibility (Brucker & Bordenstein 2012) or differences in the male's seminal fluid proteins (Swanson & Vacquier 2002) that may also hinder egg development. The outbreeding colonies of my crossing experiments with *Cardiocondyla* cf. *kagutsuchi* documented a significant lower founding success by frozen egg development in several colonies, indicating outbreeding depression.

*C.*cf. *kagutsuchi* is rather a complex of sibling species of southern and eastern Asia (Seifert 2003) than one well-defined species and my crossing experiment may hint at the initiating speciation of this complex. This is supported by findings of groups within *Cardiocondyla* cf. *kagutsuchi* that contain winged as well as ergatoid males, groups with ergatoid males only (like the experimental colonies I used in my experiments) and others groups where ergatoid and brachypterous males are present (Yamauchi et al. 2005). The different taxa of this complex could be separated by the sequencing of mitochondrial genes (Heinze et al. 2005). With respect to phylogeny, *Cardiocondyla* species with winged and ergatoid males are more ancient and wings in this genus were convergently lost at least twice (see Introduction and Oettler at al. 2010). The branch of the *Cardiocondyla* cf. *kagutsuchi* complex on the phylogenetic tree is neighbored exclusively by species that have lost the winged male form which makes the male's regain of wings in some *C.*cf. *kagutsuchi* species likely (Oettler at al. 2010).

Queen reproduction- lack of trade-off between lifespan and reproductive success

(Chapter 4)

The genus *Cardiocondyla* offers high species richness, idiosynchratic colony structures, specific life history traits and a perfect suitability for lab culturing. Thus working with *Cardiocondyla* permits to analyze general questions on the the social life of insects. Lifespan and reproductive output of one colony for example can easily be accessed and we used *C.*cf. *kagutsuchi* for investigating differences in sexual reproduction between colonies (Chapter 6) and to access the total reproductive success of single colonies, comparing it with the lifespan of the queen (Chapter 4).

We could verify that *Cardiocondyla* cf.*kagutsuchi* queens show no trade-off between lifespan and reproductive success (Chapter 4). On the contrary: more fecund queens lived even longer than less productive ones and also started earlier with egg production. A trade-off between lifespan and reproduction is usually very common among the animal kingdom (e.g. Snell & King 1977, Reznick 1985, Ellers et al. 2000). But why does it seem to be absent in social insects?

Usually individuals have to split their accessible energy to satisfy different needs. The energy spent on life history traits is thought to be traded off against lifespan. Each individual has to search for food and shelter, has to defend against parasites and germs as well as against predators and rivals, and has to look for mating partners. Females have to lay eggs or give birth to their offspring and eventually care for them. The costs for reproduction are thought to be huge for non-social females. The proximate mechanisms underlying the costs of reproduction are very complex and not yet well understood. Harshman & Zera (2007) described five areas for proximate mechanisms: hormonal regulation; intermediary metabolism and allocation; reproductive proteins; immune function; and defenses against stress and toxicity. At least in the three latter areas social insect queens differ from most other female animals.

In social insects, costs for immune function and defense against stress and toxicity are largely taken over by the workers as soon as a colony is established. Before the emergence of the first workers though a queen probably has to deal with a depression of the immune system as this has been proven to be true for other insects after the mating process (Rolff & Siva-Jothy 2002, Fedorka et al. 2004). Shortly after mating, queen survival against pathogens is reduced and mortality rate is high (Fedorka et al. 2007). So it might be beneficial for a social insect queen to mate only once in her life. The most critical period ends when the first workers hatch. Workers act as the "social immune system" as they reduce the germ load inside an ant colony by grooming off pathogens (Cremer at al. 2007). Under these circumstances a queen can probably down regulate her immune system and use the energy for persistence of her life. In humans, for example, a genetically based low inflammatory response inside an environment with low pathogen burden, can result in a higher chance of a long living (Candore et al. 2006). In Drosophila the reversal scenario is found, hinting at the same basic mechanism. Here, chronic activation of immune response leads to an inflammatory state and reduced lifespan (Libert et al. 2006).

Workers in social insects not only defend the nest against pathogens and predators but also provide the queen with food and do all the brood care, thereby establishing a constant stress reduced

environment. The costs for the remaining duty of a queen, namely the egg production, can probably be neglected. In *Caenorhabditis elegans* there seems to be no trade-off between gamete production and longevity, but there is a trade off between mating and longevity (Gems & Riddle 1996).
Another reason for the trade-off between life-span and reproduction in social insects might be due to reproductive proteins: these proteins are transferred from the male to the queen's body during mating and are responsible for a numerous physiological and behavioral post-mating changes in females (Avila et al. 2011). In fruit flies a male can augment its fitness through stimulating the female's egg laying rate at the expense of her lifespan, so that a maximum of the female's resources is put into his offspring before the female will mate again (Wolfner 1997). In ants however, where females mate only at the beginning of their reproductive phase and use the stored sperm for fertilization of their eggs, males gain more fitness from a long living female (see Introduction).

Consequently, the costs for reproduction and lifespan can probably be disentangled in social insects because males and queens have both a strong interest in a long living queen, and queens shift the major costs for reproduction towards their workers. Interestingly a similar scenario was found in a study of zoo animals, precisely in several species of birds and mammals (Ricklefs & Cadena 2007). No trade-off between lifespan and reproduction could be found in females. Similar to social insect colonies, many costs that reproducing females usually have to bear in the wild, in zoos are overtaken by zookeepers: humans care for clean parasite less surroundings, attend sick individuals, provide food, look out for stress reduced environment and even help with raising the offspring if necessary.

Colony founding and the special strategy of *Cardiocondyla "argyrotricha"*

(Chapter 5)

The distribution ability of the genus *Cardiocondyla* is exceeding. Polygynous tramp species are distributed worldwide and new colonies arise from young inseminated queens that disperse on foot accompanied by some worker and brood from the maternal nest, a process called "budding" (Heinze & Delabie 2005, Heinze et al.2006). Also in non-tramp species a great ability of even establishing a flourishing nest out of single workers with adequate brood items can be found.

The most minimalistic requirement for the foundation of a prospering colony can be found in the monogyne species *Cardiocondyla "argyrotricha"* (Chapter 5). An uninseminated queen accompanied by some workers can establish a normal colony by mating with the first male, developing from her laid haploid eggs. Mother-son mating has been reported for some haplodiploid species (Adamson & Ludwig 1993) but in the few reports of oedipal mating in social insects it has never been shown that sperm was actually transferred (Garfalo 1973, Liebig et al. 1998). We could not only observe one mating between a virgin *Cardiocondyla "argyrotricha"* queen and her son, but also verify afterwards through dissection of the spermatheca that the queen received the sperm of her son. How frequent mother-son matings in this species are under natural conditions remains an unanswered question. The natural environment of *Cardiocondyla "argyrotricha"* suggests that colony founding could be a difficult task for this species: nests were only found in clefts of big dark lava stones, in small forest streams or on the beach (Fig.1). Stone gaps are not abundant and most of them were found already inhabited by ants. In addition many stones were surrounded by water. This might limit queen dispersal and ultimately increase queen mortality rate. Males in this species are very long lived and therefore rarely produced. In case of death of the colony male, virgin queens stay uninseminated. If they are lucky though to make it to an adequate nesting site they will be nevertheless able to establish a colony on their own.

Fig.1: Natural habitat of *Cardiocondyla "argyotricha";* colonies were found in stone clefts on the beach (left) and in small forest streams (right).

Polygyny is the ancestral state in *Cardiocondyla* and prevails in the whole genus (Schrempf & Heinze 2006). So why has monogygy evolved in *Cardiocondyla "argyrotricha",* a species dwelling in habitats that do not seem to favour easy dispersal? Studies reveal that monogyny evolved independently at least two times in *Cardiocondyla*: withing the monophyletic palearctic group of xerothermic seasonal species with tolerant males and also in *Cardiocondyla "argyrotricha".* Concerning xerothermic seasonal species, Oettler et al. (2010) hypothesize that the high winter mortality of mayor colonies, reported for one species (Lenoir et al. 2007), may have promoted the seasonal production of large numbers of sexual offspring as well as the evolution of monogyny. Winter mortality, however, does not exist in *Cardiocondyla "argyrotricha"* which inhabits tropical areas. Here mass destruction of colonies is more likely to be triggered by different climatic factors. One possibility could be heavy precipitation, followed by floods which might drown ant nests within formerly dry and save stones. The same could be visualized at the beach during heavy motion of the sea. For a population a higher number of small colonies may be more beneficial than few big ones, as in case of flooding the amount of surviving colonies might be higher.

Queen reproduction- a heritable component for caste determination

(Chapter 6)

During her life an ant queen can produce a great quantity of sexual offspring and workers. In Chapter 6 I presented an experiment analyzing the question whether environmental or heritable components determine female caste in the ant species *Cardiocondyla* cf. *kagutsuchi.* We established two series of experimental colonies. The series differed by the origin of the queens, either stemming from a strong or a weak queen biased colony. Males from a third colony were allowed to mate with queens from each series. Although both treatments did not differ in the sum of produced female sexuals and workers, queen bias was significantly different in both lines. As the experimental environment was exactly the same for all colonies, we claimed that there must be a strong heritable component for caste determination (see also Introduction).

There is a growing number of reports about eusocial hymenoptera where caste determination has a more or less strong genetic component (e.g., Hayashi & Kitade 2007, Sun & Sun 2007, Anderson et al.2008, Schwander & Keller 2008). Actually genetic caste determination was thought to be unlikely to evolve. Both, kin selection and optimal resource allocation theory were believed to argument for environmental caste determination (Schwander et al. 2010).

However, kin selection theory does not really contradict genetic cast determination. Insights into different mechanisms of genetic caste determination show that the machinery of inheritance however is just far more complex.

In some harvester ants (*Pogonomyrmex*) for example, caste determination results from combination of reproductively isolated dependant lineages. Individuals that are homozygous at one special locus turn into queens, whereas heterozygosity at that locus turns them into workers. That mode of caste determination affords multiple mating of the queen with both types of males: with males carrying the same allele as the queen as well as with males carrying a different allele at the "caste" locus (Volny & Gordon 2002, Cahan et al. 2002, 2004). In *Wasmannia* male and female gene pools are nearly separated (Fournier et al. 2005) with workers evolving from normal sexual reproduction and female sexuals being clonally produced by the colony queen.

Optimal resource allocation theory claims that in social insect colonies sexuals should only be produced after the colony has reached a certain size. Therefore phenotypes should stem from differences in gene expression patterns, which means an environmental determination mechanism. In the monogynous harvester ant *Pogonomyrmex barbatus* observations showed that sexuals were only produced after a colony has reached reproductive maturity after about 5 years (Gordon 1995). A queen should therefore be able to select inter lineage sperm for insemination of her eggs to produce workers (Hosken & Pitnick 2003). On the other hand there are recent critical votes from Wiernasz and Cole (2010) that "caste determination may result from temporal variation in sperm use, rather than from fertilization bias among male ejaculates". It is also feasable that queens and workers can distinguish among pure and interlineage brood and selectively raise interlineage brood as workers (Volny & Gordon 2002). In fact, there is evidence that in incipient lab colonies of *Pogonomyrmex rugosus* gyne-destined eggs either die, are culled by workers or develop poorly during colony founding stage (Clark et al.2006b).

How castes are fixed in *C.*cf. *kagutsuchi* is not yet discovered. Our experiments hint at a maternal or genetic caste determination system. We concluded that larvae or eggs themselves differed in their likelihood of developing into sexuals or workers because although the first brood was uniformly raised by the same unrelated workers of another colony, queen bias of the first pupae was different in the two experimental series. The underlying processes of caste determination in *Cardiocondyla* cf. *kagutsuchi* still await to be discovered.

Let's get fascinated!

Keller states (2007) that reproductive systems of ants and social insects are much more diverse than generally appreciated and several interesting observations were done just out of serendipity. He pleads for a shift in values from scientific productivity to scientific creativity. I am also convinced that only an unbiased unconstrained approach - an open minded observation- not aiming at a certain result, allows us to see the whole spectrum of questions, and may hint at some corresponding possible answers. The ideas for nearly all my experiments presented here in this PhD thesis arose from some "casual" observations after spending my time magnetized just watching the fascinating colony life of ants. I want to end this work with an encouragement for more space for astonishment and curiosity as a driving force for questions in science. Let’s get fascinated like Donald Duck when he was discovering the strength of a single ant.

"Fascination recruits ambition and paired with time it may lead to knowledge."

Summary

The ant genus *Cardiocondyla* is estimated to comprise at least 100 species and is distributed worldwide. All *Cardiocondyla* species exhibit a wingless, "workerlike" ergatoid male form that shows a species-specific mating pattern. Whereas in some phylogenetically older species winged and ergatoid males exist, the peaceful winged male form is convergently lost in several more recent species.

Ants of the genus *Cardiocondyla* are tiny and inconspicuous and are frequently overlooked. Increased collecting intensity however revealed more and more species during the last decade. One of the most recently described species is *Cardiocondyla pirata*, collected on the Philippines on a rock in a shady forest stream bed (**Chapter 1**). Colonies of *C. pirata* are polygynous and presumably contain only one ergatoid fighter male that kills other eclosing males. This species shows a unique coloration pattern with translucent body parts and a black eye stripe. The function of this coloration remains speculative.

The reproductive strategies of ergatoid *Cardiocondyla* males vary among species. One monophyletic group of monogynous species with several mutually tolerant males can be distinguished from species with fighting males where one male monopolizes all virgin females by killing its rivals. Species with ergatoid fighter males use one out of two main strategies with the males' mandibles adapted towards their strategy. *Cardiocondyla venustula* stands phylogenetically between the species group of tolerant and a clade of species with fighting males and its males show a new, intermediate reproductive tactic (**Chapter 2**). Based on the seasonal production of sexuals one male cannot execute all rivals and mate with all eclosing females. Males therefore establish territories that they defend against other males and are able to mate with virgin queens inside their area.

In the whole genus *Cardiocondyla* all ergatoid males mate inside the nest and consequently most matings occur between closely related individuals or siblings. New colonies are usually found in close vicinity of the mother colony because queens are bad flyers or disperse on foot. Nevertheless colonies do not suffer from inbreeding depression due to the absence of a single locus complementary sex determination system. On the contrary: In crossing experiments, inbred *Cardiocondyla* cf. *kagutsuchi* queens (that mated with a brother) succeeded more frequently in colony foundings and had a slightly higher lifespan than outbred queens that mated with an unrelated male (**Chapter 3**). This may be founded on the good fit of genomes of the mating partners

and the close coevolution of the mates which lead to highly adapted seminal fluid proteins that favor a long life of the queen. In inbred *Cardiocondyla* cf. *kagutsuchi* colonies more fecund queens also lived longer which supports the assumption that there is no trade off between fecundity and lifespan in social insects (**Chapter 4**).

Cardiocondyla species show effective dispersal ability. Even some workers with an appropriate set of brood can raise a whole colony. The most minimalistic requirement of a colony founding could be observed in *Cardiocondyla "argyrotricha"*, a monogynous species of Southeast Asia (**Chapter5**). Virgin queens are able to found a new colony alone, only accompanied by some workers. After the first son has eclosed out of the laid haploid eggs, the queen mates with him and starts to lay diploid eggs. In such manner a complete normally functioning colony develops.

In *Cardiocondyla* colonies offspring sex ratio follows local mate competition theory, which means males are produced far less frequent than queens. In *Cardiocondyla* cf. *kagutsuchi* there is also a strong heritable component concerning sex ratio (**Chapter 6**). Established daughter colonies from two colonies that showed a great difference in the amount of produced male and female sexuals exhibited the same discrepancy in sexual production that did their mother colonies. However the number of diploid versus haploid larvae did not differ significantly. Instead the likelihood of diploid larvae to become a queen or a worker varied, which means that determination of female caste has a strong genetic or maternal component in *C.*cf. *kagutsuchi*.

Zusammenfassung

Die Ameisengattung *Cardiocondyla* ist weltweit verbreitet und umfasst schätzungsweise über 100 Arten. Bei allen *Cardiocondyla* Arten findet man eine flügellose "arbeiterähnliche" ergatoide Männchenform die ein artspezifisches Fortpflanzungsverhalten zeigt. Während in einigen phylogenetisch älteren Arten geflügelte und ergatoide Männchen vorkommen, ist in vielen jüngeren Arten die geflügelte Männchenform konvergent verloren gegangen.

Ameisen der Gattung *Cardiocondyla* sind winzig und unauffällig und werden daher häufig übersehen. Eine vermehrte Sammelaktivität hat jedoch in den letzten 10 Jahren dazu geführt, dass viele neue Arten entdeckt wurden. Eine der erst kürzlich beschriebenen Arten ist *Cardiocondyla pirata*, die auf großen Steinen in einem Flussbett auf den Philippinen gefunden wurde (**Kapitel 1**). *C. pirata* Kolonien sind polygyn, aber in jeder Kolonie findet man nur ein einziges ergatoides Männchen, das alle frisch schlüpfenden Rivalen tötet. Diese *Cardiocondyla* Art besitzt eine einzigartige Färbung mit durchsichtigen Körperteilen und einem schwarzen Augenstreifen. Die Funktion dieser Färbung bleibt spekulativ.

Das Fortpflanzungsverhalten ergatoider *Cardiocondyla* Männchen unterscheidet sich von Art zu Art. Es existiert eine monophyletische Gruppe monogyner *Cardiocondyla* Arten mit mehreren, sich gegenseitig tolerierenden Männchen, im Gegensatz zu anderen Arten mit kämpfenden Männchen. Kämpfende Männchen monopolisieren alle unbegatteten Königinnen indem sie ihre Rivalen töten. Das Kampfverhalten ergatoider Männchen kann je nach Art in zwei Hauptstrategien mit entsprechend angepassten Mandibelformen eingeteilt werden. Die Art *Cardiocondyla venustula* steht phylogenetisch zwischen der Artengruppe mit toleranten und der mit kämpfenden Männchen. *Cardiocondyla venustula* Männchen zeigen eine neue dazwischenliegende Fortpflanzungstaktik (**Kapitel 2**). Ein Männchen alleine kann aufgrund der saisonalen Produktion von Geschlechtstieren nicht alle Rivalen umbringen und sich gleichzeitig mit allen Jungköniginnen paaren. Die Männchen errichten deshalb Territorien, die sie gegen andere Männchen verteidigen. Innerhalb dieser Gebiete verpaaren sie sich mit den frisch geschlüpften Königinnen.

Innerhalb der gesamten Gattung *Cardiocondyla* kopulieren die Männchen im Nest wobei folglich Paarungen meist zwischen eng verwandten Tieren oder Geschwistern stattfinden. Aufgrund des schlechten Flugvermögens von *Cardiocondyla* Königinnen und der Tatsache dass junge Königinnen oft zu Fuss neue Kolonien gründen, findet man viele Kolonien oft in nächster Nachbarschaft. Die Gattung *Cardiocondyla verfügt* nicht über einen komplementären Mechanismus der

Geschlechtsbestimmung mit nur einem einzigen Lokus, daher findet man keine Inzuchtdepression in den Kolonieen. Im Gegenteil: In Kreuzungsexperimenten waren *Cardiocondyla* cf. *kagutsuchi* Königinnen, die mit einem Bruder verpaart wurden, erfolgreicher bei der Koloniegründung und lebten etwas länger als Königinnen die sich mit einem unverwandten Männchen paarten (**Kapitel 3**). Das kann möglicherweise mit dem guten Zusammenpassen der Genome der Paarungspartner begründet werden. Enge Koevolution der Paarungspartner führt zu bestmöglich angepassten Samenflüssigkeitsproteinen, welche ein langes Leben der Königin begünstigen. Das längere Leben fruchtbarerer Königinnen in ingezüchteter *Cardiocondyla* cf. *kagutsuchi* Kolonien stützt die Vermutung dass es in sozialen Insekten keinen Kompromiss zwischen Fruchtbarkeit und Lebensalter gibt (**Kapitel 4**).

Cardiocondyla Arten zeigen eine effiziente Ausbreitungsfähigkeit. Sogar wenige Arbeiter mit einer geeigneten Zusammensetzung an Brut können eine ganze Kolonie errichten. Die minimalistischste Voraussetzung für eine Koloniegründung findet man bei *Cardiocondyla "argyrotricha"* einer monogynen Art aus Südost Asien (**Kapitel 5**). Jungfräuliche Königinnen können eine neue Kolonie alleine gründen, nur begleitet von einigen Arbeitern. Nachdem der erste Sohn aus den gelegten haploiden Eiern geschlüpft ist, paart sich die Königin mit diesem und fängt an diploide Eier zu legen. Auf diese Art entsteht eine vollständige normal funktionierende Kolonie.

In *Cardiocondyla* Kolonien folgt das Geschlechterverhältnis der Nachkommen der Theorie der lokalen Paarungskonkurrenz, das heißt, Männchen werden viel seltener produziert als Königinnen. In *Cardiocondyla* cf. *kagutsuchi* gibt es außerdem eine starke genetische Komponente hinsichtlich des Geschlechterverhältnisses (**Kapitel 6**). Künstlich errichtete Tochterkolonien von zwei Ausgangskolonien die einen großen Unterschied in der Anzahl der produzierten Königinnen und Männchen aufwiesen zeigten dieselbe Diskrepanz in der Produktion von Geschlechtstieren wie ihre Mutterkolonieen. Die Anzahl von diploiden und haploiden Laven unterschied sich jedoch nicht signifikant. Stattdessen war die Wahrscheinlichkeit von diploiden Larven entweder Königin oder Arbeiter zu werden unterschiedlich. Folglich wird die weibliche Kaste in *Cardiocondyla* cf. *kagutsuchi* stark durch genetische oder maternale Komponenten festgelegt.

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The journey is the destination

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